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Review

The Role of the Separation Sciences in the 21th Century

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Esta revisão examina, de um ponto de vista crítico, o papel das ciências da separação no novo século, considerando a complexidade dos problemas a serem enfrentados. De forma a tornar a exposição mais dinâmica, uma determinação típica de um analito orgânico hipotético em uma matriz complexa é usado para introduzir criticamente as principais técnicas de separação empregadas atualmente. Técnicas as quais promovem uma redução ou mesmo a total eliminação do uso de solventes tóxicos são enfatizadas. Após esta avaliação o autor discute as tendências futuras nesta área, com ênfase na miniaturização das técnicas; a automação total da análise, o uso de técnicas hifenadas, o papel ainda sub-explorado dos solventes em técnicas de separação, e o desenvolvimento da cromatografia de transição de fase e da cromatografia unificada.

This review examines, from a critical point-of-view, the role of the separation sciences in the new century considering the complexity of the problems to be faced. To make the exposition more dynamic the determination of a typical (hypothetical) organic analyte in a complex matrix is used to critically introduce the major separation techniques used nowadays. Techniques that promote the reduction or that fully eliminate the use of toxic solvents are stressed. After this evaluation the author discusses the future trends in this area with emphasis on the miniaturization of the instrumental techniques, the full automation of the analyses, the use of hyphenated techniques, the under-explored role of the solvents in separation techniques, the development of transition phase chromatography and unified chromatography.

Keywords: analytical chemistry, separation sciences, chromatography, extraction, unified chromatography, sample preparation

1. Introduction

During the last half of the 20th Century a tremendous growth in the demand for more complex analytical separations was observed. While in the previous centuries much less sophisticated techniques, mainly physical separations such as extracting the gold present in minerals, were required, the accelerated industrialization observed in the 20th Century demanded a huge improvement in the analytical arsenal. The birth of the petrochemical industries, food processing companies, biotechnology enterprises, improvement in pharmaceutical products and the emergence of the related environmental contaminations – to mention a few examples – changed the necessities of our society to more demanding technologies. As a result, the Analytical Sciences had to change from very simple operations, usually involving more qualitative observations and semi-quantitative analyses, to complex instrumental determinations automated via fast computers and micro-machined devices. The preparation of an experiment, previously done in a very simple way, now demands the development of sophisticated methodologies – quite often requiring the aid of chemometric tools - followed by complex validation procedures in order to make the method acceptable to governmental regulatory agencies. The complexity of the samples analyzed nowadays, particularly those involving the presence of organic analytes in micro quantities (usually referred to as trace organic analysis), has created a need for a preliminary separation step (before the separation itself) to make the sample less complex. Even so, in environmental organic analysis, as an example, very often hundreds of compounds are still present in the sample after the pre-separation step, thus requiring high resolution techniques to be able to identify and quantify the target compounds often present in minute amounts.

A typical scheme showing a general separation approach employed in complex analysis can be seen in Figure 1. Before the separation process starts, a sampling

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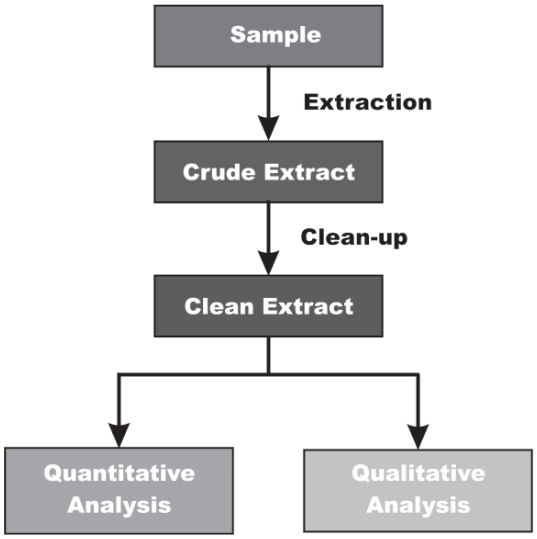

Figure 1. General separation approach employed in a typical complex analysis.

step must be performed in order to warrant that the fraction selected for the analysis is representative of the whole sample. When dealing with vegetable or fruit analysis, a number of specimens (sub-sample) have to be selected and properly sampled to assure that a small fraction of them represents the crop. There are several guidelines for sampling vegetable and fruits with the one suggested by the *Codex Alimentarius* being the most commonly used. If water is to be sampled, in addition to the care in selecting the proper container and volume to be sampled several other circumstances have to be considered, for river/sea/lake water, including the geographic description of the area, the depth from which it is sampled, the sampled volume, and so on. In general, physical properties such as temperature, pH, color, etc., have to be determined at the moment of sampling. Soil sampling also requires additional concerns, such as determination of the soil composition, sampling depth, and sampling length, weight, among other parameters. After this step the actual analytical step starts with the so-called sample preparation step. Since this step differs considerably if one is dealing with organic or inorganic analytes, in this paper we will focus our examples on the analysis of organic compounds present in minute quantities in a sample. The major goal of the sample preparation step (Figure1) is to isolate the target compound(s) from the bulk matrix, thus facilitating the next step that the analyte(s) determination. In most cases, although the compound(s) of interest is(are) isolated from the bulk matrix, several contaminants may be also extracted, as well as part of the matrix. So, further purification of the extract is required before the analyte(s) determination. This step is called the clean-up step and aims at the isolation of the target compound(s) from potential contaminants as well as getting rid of the extraction solvent and preparing the target analyte in a chemical form appropriate for its characterization and quantification. Among the several separation techniques nowadays employed for the analysis of complex mixtures, chromatography and related techniques such as capillary electrophoresis have received special attention. The analytes are individually isolated and the peak area / peak height generated by the chromatogram/electropherogram is used to quantify the analyte.

In this paper a critical review on all these steps will be addressed and discussed in the light of the recent advances in the separation sciences.

2. Sampling Techniques

Sampling is considered to be one of the most critical steps in performing an analytical determination since problems in this step will not be corrected at any further point during the analysis.[1] The idea behind sampling is to isolate from a large universe a small sample quantity that is representative of the whole sampled universe. At least two major difficulties make this step critical: sample inhomogeneity and sample size. In case of complex samples, such as environmental samples (including water, soil, and sediment), the sampling step is even more critical. River water, as an example, contains various amounts of suspended matter depending upon the depth and location of the sampling point and the geochemical characteristics. As a consequence, sampling water in one point of the river might give a different analytical answer of that obtained by sampling at another point of the same river, since the chemical constitution at the sampling moment was different (we have to remember that river water is a dynamic system instead of a static one, such as a closed water reservoir). So, in addition to depth and location, which requires multiple sampling, the time when the sampling was done is also an important parameter to be planned, since this could later be related to the rain regime of the river. In all cases, a proper sampling plan has to be created and, if possible, validated before the sampling. Several regulatory agencies, including the USEPA and USFDA, require such plans to be incorporated into the documents submitted by companies to these organizations. Soil sampling is another example of a complex and difficult sample from which to obtain a representative sub sample, due to the reasons already described: inhomogeneity of the sample and sample size. The European Community has recently carried out a strong effort to normalize sampling techniques for

soil analysis but, as it can be concluded from the published results,[2-5] they are still very far from a consensus in this field.

