CHAPTER 1

INTRODUCTION

1.1. NANOANALYSES

Separation science is the backbone of biological, biotechnological, chemical, pharmaceutical, environmental, geochemical, and agricultural disciplines and, hence, analysis is an integral part of these fields [1,2]. It is a well-known fact that almost all drugs work at low concentrations in biological systems and to carry out pharmacokinetic and pharmacodynamic studies analytical techniques should be capable of detecting drugs and pharmaceuticals at nano or lower detection limits. In spite of the curative properties of drugs, some drugs also have side effects even at low concentrations, that is, ranging from nanogram to femtogram levels [3,4]. Therefore, in the absence of techniques capable of detecting at the nanolevel we assume the absence of drug residues in the body, while these can have some bioreactions and side effects. Besides, the concentrations of some species, such as hormones, RNA, DNA, antibodies, and other proteins are very low, and require analytical techniques with low detection limits. In addition, detection of drugs at a lower concentration is required in the plasma of infants because of the availability of only limited amounts of blood samples. In addition, for some biological fluids, such as cerebrospinal fluids, only small volumes are available for sampling. Besides, high throughput screening (HTS) and drug discovery (combinatorial

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chemistry) need low level analyses. Moreover, recent advancements in proteomics and genomics compel scientists to develop nanoanalytical techniques.

Similarly, many xenobiotics, such as pesticides, polynuclear aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), plasticizers, phenols, and some other drug residues, are also toxic even at trace levels present in the earth's ecosystem [5–7]. Without analytical techniques capable of detecting them at nanolevels, we assume the absence of these pollutants in the environment, while these notorious pollutants accumulate in our body tissues resulting in various diseases and side effects such as carcinogenesis and failure of many vital body organs including the kidney, liver, and heart [8–11]. Under such situations, it is essential to have analytical techniques that can detect drugs, pharmaceuticals, and xenobiotics in biological and environmental samples at very low concentrations.

Apart from the above requirements of nanoanalyses, the need of detection at the nanolevel is also increasing continuously in dosage formulations, food products, and other chemical and biotechnology industries. Briefly, nowadays, nanoanalysis is becoming more important and scientists and regulatory authorities are asking for data on detection at the nanogram level. Among various analytical techniques, chromatography and capillary electrophoresis are the choice of scientists, academicians, and clinicians as these techniques can analyze samples of low volume or having poor ingredient concentrations. Some attempts have been made to develop nanochromatography and nanocapillary electrophoresis, which some workers call the micrototal-analysis system (μ-TAS). In this book we have replaced μ-TAS with the phrase nanoanalysis as the techniques are capable of dealing with nano amounts of samples, with detection at the nanogram level.

1.2. DEFINITION OF NANOCHROMATOGRAPHY AND NANOCAPILLARY ELECTROPHORESIS

Karlsson and Novotny [12] introduced the concept of nanoliquid chromatography in 1988. The authors reported that the separation efficiency of slurry packed liquid chromatography microcolumns (44 μm, id) was very high. Since then, many advance have been reported in this modality of chromatography and it has been used as a complementary and/or competitive separation method to conventional chromatography. Unfortunately, to date no correct and specific definition of this technique has been proposed, probably due to the use of varied column sizes (10 to 140 μm). Some definitions of nanoliquid chromatography are found in the literature based on column diameter and mobile

phase flow rates [13–15]. It has been reported that when the chromatographic separation is carried out in capillary columns of 10 to 100 μm internal diameter, the modality is called nanoliquid chromatography, whereas when capillaries of 100 to 500 μm internal diameter are used the technique is called capillary liquid chromatography [16]. On the other hand, some workers define nanoliquid chromatography as the chromatographic modality having a mobile flow rate of nanomilliliters per minute. However, no one has considered the detection aspect of this type of chromatography, which is very important in analytical science.

