Chapter 1

Advances in Low Volume Sample Analysis Using Microfluidic Separation Techniques

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Additional information is available at the end of the chapter

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Abstract

During the last decades, a great interest has been shown for miniaturised separation techniques. The use of microfluidic techniques fulfills the constant needs for increasing sample throughput and analysis sensitivity, while reducing costs and sample volume consumption. In this chapter, three microfluidic separation techniques will be addressed: capillary electrophoresis, gas chromatography and liquid chromatography. A special attention will be paid to miniaturised liquid chromatography, with a deep investigation of its advantages compared with classical liquid chromatography. Sample preparation adapted to low volumes (a few μl) will also be discussed.

Keywords: Separation, miniaturisation, microfluidics, sensitivity

1. Introduction

Separation techniques are widely used for the analysis of biomolecules as well as small molecules in various fields, as genomics, proteomics or pharmaceutical sciences. Due to the wide range of separation techniques, numerous studies have been conducted aiming to improve performances in terms of sample preparation, sensitivity, cost or analysis throughput.

Liquid chromatography (LC) is the most employed separation technique, but alternative techniques such as capillary electrophoresis (CE) and gas chromatography (GC) are nevertheless helpful to provide orthogonal separation capabilities. Ultraviolet, electrochemical and fluorescence detection are used to detect the target compounds, but mass spectrometry (MS) detection offers enhanced sensitivity and additional structural information since co-eluting compounds are differentially detected according to their mass-to-charge ratio.

Miniaturisation is a general trend common to many areas in sciences and technology. Down-scaling the separation techniques has been initiated in the 1970s, but miniaturisation has mainly experienced an exponential growth since the 1990s. Reducing the size of the separation supports brings valuable advantages as analysis time reduction, increased sensitivity and low sample and reagent consumption. However, the limited loading capacity of microfluidic devices is a drawback. Adequate sample preparation, pre-concentration and appropriate device can circumvent these inherent limitations.

2. Microfluidic separation techniques

2.1. Capillary electrophoresis

2.1.1. Instrumentation

Capillary electrophoresis (CE) is a microscale analytical technique based on the separation of compounds according to their charge-to-size ratio. The first CE device was described by Hjertén in 1967 that performed the electrophoretic separations in narrow bore tubes of 300 μm inner diameter (i.d.) for the analysis of various analytes (inorganic ions, nucleotides, proteins) [1]. In 1981, Jorgenson and Lukacs demonstrated for the first time that capillaries with a smaller i.d. (75 μm) could provide high separation efficiency using high voltages (30 kV), due to the small capillary dimensions that allowed good dissipation of Joule heat produced by such a high voltage [2].

Most modern CE instruments are very simple: a high voltage power supply, an autosampler with injection system, a capillary (25–100 μm i.d. and more often 50–75 μm, 20–100 cm length) and a detector coupled to a computer for data acquisition (Figure 1A).

Briefly, a capillary made of fused silica coated with a layer of polyimide is filled with a background electrolyte solution. When an optical detection (commonly UV or fluorescence) is employed, two electrodes are placed in buffer reservoirs to provide the necessary electrical contact between the high voltage supply and the capillary. To perform an analysis, the sample is loaded into the capillary by applying either a pressure difference (hydrodynamic injection) or an electric field (electrokinetic injection) between both extremities of the capillary. Optical detection is performed through a detection window directly on the capillary.

The hyphenation of CE to MS was first presented in 1987 by Olivares et al. that proposed an interface between CE and MS with an electrospray ionisation source (ESI) [3]. Alternative ionisation method has been described for CE-MS [4], as continuous flow-fast atom bombardment ionisation (CF-FAB) [5], atmospheric pressure chemical ionisation (APCI) [6] or atmospheric pressure photochemical ionisation (APPI) [7]. The CE-MS coupling provides structural information, enhanced sensitivity and selectivity compared with an optical detection. The CE-MS instrument configuration is modified to allow the direct entrance of the analytes into the mass spectrometer (Figure 1B).