Although very important to the analytical results, sampling is considered in many cases as a "pre-analytical" step since, in most cases, different people than those involved in the analytical laboratory do the sampling. Sampling a representative number of sub-samples of river water, soil and sediment will require the use of several specific devices not usually available in the analytical laboratory. As a consequence, it is very common that specialized companies that already have experienced people and proper equipment for large and difficult sampling work do this step.

After sampling, and still before the laboratory work, the sub samples have to be properly transported to the laboratory. Otherwise the analytical results can be invalidated since several processes can occur that may modify the sample composition, including adsorption, chemical reactions, microbial decomposition, photochemical reactions and so on. More critical will be the lost of volatiles, in the case of the analysis of volatile organic compounds (VOCs such as chloromethanes) from water, since this may completely alter the analytical results. Care should be also taken in properly storing the samples in the laboratory, since inadequate temperatures and improper materials (such as adsorbing glasses) may result in sample integrity deterioration.

3. Extraction Techniques

After the sampling step, sub samples ideally representative of the whole sampled material will be available for the qualitative and quantitative analysis scheduled to be performed. However, when dealing with complex samples usually these sub samples contain, in addition to the target analyte(s), also several other chemical compounds existing in the matrix, as well as contaminants that were accumulated into the matrix and that may interfere with the analyte(s) determination. As an example, in the analysis of organic micropollutants in river water the presence of humic substances suspended in the water may interfere in the determination of several classes of pollutants. Another important factor is that the target analytes might be present in minute concentrations in water, thus making their quantitative determination as well as the confirmation of their chemical identities difficult. It is also common that the analyte of interest is present in the matrix in a chemical form not appropriate for its determination using the desired analytical technique (as an example the analyte might not be thermally stable or not present enough vapor pressure to be analyzed by gas chromatography), thus requiring its conversion to a more appropriate chemical form. To overcome these problems, an extraction step is included in the methodology aiming to isolate the analyte of interest from the matrix, to concentrate it and to modify it to a chemical form ideal for further analysis. There are several extraction techniques in use nowadays depending upon the physical state, the chemical composition and the complexity of both the sample and the target analytes. We will elaborate on the most common techniques in use for this purpose.

3.1. Extraction of solid samples

There are a huge number of analytical techniques already used for the extraction of organic compounds from complex solid samples, the most common being Soxhlet extraction, microwave extraction, sonication and similar techniques, and several pressurized solvent extraction techniques.

Soxhlet extraction, although being probably the most widely used extraction technique for organic solid samples in the past, as still practiced presents as major disadvantages the use of large volumes of organic solvents and long extraction times (usually over 24 hours of continuous operation at the solvent boiling point), which may degrade thermally labile compounds.

Microwave irradiation has as its major goal to avoid or minimize the problem of thermal degradation since the extraction time is reduced, thus also reducing the deleterious effects of using organic solvents at high temperature. Although very simple in operation the *shake flask* technique (with or without microwave irradiation), in which the solid sample is mixed with a solvent in a flask which is shaken during the extraction, usually does not provide reasonable extraction yields.

Sonication techniques usually provide better recoveries than those obtained using the shake flask technique, but still present similar limitations.

More recently the attention of researchers and industry analysts has been focused on the use of more sophisticated instrumental techniques, particularly those using higher pressures and lower temperatures to avoid degradation of labile analytes and to reduce the extraction time.

Supercritical fluid extraction (SFE) is now a mature extraction technique that employs a solvent above its critical temperature and critical pressure. Although several solvents were investigated in the early 80's, almost all SFE are now performed using carbon dioxide as the extracting solvent. This is due to its several advantages over other fluids including: low cost, ease of disposal, very low

toxicity, easy of purification, ready availability, relatively low critical conditions (T_c= 43 °C; P_c= 73 bar), among others. One major disadvantage of CO_2 as the extraction fluid is its low polarity, which makes the extraction of medium to high polarity analytes difficult, even using high pressures. One alternative to this problem has been the addition of modifiers or co-solvents (usually organic solvents such as methanol, acetone, or toluene) to the majority fluid (usually CO_2), in order to modify its polarity and solvation power.

Figure 2 shows a schematic diagram of a supercritical fluid extraction apparatus. The basic instrument consists of a fluid reservoir, a system to pressurize the fluid to the desired value, an oven to control the temperature of the extraction cell where the sample is loaded, a restrictor to maintain the supercritical pressure inside the system and to control the fluid flow rate, and a collection vessel. If a gas such as carbon dioxide is being used as the fluid, the gas cylinder as supplied by the companies can be directly used. Liquids, such as alcohols, acetone, water and others, have also been used as extracting fluids for SFE.[6,7] In this case a properly designed vessel has to be built in order to support the high pressures used. To pressurize the fluid to the desired value, a high-pressure pump is used; alternative ways to pressurize the fluid without a high-pressure pump were developed in our laboratory[8-12] and widely used in the last ten years.[13-20] The oven can easily be homemade[9] or adapted from other instruments, mainly from gas chromatographs that have good temperature control. The extraction cell is made from a stainless steel block that can be drilled to the specified values, while an empty HPLC column (either analytical or preparative, depending upon the sample volume) can also be used. The restriction at the end of the extraction cell can be made in several ways, the most popular being either fused silica or stainless steel tubing with an appropriate internal diameter and cut to the appropriate length, or a needle valve (more expensive but also more flexible). Extract collection can be done using a simple test tube or a vial designed to operate at low temperatures, in order to avoid losses during solvent depressurization. Several other accessories may be added to this instrument, such as auto samplers, additional valves to allow different operation modes,[17,18] a sample collector, etc., but none are essential to the operation of the basic system. SFE has been used in almost all areas involving extraction from solid samples, including food, fuels, environmental and pharmaceutical samples, natural products,[21] and many others.

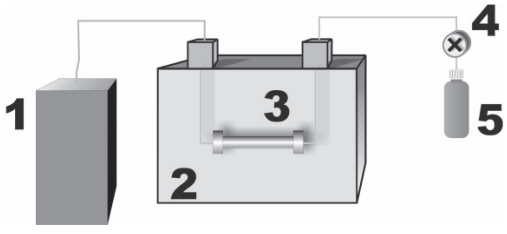

Figure 2. Schematic diagram of a Supercritical Fluid Extraction (SFE) apparatus. 1.Supercritical Fluid, 2. Oven, 3. Extraction Cell, 4. Restrictor. 5. Collector

Accelerated solvent extraction (ASE) is another pressurized solvent technique that is quite similar to SFE. In this case a liquid fluid, usually an organic solvent such as methanol, toluene or acetone, below their critical conditions but at pressures high enough to guarantee that the solvent will stay in the liquid state during the extraction process, is used. This technique offers some advantages over the classical ones, such as Soxhlet extraction, sonication and shake flask, including the use of less organic solvent, and provides faster extractions. As well, in selected cases the solvent properties can be tuned to selectively extract only certain classes of compounds. In addition to solid samples, ASE has also been used for the extraction of semi-solid matrices, such as sludge, sediments and similar samples.[22,23] The instrumentation used for ASE is very similar to that one already described for SFE, with some minor modifications.[24]