Our intention is to achieve nanolevel detection irrespective of the experimental condition as detection is the final aim in separation science. Moreover, nanolevel detection is required for low amounts of sample or samples having poor ingredients. Therefore, if the technique is capable of detecting at low or nanolevels one can analyze low volume samples or samples having poor concentrations. Therefore, detection is the most important issue in nanochromatography. Based on these requirements and logics we have defined nanoliquid chromatography more accurately and scientifically. Keeping all these facts in mind, nanochromatography may be defined as "a modality of chromatography involving samples in nanoliters, mobile phase flow in nanomilliliters per minute, with detection at the nanogram per milliliter level." This definition is a complete one and all the requirements can be fulfilled on chip-based chromatography. Therefore, a true and complete nanochromatography is only possible on a chip, which is called lab-on-chip chromatography but we simply named it nanochromatograpy (NC) broadly. In the case of liquid chromatography it may be called nanoliquid chromatography and abbreviated as NLC. The same definition is also true and complete for capillary electrophoresis, which is also possible on chips, and we have termed it nanocapillary electrophoresis (NCE).

1.3. NANOCHROMATOGRAPHY AND NANOCAPILLARY ELECTROPHORESIS

Recently, the word *nano* has become a trend in science and technology and some of us think that it is the new generation but, as mentioned above, its root is about 22 years old. NLC and NCE are gaining importance day by day. They are very useful and effective tools for samples of low quantities or having low concentrations of the analytes. Columns of low internal diameter are ideal for use in NLC and NCE, especially with detectors requiring very low flow rates, such as electrospray liquid chromatography/mass spectroscopy (LC/MS). Besides, these columns offer high sensitivity due to their low

dispersion characteristics. The microfluidic systems are more or less capable of replacing conventional "macro" systems for many applications in the life sciences. The working principle of NLC and NCE are the same as in conventional LC and CE. However, miniaturization offers many advantages over the conventional methods, including:

- Usefulness and effectiveness for samples of low volume or having extremely low concentrations of ingredients.
- Significantly reducing solvent consumption and subsequent waste production.
- Inexpensive due to low consumption of solvent, electricity, and operational time.
- Good potential portability due to a system size reduction.
- High sensitivity, speed, and reproducibilities.
- Narrowing the peak width of chromatogram/electropherogram due to better separation efficiency.
- Low mobile phase pressure in NLC.
- Simultaneous mass separation on chips.
- Good hyphenation of detectors requiring mobile phase flow.

NCE is a relatively new development in separation science, especially in proteomics and genomics. In the last two decades NCE has gained increasing importance, as can be seen from a good number of publications [17–20]. In addition to the above advantages, NCE is a suitable technique for samples that may be difficult to separate by NLC as the principles of separation are entirely different. Lower detection limits of NCE lead to the possibility of separating and characterizing small quantities of materials. Moreover, the enzymatic reactions for analytical purposes can be conducted within the capillary.

1.4. FABRICATION OF MICRODEVICES

The fabrication of microdevices in NLC and NCE is controlled by highly developed batch-processing techniques of integrated circuits. The microelectronic technology can be exploited for microflow systems with functions different from those of integrated circuits. Special processes and materials are needed, called micromachining techniques [21]. Following developments in miniaturization and integration of electronic devices, the potential of a similar revolution also emerged for mechanical and later on fluidic devices, which

led to MEMS (microelectromechanical systems) and μ-TAS (micrototal analysis systems) [22]. μ-TAS is one of the fastest growing areas covering microfluidics, material science, analytical chemistry, and biotechnology. Microfluidics refers to the science and technology dealing with minute amounts of fluids (micro-, nano-, and picoliters). Lab-on-a-chip is a miniaturization and integration of complete functionality of a chemistry or biology lab, for example, preparation, reactions, separation, and detection, onto a single chip. μ-TAS is a development involving reduced size, low power, sample, reagent and manufacture requirements and operating costs. Besides, μ-TAS can perform better services in terms of speed, throughput, mass sensitivity, and automation.

Microfluidics is the key to NLC and NCE, the miniaturized microfluidic system that can automatically carry out all the necessary functions to transform chemical information into electronic information. The first μ-TAS device was developed by Terry et al. [23] for gas chromatography, which did not gain popularity at that time, probably due to poorly developed microfluidic devices. In 1990, Manz et al. [24] introduced the concept of μ-TAS. Nowadays, μ-TAS is a popular development in various disciplines and has been reviewed by Manz and coworkers [25–28].