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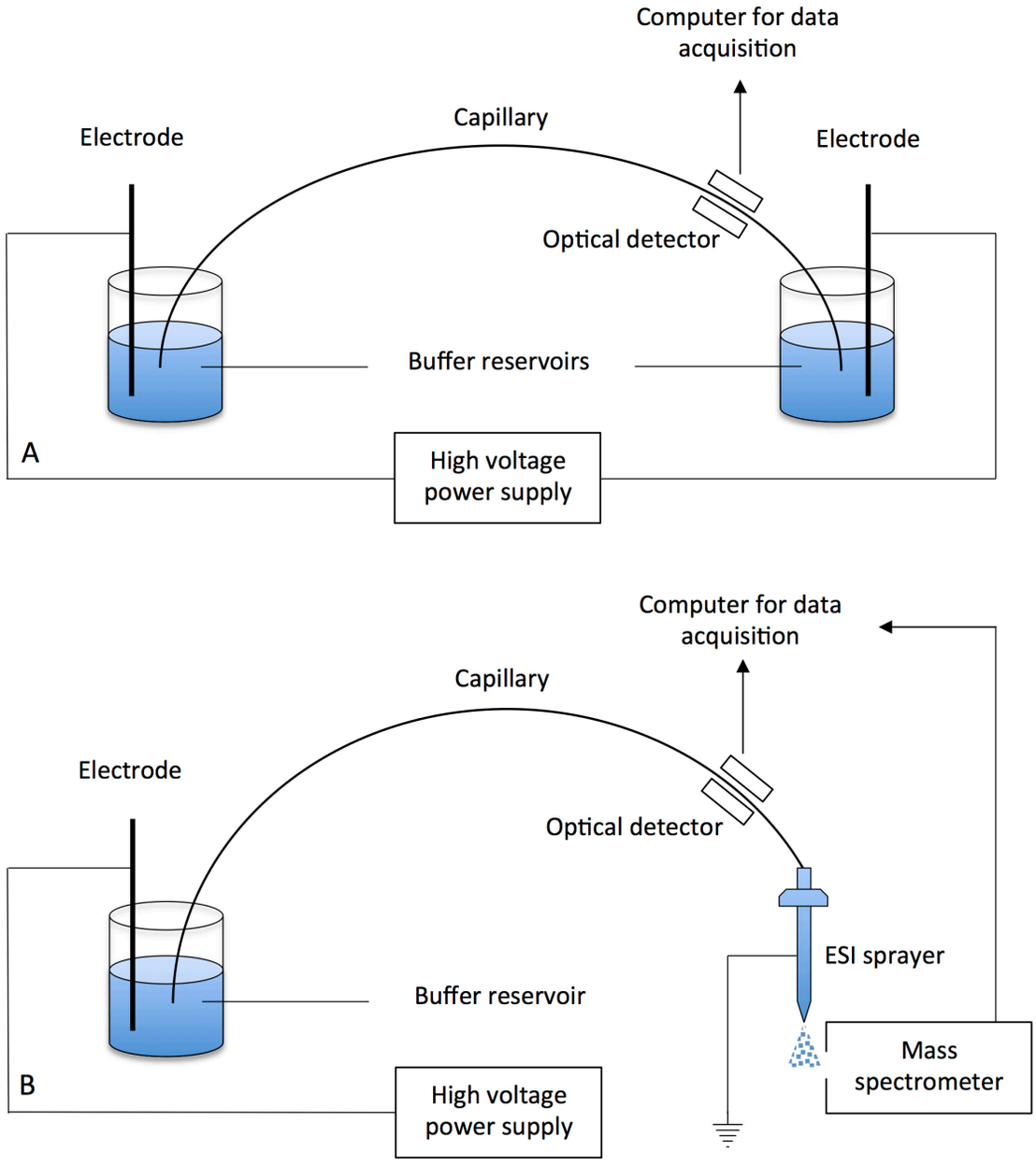

Figure 1. Schematic representation of a classical capillary electrophoresis system with an optical detection (A) and the coupling to a mass spectrometer (B).

The coupling of CE to MS can be achieved using a sheath liquid interface or a sheathless interface. The use of an additional liquid, the sheath liquid, to the electrophoretic effluent allows the formation of an electrical contact at the capillary MS output that is necessary for the electrophoretic separation, and enables the electrospray formation. In addition, the background electrolyte composition can be modified by dilution with the sheath liquid to ensure the compatibility with MS detection. However, the dilution process decreases the sensitivity in proportion with the sheath liquid flow rate.

Sheathless interfaces overcome the dilution-related sensitivity limitations encountered when using a sheath liquid interface. In this configuration, the electrical contact cannot be established through a liquid junction; the electrical contact may be established by many different techniques, e.g. by the insertion of the separation capillary into a conductive sprayer or the coating of the outlet end of the capillary by a conductive material.

2.1.2. Capillary electrophoresis on chip

In the light of the small dimensions of the separation capillary, classical CE is naturally classified into the category of miniaturised separation techniques. During the past few years, a new trend in CE instrumentation has emerged: the miniaturisation of CE into an integrated chip device for hyphenation to MS [12]. Since the introduction of the first chip-based electrophoresis device by Manz et al. [13], chip design has undergone continuous evolution from a single-channel design to more complex layouts integrating all the analytical steps on a single component. The actual classical chip design is made of two crossed microchannels, solution reservoirs for the sample and the waste, and reservoirs at the cathode and the anode for the buffer (Figure 2A) [14].

Interfacing a CE chip to an ESI-MS detector can be realised by spraying directly from the chip [15] or from a capillary sprayer attached to the chip [16] (Figure 2B).

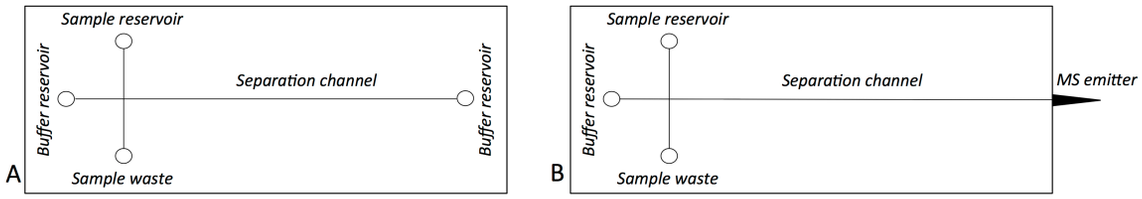

Figure 2. CE chip (A) and CE-MS (B) chip basic configuration.