Subcritical water extraction (SWE) is another pressurized solvent technique similar to SFE and ASE that uses similar instrumentation and chemical principles. Water is a universal solvent widely used in several techniques for the extraction of polar compounds from different matrices. The choice of water as the principal solvent for polar compounds is based upon its properties, including its dielectric constant, which is much higher than for most organic compounds at normal conditions of temperature and pressure. However, upon increasing its temperature and maintaining the pressure just enough to avoid its change to the vapor state, the dielectric constant of water decreases from 80 at 25 °C to 27 at 250 °C.[25] Thus, the water "polarity" concept can be changed from a polar-like solvent to a non-polar-like solvent, depending upon the selected temperature and pressure. Since these conditions usually are below the water critical pressure and critical temperature (P_c= 220 bar; T_c= 374 °C), the technique has been named subcritical water extraction (SWE). Considering also the other physical characteristics of water as a solvent, such as environmentally compatible, non-toxic, easy to find and purify and inexpensive, this technique is gaining space among the people who use extraction techniques on a routine basis. Although this technique has been used more often in environmental analyses it can be used for virtually any solid or semi-solid; its major advantage is the fact that the solvent polarity can be easily

tuned to the desired value. The instrumentation (Figure 3) used is similar to the one described for SFE and ASE.

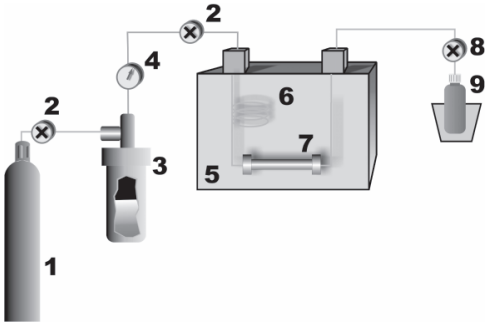

Figure 3. Schematic diagram of a Subcritical Water Extraction (SWE) apparatus. 1. N_2 Tank, 2. Valve, 3. Pressurized Solvent Vessel, 4. Pressure Monitor, 5. Oven, 6. Pre-heater, 7. Extraction Cell, 8. Restrictor, 9. Collector.

3.2 Extraction of liquid samples

In general the extraction methodology to isolate analytes from liquid samples is simpler than those described for solid samples, mainly due to the lesser complexity of most liquid matrices, such as water, gasoline and essential oils, when compared to its corresponding solid matrix counterpart (sediment, shale, plants). As an example, in most cases it is easier to promote the extraction of organic micropollutants from water samples than the extraction of the same analytes from sediments and sludge. The most popular extraction techniques used for liquid sample include liquid-liquid extraction, solid-phase extraction, solid phase micro extraction, stir bar sorption, and pressurized solvent extraction.

Liquid-liquid extraction (LLE) is still one of the most popular extraction techniques for liquid samples. In spite of its simplicity of operation, this technique lacks specificity, uses large volumes of organic solvents, demands several steps, is time consuming and difficult to fully automate for unattended operations. Considering the collective conscience developed in recent years about reduction in the use of organic solvents, this technique has been replaced by others that either minimize or eliminate this problem.

Solid phase extraction (SPE) is a technique based on a miniaturization of open column liquid chromatography. In its original version it uses the same material as used for classical and high-performance liquid chromatography (HPLC) packed into small cartridges or columns. The sample is loaded onto the top of previously conditioned small column and the analytes are eluted using an appropriate eluent with the help of either applying a small pressure on the top of the column or by decreasing the pressure at its outlet to increase the eluent flow rate.[26] More recently, several other SPE formats have being introduced, including disks, filters and pipet tips containing the solid phase.[27] Each one of these approaches presents advantages and disadvantages depending upon the sample matrix, sample volume, chemical composition of the analytes and so on.[28] One major advantage of SPE over LLE is the much smaller consumption of organic solvents required by the former, thus avoiding the problems related to this. Also, SPE is much easier to be automated than LLE; several companies commercialize mechanized, automated and even robotic versions of sophisticated systems.

Pressurized solvent extraction (PSE) techniques such as SFE, ASE and SWE, already discussed for solid sample extractions, are also utilized for liquid matrices. However the major inconvenience is that the sample has to be first homogenized on a solid matrix, such as diatomaceous earth, silica, florisil, charcoal and similar materials, before being loaded into the extraction cell. This creates several inconveniences, including adsorption of analytes onto the solid matrix, with the resulting increase in steps, and the use of additional materials that, on a large scale, might increase in a significant way the final cost of the analyses. The few experiments reported in the literature directly using pressurized solvent extraction with liquid samples are not very encouraging since the recovered yields for several analytes are small when compared to other extraction techniques used for similar purposes.[29] A major advantage of this approach is the complete elimination of organic solvents and its several benefits, as already presented.

Solid Phase Micro Extraction (SPME) was developed during the last decade of the 20th Century[30] for the analysis of organic micropollutants in water, although its application scope has been widely enlarged to include several other matrices, from plants to polymers.[31] In its original version, a fiber (*ca.* 10 cm long, having about 1 cm of the tip coated with an adsorbent material), fitted into a microsyringe, is immersed in the solution to be analyzed and the analytes of interest are sorbed onto the fiber, later being desorbed in the hot injection port of a gas chromatograph. The desorbed compounds are directed to the column with the help of the carrier gas and analyzed in the standard way, eliminating the use of solvents in the extraction step. Although very useful for the analysis of many volatile compounds in liquid matrices,[30,31] this technique still suffers from some drawbacks, the major one being the need to heat the sampled analytes in the hot injector, thus preventing the analysis of thermally labile and nonvolatile compounds. For these applications, the coupling of SPME with High Performance Liquid

Chromatography (HPLC) has been investigated. Two different approaches have been evaluated. The first one consists of dipping the fiber into the solution to be analyzed (exactly as in SPME-GC) and later desorbing the fiber by dipping it into a vial containing an appropriate solvent. After this step, the solution is introduced into the HPLC system through the sampling valve. The main drawbacks of this approach are that the solution might require a further concentration step before the analysis and the fact that it uses solvents (mainly organic ones) although to a much lesser extent than in LLE, Soxhlet extraction and similar approaches. An alternative to this approach is the use of an interface between the extraction vial and the LC injector into which the fiber is placed after the extraction step. Then, an appropriate solvent will desorb the analytes from the fiber into the LC injection valve, thus allowing the analysis to be started. The major limitations of this approach are that it also uses a solvent to desorb the sample and that the only interface commercially available presents a much too large dead volume and leaks quite frequently. A new interface concept, developed in our laboratory, is being evaluated to overcome these problems. Another important limitation at the moment is the very limited number of fibers commercially available, since this technique has been licensed by the patent holder to only one fiber manufacturer. To overcome this limitation, several laboratories have developed new fibers for their own use.[32,33]

Stir Bar Sorptive Extraction (SBSE) is a recently developed technique,[34] aiming to be an extension of SPME. It uses a set up similar to the later but instead of a fiber it uses a stir bar coated with a polydimethylsiloxane (PDMS) film. The bar is introduced into the sample, which is stirred for a defined time (depending upon the matrix and analytes) to allow the transfer of the sample from the liquid matrix to the solid bar. After this step the bar is inserted in a heating system in order to thermally desorb the extracted analytes in a similar way as done for SPME-GC. The major advantages of SBSE over SPME is that the former avoids irreversible adsorption effects already reported to happen in the SPME fiber, particularly for semi-polar and polar analytes, and the thicker film of the polymer coating over the stir bar, when compared to the SPME fiber, allows a higher concentration effect. On the other hand only one type of coating is commercially available for SBSE to the moment, thus very much limiting the scope of the technique.