The development era of microdevices was from 1970 to 1980 in the silicon microprocessors industry. Interested readers can consult textbooks on this subject [29–31]. Kim et al. [32] presented a molding protocol for patterning network channels by contacting a substrate and a patterned elastomeric master. Later on Mrksich and Whitesides [33] used microcontact printing to pattern structures of self-assembled monolayers (SAMs) on the submicrometer scale. In 1996, Zhao et al. [34] used microtransfer molding for fast fabrication of organic polymers and ceramics in three dimensions using a layer-by-layer structuring. Park and Madou [35] designed a three-dimensional electrode useful for a high throughput dielectrophoretic separation/concentration/filtration system. Clicq et al. [36] used reversed phase chromatography on a rectangular glass chip coated with C_8 silica gel. Many other attempts have been made by various workers toward fabrication of microdevices [37–42]. The design of microdevices is a very important issue, especially in NLC and NCE. Bousse et al. [43] reported a microfabricated electrokinetic device having loading and separation channels. Manz and Becker [44] developed a design in NCE useful for effective working of the system. The introduction of stationary phases in NLC is quite difficult; however, some workers have attempted this [45–48]. The stationary phases were introduced into a microchannel by coating of the inner surface of the capillary or by packing the channels or in situ polymerization of continuous beds. Some microfluidic-NLC chips have been commercialized by a few manufacturers.

1.5. DEVELOPMENTS IN NANOANALYSES

Karlsson and Novotny [12] introduced the nanoliquid chromatography (nano-LC) concept and since then it has been proposed as a complementary and/or competitive separation method to conventional liquid chromatography. Nano detection is being achieved by modified LC systems but it is still in its development stage [49]. Generally, column internal diameters in the range of 25 to 100 μm are considered under nanochromatographic systems. But the miniaturization and integration of electronic devices led to the development of microelectromechanical systems (MEMS) and micrototal analysis systems (μ-TAS), which are components of chip-based analytical machines as MEMS devices are faster, selective, sensitive, and economic in nature. μ-TAS is a fast-growing area in separation science. Of course, lab-on-chip systems are not yet fully developed but demand for them is increasing due to their economic, fast, and portable capabilities. A complete analysis can be carried out in seconds by using 1 to 5 mL of solvent. Moreover, the machines are very compact and can be carried to sites for environmental analyses.

Some important developments and advances in NLC and NCE are described in papers by Manz and coworkers [25–28]. These authors [50] presented a miniaturized open-tubular liquid chromatograph on a silicon wafer with a 5 × 5 mm silicon chip containing a conductometric detector, connected to an off-chip conventional LC pump and valves to perform high-pressure liquid chromatography. Manz et al. [24] proposed a μ-TAS concept in which silicon chip analyzers having sample pretreatment, separation, and detection played a fundamental role. In the early stage the intention of miniaturization was to increase analytical performance of the device rather than reduction in size. It was also realized that miniaturization provided the advantages of a smaller consumption of reagent and time. Moreover, μ-TAS provided an integration of separation techniques, as it is capable of performing sample handling, analysis, and detection on a single chip. Later, Jacobson et al. [51] used a microchip of fused quartz to separate complexed metal ions in polyacrylamide-modified channels. In the same year Ocvirk et al. [45] developed NLC with a split injector, a packed small-bore column, a frit, and an optical detector cell onto a silicon chip. Moore et al. [52] reported chip-based micellar electrokinetic capillary chromatography (MECC) of neutral dyes. Similarly, von Heeren et al. [53] analyzed biological samples on MECC fabricated onto a chip. Penrose et al. [54] reported a centrifugal chromatograph for reversed-phase separations. Recently, Zeng et al. [55] reported the chiral separation of amino acids on polydimethylsiloxane (PDMS) chips.