In 1999 Agilent launched the Bioanalyser 2100, the first commercial microfluidics-based platform for DNA, RNA, protein and cell analysis. Separation is performed by capillary electrophoresis in channels containing a gel matrix. This device is the miniaturised counterpart of gel electrophoresis analysis (e.g. SDS-PAGE for proteins and agarose gel electrophoresis for nucleic acids) [17, 18]. In this device, sample with a volume between 1 and 6 μl moves through the loading channels, and a fraction of this volume is injected into the separation channel filled with a gel matrix. Fluorescence detection is performed on the chip itself. Total analysis time (including sample loading, separation, staining and destaining) is 30–40 min on the Bioanalyser chip, what is much shorter than the few hours (up to 1 day) required for the classical gel electrophoresis process.

Advantages of CE downscaling are reduced analysis times (minutes to seconds), low sample volume requirements (to the picolitre range), low solvent consumption and high throughput capabilities through the possibility of performing simultaneous separations in parallel channels [12].

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Many applications using CE on chip have been developed for the analysis of a wide range of matrices and analytes: food analysis (including small molecules, organic acids, heavy metals, toxins, microorganisms or allergens) [19], amino acid analysis [20] or even intact protein characterisation [21].

2.2. Gas chromatography

Gas chromatography (GC) was first described by James and Martin in 1952. They presented a separation of volatile fatty acids on diatomaceous earth impregnated with a mixture of silicone oil and stearic acid as stationary phase, and a flow of nitrogen as the mobile phase [22]. GC underwent an explosive progression during the next decade, with applications for the petroleum industry [23], followed by biochemical applications [24, 25].

First GC separations were performed on packed columns of 1–5 mm i.d. Column length limitations due to backpressure drop led to the introduction of capillary GC columns [26, 27]. In such columns, the stationary phase is coated on the inner walls of the capillary to form a thin film (wall-coated open tubular, WCOT), or impregnated into a porous layer (porous layer open tubular, PLOT) [28]. Since capillary GC columns have less than 1 mm i.d. (typically 0.05–0.53 mm), this technique could already be considered as miniaturised.

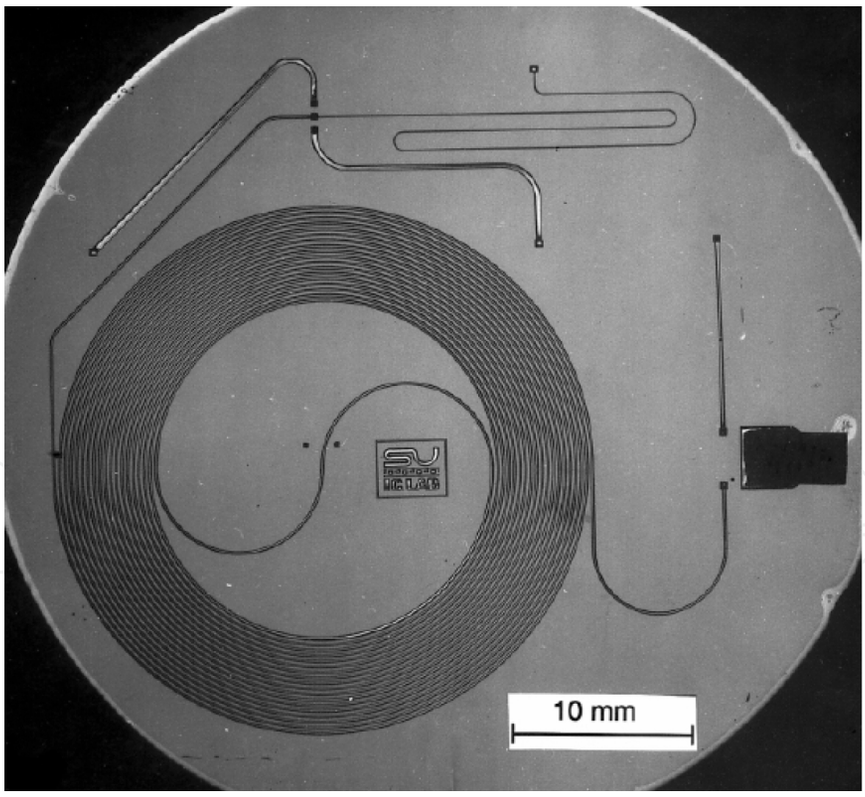

Figure 3. Angell's GC chip integrating sample injection valve, separation column and detector [29].

However, since the 1970s researchers have been trying to integrate all the components of a gas chromatographer including the detector on a single piece, or chip. In 1979, Angell proposed a

silicon wafer chip including a sample injection valve, a 1.5 m column and a detector [29] (Figure 3). In the next decades, other homemade chips were proposed, but to our knowledge no commercial version of a small and portable GC chip has been proposed so far.