3.3. Extraction of gaseous samples

Gaseous samples have been traditionally extracted by headspace techniques, the most commonly used headspace technique being the static headspace (SH) and the dynamic headspace (DH) approaches. In the static mode the sample, contained in a closed vial, is heated for a defined time (depending upon the matrix and analyte characteristics) to achieve an equilibrium between the liquid and the gaseous state. After this step the vapor is sampled, usually with a gas-tight syringe, and injected into a GC to be analyzed. In the dynamic mode, the sample is not enclosed in a sealed vial but is instead swept constantly with a fresh flow of an inert gas such as nitrogen or helium. As a result, the analytes of interest are removed from the original sample into the gas stream and, in order to concentrate the analytes before analysis, a chemical trap is placed in the DH system. The analytes concentrated into the trap are later thermally desorbed into the GC column and analyzed. Due to the use of a trap to concentrate the target compounds, this approach is usually referred as a purge-and-trap (P&T) system; since it includes a concentration step, the P&T system usually allows the detection of much lower concentration levels than the static headspace system.

Another approach in use for gaseous samples has been HS-SPME, in which the solid phase micro extraction system operates in the headspace mode. Although the name might suggest that it as a novel technique, in fact it is the same SPME with the only difference that the fiber, instead of being immersed into the liquid matrix,is suspended in the vapor phase after the headspace equilibrium has been achieved.[30] Since SPME is a concentration technique, the HS-SPME technique allows achieving very low detection limits, being easier to use and relatively less expensive, when compared to the apparatus required for instrumental static and dynamic headspace techniques.

Several other techniques are still widely used for the extraction of gases and organic vapors, including bubblers and impingers,[35] in which the analytes are usually collected in a liquid solvent. Another succesful approach in this case is the use of a SPE cartridge to trap and concentrate the analyte vapors that are later desorbed using a small volume of an appropriate solvent, thus concentrating the analytes for further anlalysis.

4. Sample "Clean Up"

In most cases, after the sampling and extraction steps, the analyte(s) of interest is (are) still present in a complex environment that usually still contains hundreds of compounds which could interfere in the determination step of the analysis. As a consequence, an additional step – usually called "clean-up"- to remove the analytical interferences, as well as to make the sample simpler for

further analysis, is required. Several options are available for sample clean-up depending upon the analytes of interest, the matrix and the potential contaminants. Among them, column liquid chromatography, solid phase extraction, and liquid-liquid extraction are the most popular at the moment.

4.1. Low and medium pressure Column Liquid Chromatography

Liquid Chromatography is one of the oldest separation techniques and is still widely used nowadays. One of its major applications today is for the clean-up of complex samples before their qualitative and quantitative determination, usually employing an instrumental technique such as High Performance Liquid Chromatography (HPLC) or High Resolution Gas Chromatography (HRGC). In most cases this technique is very simple, both conceptually and in practice, consisting of an open tube similar to a standard burette fitted with a stopcock at one end to control the flow rate of the mobile phase percolating through the system. This tube (column) is usually loaded with a solid material such as silica, alumina, florisil, and ion exchange resin and so on - termed stationary phase (SP) - which will retain certain compounds present in the sample while allowing the percolation of other analytes, thus promoting the desired sample "clean-up". Among the advantages of this technique are its operational simplicity and its versatility, since several separation modes (mechanisms) are possible by just changing the stationary phase characteristics: from adsorption to absorption, from ion exchange to size exclusion. Although the SP particle sizes (usually larger than 40 μm) used in this technique are considered to be too large to achieve high column efficiencies, compared to the ones achieved in HPLC (that uses particle sizes smaller than 10 μm), low and middle pressure LC are still very popular as clean-up techniques due to the fact that the clean- up step does not usually intend to promote a complete separation among the analytes, but instead to induce the removal of interferences from the sample.

4.2. Higher Pressure Liquid Chromatography (HPLC)

Since the boundary between medium pressure and high pressure liquid chromatography is not always easy to be recognized, the use of just Liquid Chromatography to designate this clean-up technique has been preferred in many cases. All forms of Liquid Chromatography, independent of the pressure, particle size and instrumental approaches, have been used for sample clean-up, the major advantages of higher pressure LC over low pressure ("classical") liquid chromatography being its separation power, as measured by the column efficiency or plate numbers, and its miniaturization, which allows the use of smaller columns and instrumentation.

4.3. Multidimensional Liquid Chromatography

As a consequence of their higher efficiency and miniaturization, small, closed LC columns may be coupled on-line with analytical columns allowing us to set up very powerful and versatile analytical devices usually referred to as multidimensional liquid chromatography. In this case two or more columns operating under different mechanisms may be coupled to one other in such way as to improve the separation power. When coupling two columns on-line, the first column usually acts as a clean-up or selector column while the second is used for the analytical separation. As an example, the separation of pesticides found in fruits may be done by using two columns coupled on-line, with the first being a gel permeation column for the clean-up step and the second being a reversed-phase column for separating the pesticide(s) of interest from other compounds present in the sample. In this approach the crude fruit extract is introduced through a high pressure valve onto the top of the GPC column and the high molar mass endogenous compounds are first eluted and isolated from the pesticide(s) of interest while the lower molar mass compounds are more retained by such a column. When the target compound(s) leave(s) the first column, the flow rate is diverted through a valve to the reversed phase column and the analyte(s) is (are) then separated from the other compounds eluted in this "time window". Similar approaches may be used to clean up drugs from biological fluids, proteins in complex matrices (such as for proteomics analyses), low concentration compounds in liquid fuels (such as PAHs in gasoline) and so on. Considering its tremendous separation power, allied to excellent versatility, multidimensional chromatography should perform an important role in separation sciences in this new Century.

4.4. Other clean-up approaches

Several other separation techniques already described in this review are also used for sample clean-up, particularly the extraction techniques such as SPE, LLE, SPME and SFE. In this case the extraction technique may be used in different ways so as to perform extraction and clean-up in one step, as occurs in SPME, or just as clean up, as in SPE. In SFE the clean- up may be performed in

certain analytical conditions and, after removing most endogenous analytes, the conditions are changed and the target compound is extracted. An extraction technique may also be coupled on-line with a separation technique such as chromatography or electrophoresis, thus allowing on-line extraction and analysis. A pioneering work in this field was done by the author's laboratory by successfully coupling on-line supercritical fluid extraction with capillary electrophoresis[36] for the extraction, concentration and analysis of complex samples. This clean-up approach, involving on-line coupling of analytical techniques, will certainly find many more applications in these coming years.