Chip-based technology is successful in NCE over NLC due to difficulties of integrating on chip pumps, injectors, mechanical valves, and the lack of easy

flow control [56]. Besides, it is also difficult to install a stationary phase in NLC and sealing of the microchannels, which should be perfect for the mobile phase to flow appropriately through the stationary phase. In 1992, Manz et al. [57,58] introduced the first NCE integrated silicon and glass chips. The authors described the concept of μ-TAS in NCE by integrating injection, separation, and detection on the chip. Furthermore, some advancement has been reported in this direction by the same workers [59–62]. Woolley et al. [63] developed capillary array electrophoresis (CAE) for the analysis of different DNA samples. Since then much work has been done in this area and some quite good papers are available, which have been considered for preparing this book. Other developments in NCE have been reported from time to time and can be used for the analyses of different compounds [64–83]. Another development in NCE was the enantiomeric resolution of amines on chip-based NCE by Cong and Hauser [84]. Reviews have been published describing various aspects of microdevices [25–28,85–91].

1.6. DATA INTEGRATION

As mentioned above, the basic principle of NLC is the same as for conventional techniques. The separation is identified and characterized by measuring retention times, capacity, separation, and resolution factors. Therefore, it is necessary to explain the chromatographic terms and symbols by which the chromatographic speciation can be understood and explained. Some of the important terms and equations of the chromatographic separations are discussed below. The chromatographic separations are characterized by retention (k), separation (α), and resolution factors (Rs). The values of these parameters can be calculated by the following standard equations [92].

$$k = (t_r - t_0)/t_0 \tag{1.1}$$

$$\alpha = k_1/k_2 \tag{1.2}$$

$$Rs = 2\,\Delta t_r/(w_1 + w_2) \tag{1.3}$$

where t_r and t_0 are the retention time of the separated species and dead time (solvent front) of the column in minutes/seconds, respectively. Δt, w_1, and w_2 are the difference in the retention times of two peaks of the separated species, the base width of peak 1 and peak 2, respectively. If individual values of α and Rs are 1 or greater the separation is supposed to be complete. If the individual values of these parameters are lower than 1 the separation is understood as partial or incomplete.

The number of a theoretical plate (N) characterizes the quality of a column/chip. The larger N is, the more complicated the sample mixture that can be separated with the column. The value of N can be calculated from the following equations:

$$N = 16(tr/w)^2 \tag{1.4}$$

or

$$N = 5.54[(tr)/w_{1/2}]^2 \tag{1.5}$$

where t_r, w, and $w_{1/2}$ are the retention times in minutes or seconds of the peak, peak width at the base, and at half the height of the peak, respectively. The height equivalent to a theoretical plate [HETP (h)] is a section of a column or chip in which the mobile and the stationary phases are in equilibrium. Since a large number of theoretical plates are desired, h should be as small as possible. Naturally, there are no real plates in a column or chip. The concept of a theoretical plate is a variable and its value depends on particle size, flow velocity, mobile phase (viscosity), and especially on the quality of the packing h can be calculated from the following equation:

$$h = L/N \tag{1.6}$$

where L is the length of the column used.

The mechanism of separation in NCE is based on the difference in the electrophoretic mobility of the separated species. Under NCE conditions, the migration of the separated species is controlled by the sum of the intrinsic electrophoretic mobility (μ_{ep}) and the electroosmotic mobility (μ_{eo}), due to the action of electroosmotic flow (EOF). The observed mobility (μ_{obs}) of the species is related to μ_{eo} and μ_{ep} by the following equation:

$$\mu_{obs} = E(\mu_{eo} + \mu_{ep}) \tag{1.7}$$

where E is the applied voltage (kV).

The simplest way to characterize the separation of two components, the resolution factor (Rs), is to divide the difference in the migration times by the average peak width as follows:

$$Rs = 2(t_2 - t_1)/(w_1 + w_2) \tag{1.8}$$

where t_1, t_2, w_1, and w_2 are migration times of peak 1 and peak 2, and the widths of peak 1 and peak 2, respectively.

The value of the separation factor may be correlated with μ_{app} and μ_{ave} by the following equation:

$$Rs = (1/4)(\Delta\mu_{app}/\mu_{ave})N^{1/2} \tag{1.9}$$

where μ_{app} is the apparent mobility of two separated species and μ_{ave} is the average mobility of the separated moieties. Using Equation 1.9 permits independent assessment of two factors that affect separation, selectivity and efficiency. The selectivity is reflected in mobility of the analytes while the efficiency of the separation process is indicated by N. Another expression for N is derived from the following equation:

$$N = 5.54(L/w_{1/2})^2 \tag{1.10}$$

where L and $w_{1/2}$ are the capillary length and peak width at half height, respectively. Here it is important to point out that it is misleading to discuss theoretical plates in NCE and it is simply a carryover from chromatographic theory. The theoretical plate in NCE is merely a convenient concept to describe analyte peak shape and to assess the factors that affect separation.