A few applications have been developed on microbore GC systems, but research is still more dedicated to reliable miniaturised system development rather than method development [32].

2.3. Liquid chromatography

Liquid chromatography is the most commonly used separation technique with a wide range of applications. The precursor of liquid chromatography was the Russian scientific Mikhail Semenovich Tswett. He discovered that plant leave extracts poured on a column packed with particles could be separated into distinct coloured bands. In 1956, Van Deemter published his famous work about the fundamental equation of the relationship between mobile phase linear velocity and height equivalent to a theoretical plate (Figure 4) [33]. The modern appellation high pressure (now interchangeable with performance) liquid chromatography was first introduced by Horvath in 1970 to designate liquid chromatography performed on reduced (<10 μm) porous particles. Since the 1970s, LC underwent an explosive popularity to become a standard separation technique with continuous progress in stationary phase variety and performances, hardware features and fields of applications.

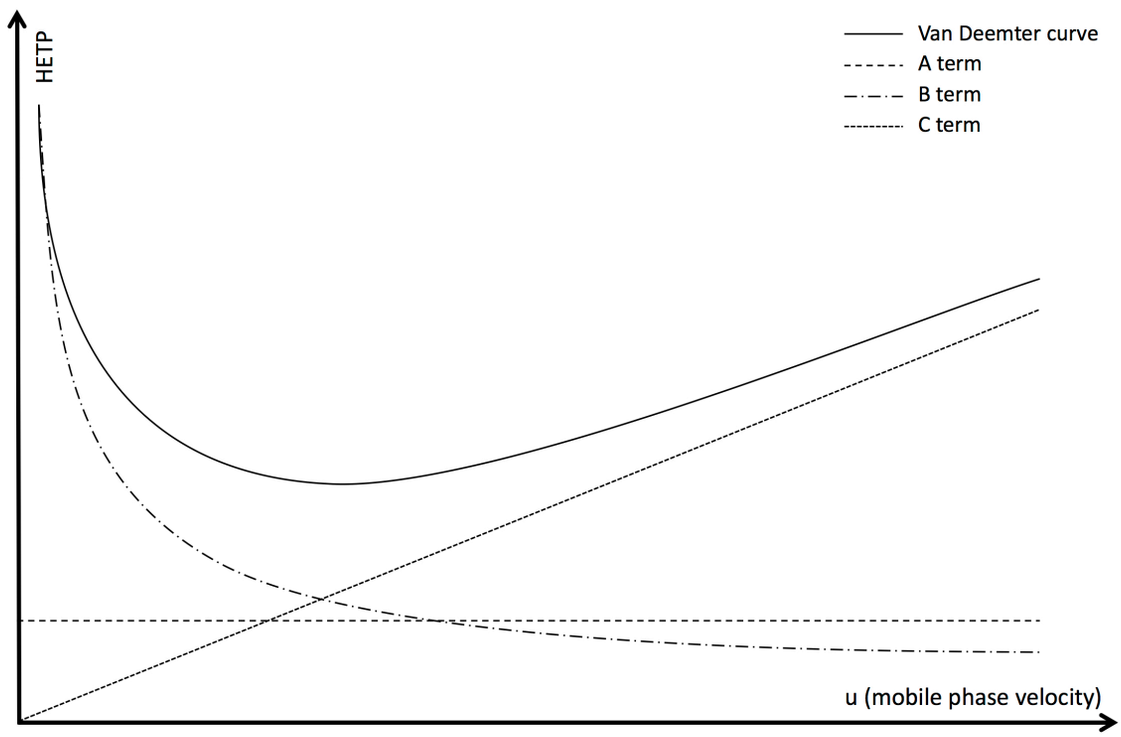

Figure 4. Van Deemter plot deconvolution: (A) Eddy diffusion term; (B) longitudinal diffusion term; (C) resistance to mass transfer term.

Two major research axes of LC have been developed (Figure 5) to comply with the growing needs in increasing the analysis throughput, enhancing sensitivity and reducing analysis cost and environmental footprint through a decrease in solvent consumption [35].

2.3.1. Stationary phase particle size reduction

Considerable gains in terms of sensitivity and analysis time (or chromatographic resolution) could be obtained by reducing the stationary phase particle size to less than 2 μm, giving rise to ultra-high performance liquid chromatography (UHPLC). The use of smaller particles can significantly reduce the height equivalent of a theoretical plate (HETP) generated in a separation.

$$HETP = A + \frac{B}{u} + Cu \tag{1}$$

where u is the mobile phase velocity, A is the Eddy diffusion term, B is the longitudinal diffusion term and C is the resistance to mass transfer term.

The C term mobile phase component C_m can be expanded to the following relationship, showing its dependency on the square of particle size:

$$C_m = \omega \frac{d_p^2}{D_m} \tag{2}$$

where k is the retention factor, d_p is the particle diameter, D_m is the diffusion coefficient of the solute in the mobile phase and ω is the pore size distribution, shape and particle size distribution coefficient.