5. Analyte Determination: Qualitative and Quantitative Analysis

After the clean-up step has been completed, the sample is finally ready for the actual goal of the analytical methodology: the qualitative and quantitative determination of the compound(s) present in the sample. In most cases, the first step consists of the qualitative determination of the compound(s) under investigation, before their quantification. At this moment the analyst will be faced with several real situations between two extremes: at one extreme the analyte to be determined is well known (target) and the analytical conditions are well established, just requiring that the analysis be performed, such as in routine analysis laboratories. At the other extreme is the situation in which the sample is not known and the analytes to be determined are also unknown, such as often occurs in research laboratories. In the latter situation the compounds will have to be well-isolated one from the other and then identified before any quantitative work can be done. Several different approaches may be used in these cases, with chromatographic and electrophoretic techniques the ones receiving more attention at the moment.

5.1. Chromatographic techniques

Chromatographic techniques are among the separation techniques that expanded most in the last Century. Developed in the beginning of the 20th Century, this group of techniques received a major impulse ca. a half century later (middle of the 50's) with the development of Gas Chromatography (GC), followed by another advance in the late 60's with the introduction of High Performance Liquid Chromatography (HPLC). Supercritical Fluid Chromatography (SFC) became a useful analytical tool only in the 80's, particularly after the commercial availability of fused silica tubing that allowed the development of open tubular SFC columns. The chromatographic techniques are usually named according to the physical state of the mobile phase (Figure 4), being classified as gas, liquid and supercritical fluid chromatography.

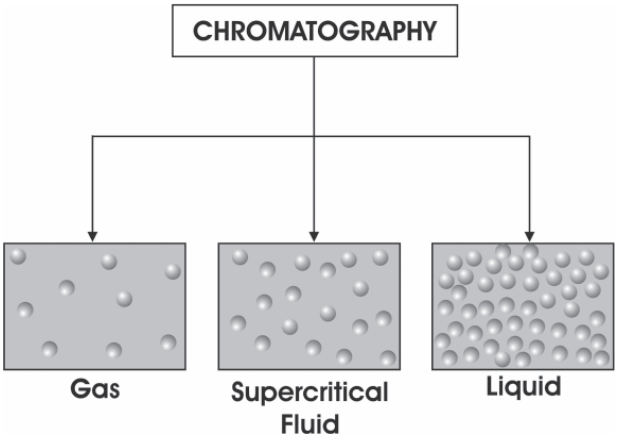

Figure 4. Classification of the chromatographic techniques according to the physical state of the mobile phase.

Gas Chromatography (GC) is now a mature analytical tool particularly useful in the analysis of volatile and thermally stable analytes. The development of open tubular columns, also called capillary columns, in which a stationary phase is coated on the inside wall of a fused silica tube, allowed this technique to achieve very high efficiencies. The major advantage of these columns for most separations is the fact that they are not packed, thus avoiding the many problems related to the use of solid packing materials. Thus, open tubular columns have very low pressure drops (difference between the pressure at the column head and at the column end), allowing us to prepare long columns with small internal diameters. This allows the production of columns having a large number of plates (close to 1 million plates for a column 100 m long with a 100 um id). Since these high efficiency columns also lead to higher resolutions, this technique is often referred as High Resolution Gas Chromatography (HRGC). A limitation of this technique is that the sample has to be volatile and thermally stable under the analytical conditions.

High Performance Liquid Chromatography (HPLC) complements GC in the sense that it does not require that the sample is volatile or thermally stable as it usually operates at ambient or sub-ambient temperatures. The development of silica-bonded phases with small particle diameters allows the production of the very stable and inert reversed phases widely used nowadays. Since these columns are packed with small particles, the pressure drop along the column is fairly large, thus preventing us from preparing long columns that would show higher

efficiencies. Although this limitation is compensated by the use of smaller particles, even so the efficiency per column is much lower than that for open tubular GC columns, making GC the technique of choice for the separation of volatile and thermally stable analytes. Although termed High Pressure Liquid Chromatography in the early days, when the instrumental version of column liquid chromatography was developed (*ca*. 30 years ago), this technique has received several names including High Performance Liquid Chromatography, Column Liquid Chromatography and just Liquid Chromatography. Anyway, the symbol HPLC has been used to identify this instrumental version of the technique as opposed to "classical" low-pressure liquid chromatography.

Supercritical Fluid Chromatography (SFC) received some attention in the 80's and 90's but never reached the popularity of either GC or HPLC. Very often claimed to be the panacea for all separation problems in the 80's, particularly due to commercial interests in promoting the technique, it has emerged in this new century as an option for specialized separations. Two approaches have been developed: from one side, the use of GC-like instruments and small internal diameter open tubular coated columns and, from the other, the use of HPLC-like instruments and larger diameter packed columns. A good example for which SFC presents advantages is in the quality control analysis of chiral drugs using packed SFC chiral columns with CO_2 as mobile phase. This separation may be done faster using SFC than with either HPLC or GC, with the necessary resolution.

5.2. Electrophoretic techniques

Capillary electrophoresis (CE) was introduced at the beginning of the 80's and immediately created a great many expectations about this new format of an already well know separation technique, which had usually been restricted to the analysis of biological samples. The advent of fused silica columns for GC allowed the preparation of more flexible and inert columns and the advances in detectors, particularly for capillary LC, contributed to the development of detection systems for CE. Several modes and mechanisms are available for the interested analyst, including: free solution capillary electrophoresis (FSCE) , also called capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MECC), capillary gel permeation electrophoresis (CGPE) and capillary isotachophoresis (CIP), to mention only some. It will still take some time before most of these techniques become part of the routine in laboratories outside the academic environment. Several problems contribute to this. Commercially available equipment is more expensive than an equivalent GC or HPLC and, excluding the laser fluorescence detector (LIF) - which is very specific and expensive – CE detectors are not as sensitive as the ones routinely used for GC and HPLC, as well as the need for use of fairly high voltage sources (30,000 volts or even more). Sample introduction reproducibility has been a problem for long time and migration time reproducibility is poor, when compared to LC and GC. As a result of these limitations, and in spite of its great potential, CE techniques have not yet found a real space outside that already covered by its lower voltage counterpart (conventional electrophoresis), which is in the analysis of biomolecules such as proteins, polycarbohydrates, DNA sequencing, and similar niches.

5.3. Qualitative analysis

After the isolation of the analytes of interest they should be properly identified in order to be later quantified. In most cases dealing with complex samples this is the most demanding task, since these samples may still have – even after all the steps already discussed – several analytes present in a wide concentration range (from very low to quite high concentrations). The first and most widely used identification procedure is to match the retention time of the analyte with the retention time of a "pure" analytical standard. This practice should not be overemphasized since it is susceptible to several errors, the most common being the fact that the retention time might be characteristic of a compound under certain analytical conditions but it is not unique; this mean that more than one compound may have the same retention time and lead us to an equivocal identification. There are several practical ways to improve this picture a little bit, including the use of "spiking" techniques; relative retention and retention index systems.[37] However, since they all use retention time they all suffer from the same problems. Since retention times are more difficult to reproduce in LC (and SFC and CE) than in GC, the problems associated with this approach are even more critical with such techniques. Another way to improve this situation is the use of more selective detectors such as the electron capture detector (ECD) in GC and the fluorescence detector in HPLC. In these cases some of the co-eluting peaks might not be detected by these more specific detectors, even when eluted together with the target analyte. Spectrophotometric detectors, such the Photodiode Array Detector (PDA) and Scanning Fluorescence Detectors (SFD) used in HPLC, also supply the spectra of the compound, thus providing an additional way to confirm the peak identity.