HETP may be considered as the function of the capillary occupied by the analyte and more practical to measure separation efficiency in comparison to N. σ^2_{tot} is affected not only by diffusion but also by differences in the mobilities, heating of the capillary in joules, and interaction of the analytes with the capillary wall, and hence σ^2_{tot} can be represented as shown in Equation 1.11.

$$\sigma^2_{tot} = \sigma^2_{diff} + \sigma^2_{\mathrm{T}} + \sigma^2_{int} + \sigma^2_{wall} + \sigma^2_{Electos} + \sigma^2_{Electmig} + \sigma^2_{Sorp} + \sigma^2_{Oth} \tag{1.11}$$

where the values represent the square roots of the standard deviations of total, diffusion, heat, injection, wall, electroosmosis, electromigration, sorption, and other phenomena, respectively.

Practically, velocity (v) and observed mobility (μ_{obs}) of the analyte, electrophoretic mobility (μ_{ep}), and electroosmotic mobility (μ_{eo}) can be calculated by the following equations.

$$v = \mu_{obs}E \tag{1.12}$$

where E is the strength of the electric field in V/cm.

E is described by the following equation:

$$E = V/Lt \tag{1.13}$$

where Lt is the total length of the capillary or channel.

By putting the value of Equation 1.13 into Equation 1.12

$$v = (\mu_{obs} V)/Lt$$

or

$$\mu_{obs} = (vLt)/V \tag{1.14}$$

We know that the velocity (v) of the analyte equals distance divided by time and, hence, velocity in NCE may be written by the following equation:

$$v = Ld/td \quad (\text{cm sec}^{-1}) \tag{1.15}$$

where *Ld* and *td* are the distance of the capillary or channel up to the detector point and the time of migration of the analyte up to the detector, respectively.

By putting the value of v from Equation 1.15 into Equation 1.14

$$\mu_{obs} = (Ld/td) \times (Lt/V) \tag{1.16}$$

or

$$\mu_{ep} + \mu_{eo} = (Ld/td) \times (Lt/V) \tag{1.17}$$

For benzene the value of μ_{ep} is zero and hence:

$$\mu_{eo} = Ld/td \times Lt/V \quad (\text{cm}^2\text{V}^{-1}\text{sec}^{-1}) \tag{1.18}$$

Equation 1.17 may be written in the following form:

$$\mu_{ep} = Ld/td \times Lt/V - \mu_{eo} \quad (\text{cm}^2\text{V}^{-1}\text{sec}^{-1}) \tag{1.19}$$

By using all these equations, we can calculate all parameters of NCE required in any analysis.

1.7. PROTOCOL OF NANOANALYSES

Of course, conventional chromatographic and capillary electrophoretic methods are popular for the analyses of drugs, pharmaceuticals, and pollutants in biological and environmental matrices. In the last few decades, new developments in nanoanalyses have emerged in the literature due to their high separation efficacy, speed, inexpensive running cost, and small volume requirement, which are major