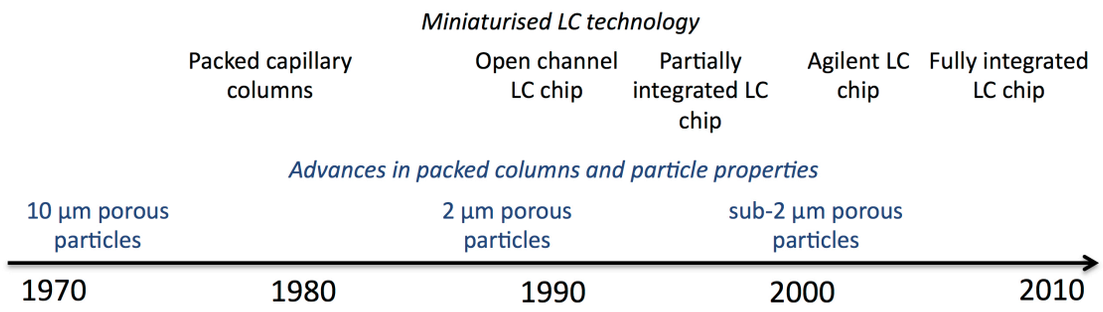

Figure 5. Historical trends in development of HPLC and on-chip LC (adapted from Lavrik et al.'s review [34]).

Particle size reduction has been initiated since the beginning of the spreading of HPLC as a separation technique, but technical limitations related to the pressure drop caused by particle size reduction delayed the commercialisation of sub-2 μm particle columns with classical

dimensions [36]. The first pumps able to face ultra-high pressure were presented by Jorgenson [37] shortly followed by Lee et al. in the late 1990s. In 2004, Waters commercialised the first UHPLC system that was design to deliver pressure up to 1000 bar [41].

2.3.2. Column inner diameter reduction

In parallel with the reduction of particle size, the miniaturisation of LC columns in terms of inner diameter encounters a growing interest since the late 1970s [42].

As previously developed, downscaling the inner diameter of the separation support increases sensitivity with up to 3–4 orders of magnitude in a reduced analysis time. In addition to the advantages of UHPLC, LC miniaturisation reduces drastically the requirements in terms of sample and mobile phase volume.

Miniaturised columns operated on classical LC systems have been described, but void volumes that are very large compared with flow rates and column volumes are responsible for peak dispersion. For that reason, the integration of chromatographic components on a chip (separation channels and electrospray emitters for MS detection, but also additional channels, connections and microvalves) has rapidly been the major strategy to minimise void volumes and efficiency drop [43].

In 1978, Tsuda and Novotny experienced with the performances of packed glass capillaries with 50–200 μm inner diameters.

During the next years, research on chip technology was mainly focused on electroosmosis- or electrophoretic-driven separations due to the technical challenge represented by the connection between LC pumps and chips.

2.3.2.1. Open-channel chromatography

The simplest way to perform miniaturised liquid chromatography on chip is to coat the inner walls of the channels with chemical groups that may interact with the compounds of interest, i.e. to perform open-channel chromatography. In 1990, Manz et al. proposed the first chip prototype for open-tubular liquid chromatography made of silicon and coupled to a miniaturised conductometric detector connected to a classical LC pump [46]. Jacobson et al. proposed the first open-channel separation application on a glass chip coated with octadecylsilane chains in 1994, with low theoretical plate heights (4.1–5 μm) [47]. Due to the small specific surface of such systems, researchers conceived coating modifications to increase the phase ratio (ratio between the volume of stationary phase and the volume of mobile phase): porous layer open-tubular (PLOT) columns, functionalised particles embedded in a porous layer [50] or immobilisation of nanoparticles onto the walls [51].

Open-channel chromatography (with an ideal i.d. of 10–20 μm) provides high efficiency since the molecular diffusion is the only contributor to band broadening. However, due to its limited specific surface, column capacity stays low even with stationary phase modifications.

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2.3.2.2. Micropillars, collocated monolith support structures and nanotubes

Micropillars, collocated monolith support structures (COMOSS) or nanotubes may combine small channel dimensions and large specific surfaces. COMOSS were introduced in 1998 by He and Regnier in response to the difficulty to produce chromatographic columns from wafers [52]. They proposed an approach where the stationary phase is not created by polymerisation *in situ*, but by etching the chip material (e.g. quartz, polydimethylsiloxane (PDMS) or cyclic olefin copolymer (COC)) that may be further functionalised. The result is a highly well-ordered structure (Figure 6) obtained as separation support. Eddy diffusion term in the Van Deemter equation is consequently much reduced, leading to high separation efficiency.