By far the most important and reliable identification system for chromatographic and electrophoretic peaks at the moment is the group of techniques called Mass Spectrometry (MS). When coupled with a chromatograph or an electrophoresis system these detection systems generate two dimensional data, one being the chromatographic (electrophoretic) information and the other the mass spectrum of the analyte. Combined, this approach will successfully identify the compound of interest in most situations.

Figure 5 displays a general drawing of a typical Mass Spectrometer. As can be seen, the first step consists in the sample introduction that, in our case, will be done though the chromatograph; the sample is ionized in the ionization source and the ions produced will be separated in the analyzer section and sent to a detector. The signal reaching the detector will be stored under the control of a computer that can be opened later for various data manipulations, to prepare standard or customized reports. The most popular MS systems in use nowadays are referred to by their type of analyzer: quadrupole, ion trap and time of flight mass spectrometers. All these systems are capable of operating in different MS modes, such as full scan, single ion monitoring (SIM) and extracted ion mode, and generate reconstructed chromatograms and mass fragmentograms. Together with the chromatographic information and with the addition of computer matching techniques using a previously prepared "library", the MS data will, in most cases, provide a positive identification for many target compounds and will be a valuable tool in the identification of unknown compounds. Other spectroscopic techniques, including Fourier Transform Infrared (FT-IR) and Nuclear Magnetic Resonance (NMR) are becoming more popular as detection systems for chromatography. The recent introduction of commercial LC/NMR equipment has increased the expectation that other new hybrid instruments will be available very soon.

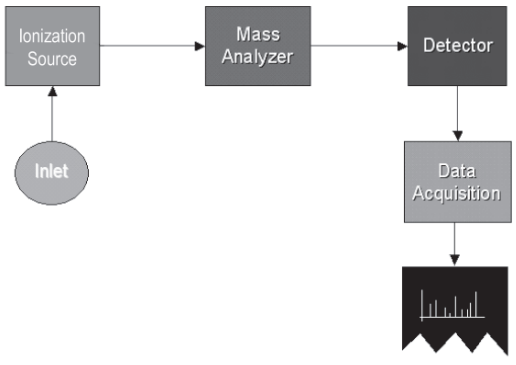

Figure 5. Schematic diagram of a Mass Spectrometer.

5.4. Quantitative analysis

Once the compounds are properly separated and identified using the procedures just described, quantitative analysis is often the simplest step of the analytical methodology, since the advent of data systems has made this step much easier than when the use of electronic integrators or manual calculations were required. Among the several procedures for quantitative analysis, the standard ones involving external standard and internal standard methodologies are still the most used. Depending upon the sample complexity, analyte structure and other factors, such as the requirements of the different regulatory agencies (in general environmental agencies suggest the use of the external standard procedure while public health agencies demand the use of the internal standard procedure, particularly for generic drugs) a particular quantitative procedure has to be selected. Anyway they are very well established now and a good chromatographic separation will make the quantitation step very easy.

6. Future Trends in the Separation Sciences

Considering the difficulty of the analytical problems to be solved and the complexity of the matrices to be studied (Proteomics is an example) the Separation Sciences will continue to have a bright future and a special role in Analytical Chemistry in this new Century. In this section future trends in the Separation Sciences are addressed from the author's point of view.

6.1. Miniaturization

A recent trend, already observed at the end of the 20th Century, was a decrease in the size of both analytical instrumentation and its accessories. It has to be understood that this miniaturization is not only related to saving space - although this will be an extra benefit. In fact, miniaturization can bring several advantages; in liquid chromatography the decrease in the column diameter and length will decrease the mobile phase flow rate and, as a result, its consumption. As a consequence, less solvent (usually toxic organic solvents such as acetonitrile are used) will be used and discarded, representing an important savings and also exposing the analyst to lesser quantities of hazardous solvents. In addition, the miniaturization of LC columns leads to an easier coupling with other instruments such as MS and NMR, expanding the possibilities of the technique. The use of lower flow rates also improves detection when using concentration-dependent detectors (such as UV-VIS) since the dilution of the analyte in the mobile phase is lower.

Figure 6 illustrates an in-scale comparison among the different columns used in GC in the last 50 years, going from the traditional packed columns, with an internal diameter of at least 4 mm, to the latest commercially available 0.1mm id open tubular columns. As can be seen, a tremendous decrease in column id (and, as a consequence, in the mobile phase, sample, and so on) is noticed. A similar situation is also found in the miniaturization of HPLC columns (Figure 7): from the traditional 4.0 mm id columns (still widely used) to open tubular capillary LC columns. Although receiving more benefits than in the case of GC,

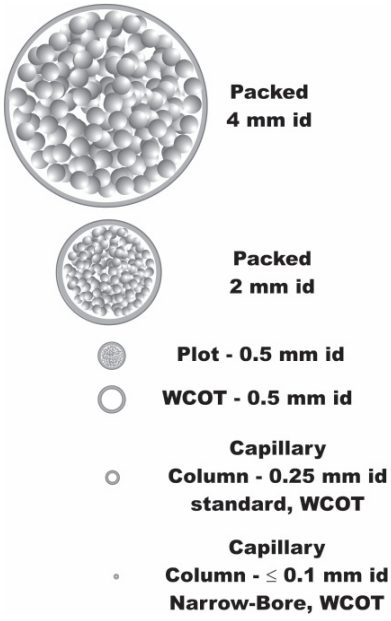

Figure 6. Comparison among GC columns (in scale)

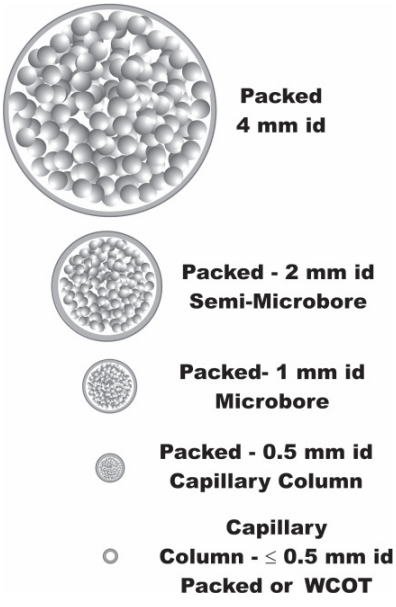

Figure 7. Comparison among HPLC columns (in scale)

HPLC miniaturization has been much slower and has not yet achieved an equivalent maturity. It is well established now that, when this occurs, several additional benefits will arise, such as a better and easier coupling to MS, similar to what now occurs with HRGC.