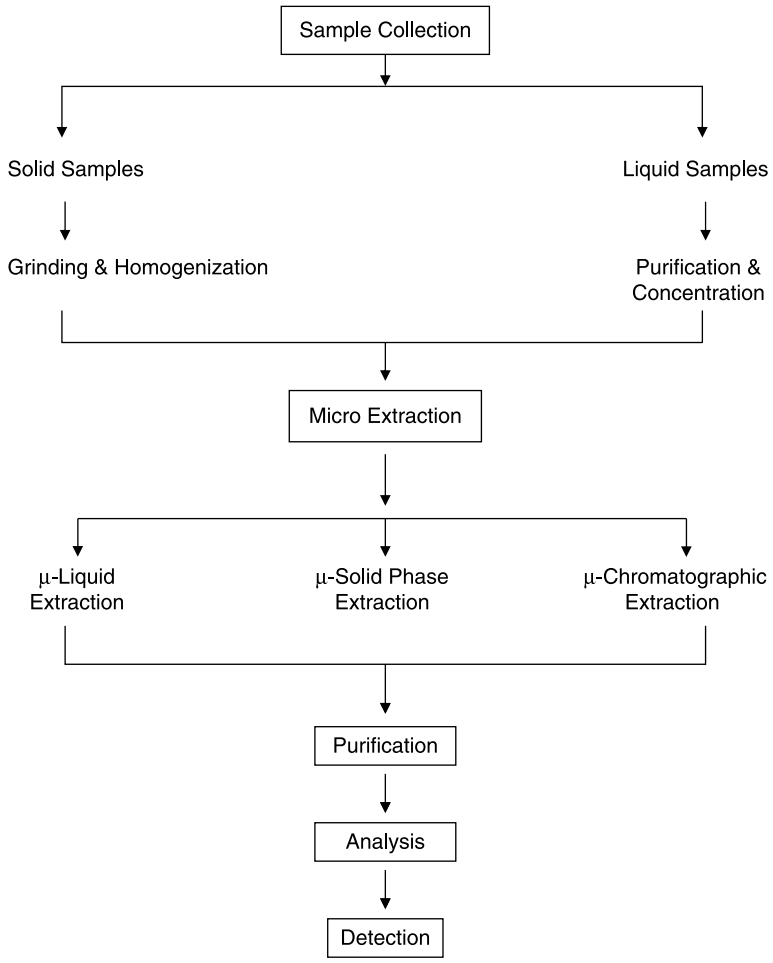

Figure 1.1 Protocol for analyses at nanolevels.

trends in analytical science. The number of publications in separation science using this development have increased. Use of these techniques is difficult because it involves micro or nano amounts of samples. A well-trained operator is required for handling NLC and NCE. A good protocol is always useful to work with these modalities of chromatography and electrophoresis. The working protocol for NCL and NCE is shown in Fig. 1.1 and a careful perusal of this figure indicates that sample preparation is a very important issue in these modalities, especially in unknown matrices. Our experience dictates that online sample preparation devices are required to avoid any error of methods due to small sample quantities. Optimization and other issues related to nanoanalyses are discussed in later chapters of the book.

1.8. SCOPE OF THE BOOK

This book explains the importance and applications of nanoanalytical techniques with special emphasis on separation of drugs, pharmaceuticals, and xenobiotics in biological and environmental matrices. A thorough search of the literature was carried out and many papers are available on nanoanalyses. As per the definition of NLC and NCE, microchip-based techniques are true nanoanalytical methods but only a few papers are available using these modalities, which have been included in this book. Some publications are also available on chromatography and capillary electrophoresis with columns with internal diameters ranging from 25 to 100 μm and flow rates of 25 to 4,000 nL/minute. In addition to this, a few papers describe detection at the nanogram level by using conventional LC and CE methods. These papers describing separations on microbore columns and normal columns with detection at the nanogram per milliliter level have been included in this book to make it more useful for the reader. The intention behind this is to cover all the analyses dealing with nanolevel detection, which is the last destination of nano world separation science. Arbitrarily, publications having 0.5 ng/mL (500 ng/L) or lower as the detection limits for the analyte(s) were considered as nanoanalyses and included in this book. However, the main emphasis has been given to the instrumentation and analyses on chip-based NCE and NLC.

1.9. CONCLUSION

The development of microdevices led to a new area in separation science called μ-TAS or lab-on-chip technology. But we have given a new term, nanoanalysis, to this type of analysis. The chip-based microfluidic devices are well recognized and used in many areas of biological sciences [93]. This book discusses fabrication, instrumentation, detection, nanoliquid-chromatography (NLC), and nanocapillary electrophoresis (NCE) since these techniques deal with nano amounts of sample, flow rate, and detection as well. We hope that this text will be useful for graduate students, researchers (biological and environmental sciences), and professionals of pharmaceutical, agrochemical, and other chemical industries.

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