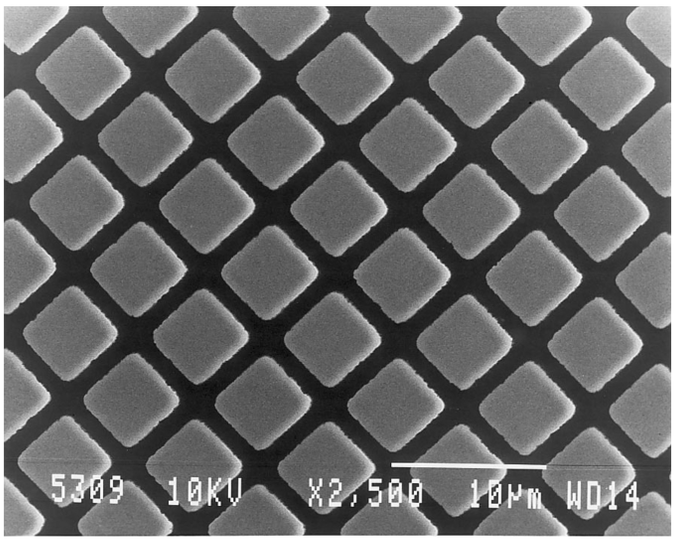

Figure 6. SEM image of a COMOSS organised structure [52].

PDMS can be considered as a C_1 phase, but its hydrophobicity is too low to perform adequate separation; PDMS monolithic pillars could therefore be functionalised by octasilane, octadecylsilane or other groups to improve analyte separation (Figure 7).

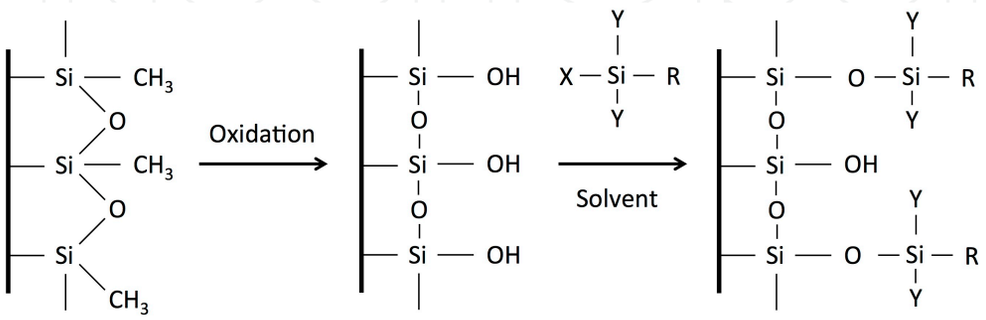

Figure 7. PDMS functionalisation.

COC stationary phase has been presented for the first time by Gustafsson et al. in 2008 [55]. This material presents interesting features in terms of chemical inertness and stability in hydro-organic solvents. The hydrophobic character of COC allows it to be used as chip substrate and stationary phase. COMOSS chips made of on-porous materials as PDMS and COC have high separation efficiency, but low sample capacity due to the low interaction surface.

In addition to non-porous materials as PDMS or COC, superficially porous pillars have been proposed to circumvent the low sample capacity. Two orders of magnitude could be gained in terms of specific surface, increasing the chip sample capacity [56]. Another approach is the *in situ* growth of nanotubes on a COMOSS structure. Increased sample loading capacity and better retention than with C_{18}-functionalised pillars could be obtained [43].

2.3.2.3. Miniaturised monolithic columns

Monoliths are continuous stationary phase beds generated by *in situ* polymerisation of monomers in the presence of porogen agents, resulting in a bimodal structure that exhibits macropores (>50–100 nm) that allow the mobile phase to pass through the column, and mesopores (<20 nm) that offers a high interaction surface for analyte retention [57]. Monolith retention properties can be defined before the polymerisation process by adjusting reagent nature and proportion, or by functionalising the polymer bed. Monoliths present undeniable chromatographic features and deserve to be more thoroughly understood in terms of synthesis parameters and their impact on chromatographic properties [43].

2.3.2.4. Packed particles

Besides the above-mentioned novel LC-chip stationary phases, silica particles can also be employed with the advantage of being well-known due to their broad utilisation for decades in classical LC; a wide range of particle functionalisation types and specifications have been commercialised for a long time. However, special attention has to be paid to particle packing homogeneity and immobilisation of the particles inside the microchannel.

Different column packing procedures have been developed to find the best way to obtain homogenous particle beds. Particles could be brought into chromatographic channels and trapped between weirs or frits that prevent further particle movements. Micromachined frits demonstrate better efficiency than sintered frits that generate more band dispersion [61]. Another procedure was developed for the first time in 2002 by Ceriotti et al. [62]. They proposed a fritless configuration where the particulate bed is retained in the chromatographic channel by a tapered profile at the end of the column. Improvements to this concept were proposed by Gomez et al. that presented a packing process with increased particulate bed stability.

2.3.3. The Agilent HPLC-chip

In 2005, Agilent developed and commercialised a miniaturised HPLC-chip system designed for direct coupling to a mass spectrometer [60]. Polyimide was chosen as chip substrate material due to its chemical and physical inertness, and the low MS background generated.

The fabrication process consists in laser ablation of polyimide film to form the microfluidic channels, ports, chambers and columns followed by deposition of electrical contacts for the electrospray. The last step is the packing of the sample enrichment column and LC column with the stationary phase [65]. This latter operation is performed by introducing isopropanol particle slurries into both channels under a pressure of 120 bar. A wide range of particle chemistries, dimensions and porosities are available in classical Agilent LC columns that can be packed into the chip device.