In terms of instrumentation, a recent trend, which might expand in the next decades, is the use of microchips for producing most analytical devices, including GC, LC and CE instruments.[38-40] These new instruments (in some cases they reach the size of a coin) will certainly be very attractive in cases where space must be saved, such as in interplanetary probes, field laboratories and similar applications. However the systems available at the moment are not yet adequate to handle highly demanding analytical problems where high efficiency is required.

The author believes that it would have been much more interesting at the moment to have had an intermediate solution - such as the one faced during the miniaturization GC and LC columns - while "lab-on-a-chip" technology is being fully developed and its benefits can be well proven. Meanwhile, there is still room for intermediate miniaturizations in column dimensions, oven size and heating/cooling speed, injectors – particularly GC injectors for low id capillary columns - and detectors. In HPLC, open tubular capillary LC columns still have to be fully developed and implemented in commercially available instruments. These modifications will bring a tremendous improvement in the existing chromatographic systems without demanding too many technology changes, as will be required for microchip production and application.

6.2. Neither temperature, nor pressure: electrical field

While GC uses the column temperature to selectively elute the compounds and HPLC uses a pressure gradient for the same purpose, there is a group of techniques, named electro driven separation techniques, that uses an electrical field to obtain similar results. They are also called electrophoretic techniques, although in, some cases, chromatographic principles and instrumentation might be also involved. Among these techniques, two are gaining popularity outside the area of bioanalytical chemistry: Micellar Electrokinetic Capillary Chromatography (MECC) and Capillary Electrochromatography (CEC). As the names of these techniques already imply they are both hybrid techniques that use aspects of chromatography as well as aspects of electrophoresis. MECC is a technique that uses an experimental set up similar to capillary zone electrophoresis (CZE), but includes in the buffer a compound that has the capability to form micelles under certain conditions. These micelles will selectively retain certain compounds

over others, thus promoting the desired separation (Figure 8). Since the micelles act as a "pseudo stationary phase", this technique has usually been referred to as a chromatographic technique, although many researchers do not agree with this classification. Another technique is electrochromatography, which also uses the instrumental set up of capillary electrophoresis while the separation is done in either a packed or an open tubular capillary column. In this case the separation is similar to the one obtained using capillary LC, but employs voltage instead of pressure to move the mobile phase. Figure 9 despicts a schematic drawing showing a packed and an open tubular CEC column. A major advantage of this technique is that it uses the selectivity of the LC columns allied to the high efficiency of CE columns, thus resulting in much more efficient separations using CEC columns.[41,42] This fact should increase the popularity of CEC and capillary LC columns over pure electrophoretic techniques in applications outside the scope of bioanalytical chemistry.

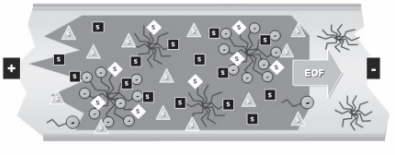

Figure 8. Schematic drawing of a MECC column emphasizing micelle formation inside the column. The micelles will show preference to retain some analytes while not retaining (or retaining less) the others, according to their relative affinities represented here by different shapes.

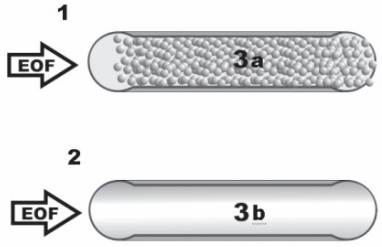

Figure 9. Capillary Electrocromatography (CEC) Columns. 1. Packed CEC column, 2. Open Tubular CEC column, 3a. Packing Material, 3b. Wall Coating.

6.3. Hyphenated systems

Another way to improve a difficult separation is to couple analytical techniques that provide complimentary selectivity to each other. Since the representation of these coupled techniques is through a hyphen they have been called hyphenated techniques. This may involve similar techniques, such as LC coupled to LC (LC-LC) or dissimilar techniques, such as SFE coupled to CE (SFE-CE). In the first case, two HPLC columns employing different separation mechanisms are used (as an example the first column could be an RP-18 and the second a chiral one) to improve the selectivity of the separation and to eliminate interfering compounds; the other example uses an SFE system to on-line extract and transfer the analyte from the original sample to a CE vial for analysis. Recent improvements in hyphenating HRGC columns has permitted the arrival of Comprehensive Multidimensional Gas Chromatography,[43] a technique that has found a place in liquid fuel analyses. Descriptions of hyphenated systems have been published,[44-46] including SFE-SPE-HRGC, LC-GC, SPME-GC-MS, SFE-SFC, SFE-CE and so on. Considering the high selectivity and efficiency of such systems we can envisage that hyphenated techniques will definitively have an important role in analytical separations in this new century.

6.4. The role of the solvent in separation sciences

Although the solvent plays an important role in extraction and separation techniques, only recently has this started to be better investigated. Until the middle of the past century, most analytical separations were restricted to the use of liquid solvents, with eventual use of a gas. As soon as the use of supercritical fluids, particularly CO_2, became more popular, this situation was expanded to also include supercritical fluids (SF) as analytical solvents. However, in between a gas and a liquid there are states other than SFs that might be useful for analytical applications (Figure 10). As can be seen, there are several other possibilities in terms of solvents still underexplored, waiting for more research. A still even less explored and understood situation is that involving a phase transition

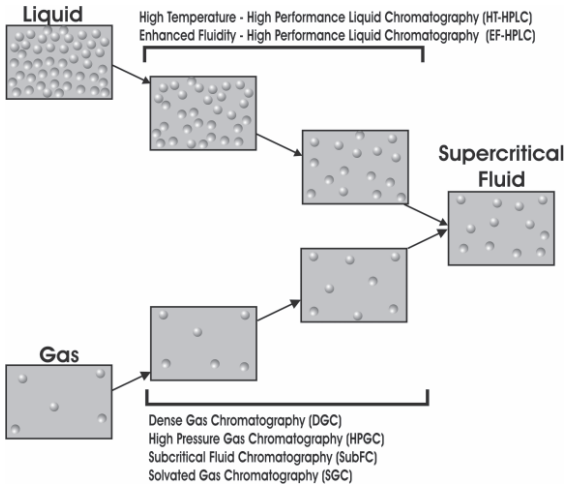

Figure 10. The role of the solvent in the separation sciences, with emphasis on various intermediate chromatographic techniques

during the separation, as is the case when the analysis starts with the mobile phase in the supercritical state, which is later changed to a gas near the end of the column. The use of this technique, named Transition Phase Chromatography (TPC) presents several advantages over GC, including the fact that it can elute higher molar mass compounds, since it starts in the SFC mode, but still uses highly efficient capillary columns. This technique is very new and is still in the developmental stage although several advantages can already be perceived.[47,48]

6.5. High speed separation techniques

The increasing demand for faster analyses aiming to enlarge laboratory productivity has resulted in a tremendous development in the speed of analyses, particularly those involving chromatographic techniques. The miniaturization of GC columns has allowed the use of 50 μm (or even less) id open tubular columns of short length (one meter or less) coated with a very thin film of a stable polymer. With these conditions very stable and fast columns are produced that can rival the standard 25 m x 0.25 mm id columns in terms of efficiency but result in much shorter analysis times, typically seconds instead of minutes.[49] One drawback in using these columns is that the injectors of most commercially available GCs are not prepared to handle the minute quantity of sample required for this separation technique in order to avoid column overload. More research has to be done in the field of sample introduction, in order to make these miniaturized columns a practical tool for the analytical separation of real samples. In LC, the separation speed follows a similar trajectory in the direction of very fast separations.[50] This has usually been achieved with the miniaturization of the stationary phase particle diameter (1.5 μm or less, instead of 5-10 μm) and of the column length (a few centimeters instead of the standard 15-25 cm). As a result, very fast separations of simple samples have been obtained in a few seconds instead of the minutes usually required. In this case, the appropriate instrumentation has to be used and particular attention has to be paid to the detector response time and the data system, otherwise the very fast peaks eluting might not be properly integrated by the data system.