Chromatographic separations on the Agilent HPLC chip are performed using pressure-driven mobile phase flow. Interfacing macrodimension pumps to nanodimension channels is made through a Chip-Cube interface in which the chip device is sandwiched between the rotor and stator (Figure 8) of a valve. Transfer capillaries from pumps, injector and to waste are connected to the valve stator, ensuring a tight and zero void-volume connection.

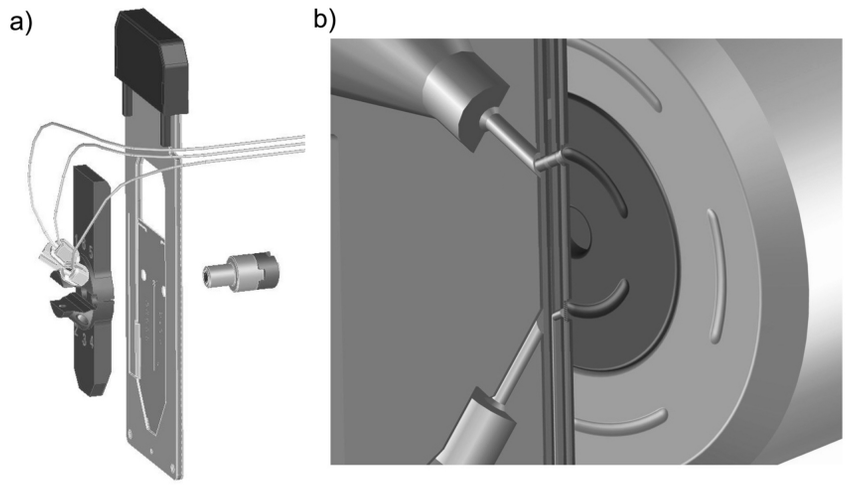

Figure 8. Valve rotor (right) and stator (left) connected with transfer capillaries (A); cut-out view of fluidic connections (left), chip (middle) and valve rotor (right) [65].

Analysis on HPLC-chip consists of sample loading on an enrichment column pushed by a first pump equipped with a split flow device and working at capillary flow rate. After microvalve switching, a second pump delivering a split nanoflow rate is employed to perform chromatographic separation, passing through the enrichment column and the separation channel.

HPLC-chip hyphenation to MS is ensured by an electrospray emitter incorporated in the chip device. The electrospray tip is formed of a prolongation of the polyimide laminated films that constitute the chip substrate. The latter is laser ablated to the appropriate shape (45 μm diameter and 2 mm long) and coated with a conductive metal.

The integrated design of this miniaturised device reduces drastically void volumes and leakage possibilities. Moreover, HPLC-chip is easy to use and compatible with classical LC modules (pumps, autosampler/injector), which opened a wide field of applications.

Since its commercialisation in 2005, HPLC-chip has been used in qualitative analysis of tryptic peptides and proteins, and quantitative analysis of small molecules and peptides [72].

3. Interests of miniaturised LC

3.1. Injection volume

As in classical HPLC, the maximal volume that can be injected without causing a chromatographic band distortion is expressed by the following equation :

$$V_{max} = \frac{\theta.D.\pi.L.d_c^2.\epsilon_c.\left(1+k\right)}{\sqrt{N}} \quad (3)$$

where θ is the fractional loss of the column plate number caused by the injection, D is the constant describing the injection profile, L is the column length, d_c is the column i.d., ϵ_c the is column porosity, k the is retention factor and N the is column efficiency expressed by the theoretical plate number.

As shown in this equation, V_{max} is the proportional to the square of d_c, and the following relationship can be established:

$$\frac{V_{max^{miniaturised}}}{V_{max^{classical}}} = \frac{d^2{}_{miniaturised}}{d^2{}_{classical}} \quad (4)$$

For two columns that have the same length, efficiency and porosity but differ by their inner diameter (4.6 mm for classical dimensions and 75 µm for the miniaturised version), a theoretical injection volume reduction factor of 3762 should be observed (e.g. 10 µl onto a conventional system to approximately 2.5 nl on a nano-LC column) while keeping the same chromatographic performances. Such a reduction of the required injection volume represents an undeniable advantage of miniaturised LC systems, since a growing interest is brought, for instance, to the analysis of biological matrices that are often available in limited volumes.

In practice, a great sensitivity gain can be obtained by injecting higher volumes onto the miniaturised chromatographic system, without causing peak distortion due to an overload. In the case of micro-LC, different peak compression techniques have been studied, such as on-column concentration or sample plug bracketing. In nano-LC, a trapping column is often connected to the analytical column by a valve, allowing large sample volumes to be loaded onto the system and the sample to be pre-concentrated.