6.6. Automation of the separation techniques

Another important improvement recently introduced in the separation sciences is automation capability, particularly in the sample extraction and concentration steps.[51] Several options are now commercially available to fully automate the analysis from sample extraction to the quantitation step. As an example, Figure 11 shows a fully automated approach to a complex analytical problem: the analysis of organic micropollutants in river water. The sample is loaded into the auto sampler and a robotic arm will transfer an aliquot of the sample to the solid phase extraction system. The analytes are concentrated in the cartridge and later automatically desorbed using an appropriate solvent. A representative portion of the extract is then transferred to the GC-MS system through an automated injection system (ATAS), which allows the injection of large volumes into the GC system (typically 100-200 microliters). The MS spectrum of each compound is acquired and the quantitative analysis is automatically done as described in the analytical protocol. This system is a fully automated on-line SPE-LVI-GC-MS system whose full potential is still to be explored in other areas, such as biological fluid analyses, petrochemical and pharmaceutical analyses, etc.

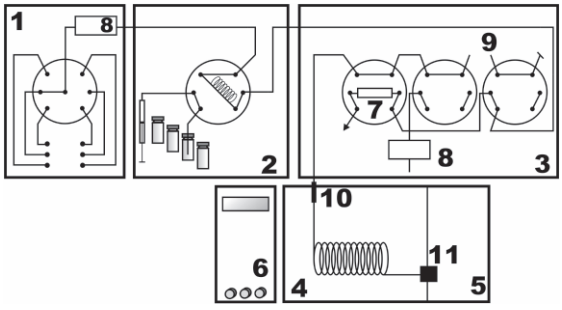

Figure 11. A fully automated analytical set up for the analysis of complex samples by on-line coupling Solid-Phase Extraction/Gas Chromatography with a Large Volume Injector and a Mass Spectrometer: SPE-LVI-GC-MS. 1. Solvent Delivery Unit, 2. Autosampler (MIDAS), 3. Automated SPE Unit (PROSPEKT) 4. GC, 5. MS, 6. Large Volume Injector (OPTIC), 7. SPE cartridge, 8. Pump, 9. Nitrogen gas, 10. PTV Injector, 11. Interface.

6.7. Unified chromatography

Due to several factors, including the commercial interests of some companies, the family of chromatographic techniques had its development as completely different (and in some cases competitive) instruments. However, as pointed out by Giddings several years ago[52] the division of chromatography into these several techniques is artificial and unproductive. Giddings also postulated that gas, liquid and supercritical fluid chromatography are just different ways to present the same technique due the particularities of the mobile phase. The first group to demonstrate in practice that GC, LC and SFC are complimentary and could be performed in a single instrument was led by Ishii.[52] The practical development of this idea has been slow, mainly

due to the difficulties in producing a single column that will efficiently operate using a gas, a liquid or a supercritical fluid as mobile phase. This has recently been investigated, based on highly efficient capillary columns developed for Transition Phase Chromatography using a supercritical fluid as the packing medium. In order to quickly change from one eluent to another (gas to liquid or supercritical fluid) fast valves driven by in-house developed software have proven to be appropriate. Figure 12 shows a schematic drawing of an apparatus for unified chromatography being developed in our laboratory. As can be seen, the instrument may operate using gases, liquids or supercritical fluids as mobile phases, also being able to run transition phase chromatography. More applications have yet to be developed, in order to seduce more researchers to further investigate the tremendous potential of this approach.

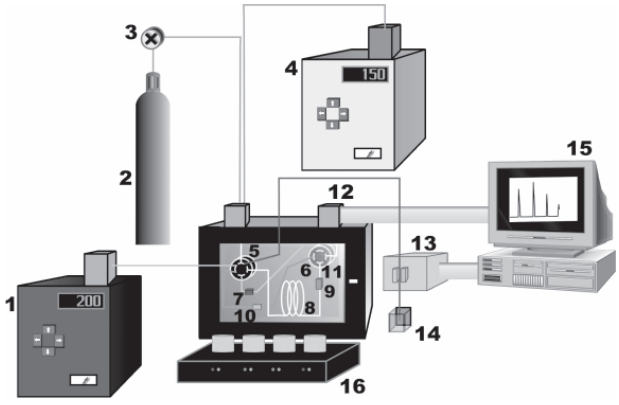

Figure 12. General view of a Unified Chromatographic Apparatus. 1. High Pressure Pump for Supercritical Fluid Delivery; 2. Gas Cylinder. 3. Pressure Control Valve; 4. HPLC Pump; 5. Selection Valve; 6. Restrictor Selection Valve; 7. Connector; 8. Column; 9. Restrictor; 10. Oven; 11. Retention Gap; 12. GC Detector; 13. LC Detector; 14. Sample Waste; 15. Data System; 16. Computerized Valve Controller System.

Conclusions

In this paper the latest significant developments in the Separation Sciences have been introduced and critically discussed. All major steps involved in a typical analytical determination of analytes present in complex samples were described. Emphasis instrumental extraction and clean up techniques that minimize or eliminate the use of toxic solvents was stressed. After this balance, the author projected, from his point of view and upon examining the related literature, the future trends in this important area of modern analytical chemistry. Miniaturization of the separation sciences in general, and the chromatographic and electrophoretic techniques in particular, will bring tremendous benefits over the present situation. This is not only a question of saving bench space but such miniaturization will also allow us to use less of the toxic solvents, with all their consequences, permit easier coupling with other analytical techniques, such as MS, NMR, and others, and the development of transition phase chromatography, hyphenated techniques and multidimensional chromatography. Miniaturization of analytical instrumentation will facilitate the development of fully automated systems that may combine extraction, clean-up, separation (GC or LC) and identification (MS) in one set up, as described herein.

Finally, based upon all these developments, we will finally be able to see the dream of the pioneers in this field become a reality: the development of unified (or universal) chromatography. This will allow us to work with only one instrument, independent of the mobile phase to be used and sample (matrix/analyte) characteristics. Also, this system will be compatible with sequential analyses (first GC, then SFC and finally HPLC from a single injection, as an example of a possible set up) by just changing the eluent characteristics. This will represent a tremendous savings in instrument and labor and will make the chromatographic techniques even more popular than they already are.

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