3.2. Peak concentration

A reduction of the inner diameter of a chromatographic column results in a higher peak concentration at the detector (C_{max}), as shown in the following equation:

$$C_{max} = \sqrt{\frac{N}{2\pi}} \times \frac{4m}{\pi.L.V_o.\epsilon_c.\left(1+k\right)} \quad (5)$$

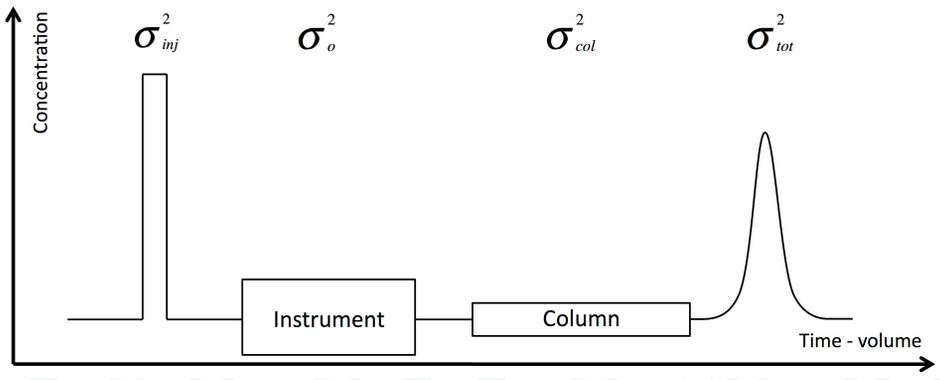

Figure 9. Schematic representation of band broadening components in a chromatographic system (adapted from Lauer [79]).

where m is the total amount of sample loaded on the column and V_0 is the column volume.

C_{max} is the proportional to m and to N, and inversely proportional to V_0. Since V_0 is directly related to d_c, C_{max} is inversely proportional to the square of column diameter. In other words, Eq. (6) can be used to illustrate the sensitivity gain that can be expected with miniaturised columns.

$$\frac{C_{max^{miniaturised}}}{C_{max^{classical}}} = \frac{V_{0^{classical}}}{V_{0^{miniaturised}}} = \frac{d^2_{c\ classical}}{d^2_{c\ miniaturised}} \tag{6}$$

Downscaling the size from classical dimensions (4.6 mm) to miniaturised dimensions (75 μm) would theoretically result in a gain factor of C_{max} of 3762.

3.3. Void volume reduction

Void volumes are detrimental to the chromatographic performances in all LC configurations. However, when working with miniaturised systems, the smallest void volume can act as a mixing chamber and result in an important loss in sensitivity and separation efficiency. The total band dispersion occurring in a chromatographic system (Figure 9) can be expressed by the total variance σ^2_{tot} that sums the variances due to the column (σ^2_{col}) and to the rest of the chromatographic system (σ^2_{ext}).

$$\sigma^2_{tot} = \sigma^2_{ext} + \sigma^2_{col} \tag{7}$$

Band dispersion due to the column, σ^2_{col}, is in particular a function of column volume (and consequently to d_c^2) and efficiency, which are physical properties that cannot be changed for a given column in order to decrease peak broadening:

$$\sigma_{col}^2 = \frac{\pi.L.d_c^2.\epsilon_c.\left(1+k\right)}{4\sqrt{N}} \tag{8}$$

However, other factors having an influence on band broadening through σ_{ext}^2 can be expressed as:

$$\sigma_{ext}^2 = \frac{V_{inj}^2}{d_c^2} + \sigma_0^2 \tag{9}$$

where V_{inj}^2 is the injection volume, σ_0^2 is the instrument variance and σ_{ext}^2 is the extra-column variance.

As shown in this equation, V_{inj}^2 and σ_0^2 are directly related to σ_{ext}^2 [79]. In other words, the minimisation of extra-column void volumes by using the smallest connection capillaries and fittings possible is clearly beneficial to avoid chromatographic band dispersion.

In the light of these considerations, systems with very low extra-column void volumes have been developed including integrated systems (see Section 2.3.3).

3.4. Low flow rate

Mobile phase flow rate F is a value that is also related to the internal column diameter as seen in Eq. (10):

$$F = \frac{\pi.d_c^2.\epsilon_c.u}{4} \tag{10}$$

where u is the mobile phase velocity.

The following relationship can be written in Eq. (11):

$$\frac{F_{classical}}{F_{miniaturised}} = \frac{d^2{}_{classical}}{d^2{}_{miniaturised}} \tag{11}$$

This drastic flow rate reduction has evident economical and ecological advantages, especially when working with pumping systems that directly deliver the right mobile phase flow rate without involving the use of a split flow system.

3.5. Retention volume

The retention volume V_R is defined as the mobile phase volume that is required to elute a compound of a given retention time t_R :

$$V_R = t_R.F = t_R.\frac{\pi.d_c^2}{4}.\epsilon_c.u \tag{12}$$

In the light of the reduced column dimensions in miniaturised LC systems compared with classical systems, the mobile phase volume that is needed to elute a compound with a specified *k* value is reduced proportionally to the square of the internal column diameter, as shown in Eq. (13).

$$\frac{V_{R^{classical}}}{V_{R^{miniaturised}}} = \frac{d^2{}_{classical}}{d^2{}_{miniaturised}} \tag{13}$$

3.6. Hyphenation to MS

When using mass spectrometry, compounds of interest have to carry a net positive or negative charge, depending on the mode that is employed. Analyte electrospray ionisation occurs in three major steps: first, charged droplets are formed from the chromatographic eluent under the action of a strong electric field. The eluent takes the shape of a cone (the Taylor cone) when a critical electric field threshold is reached. A pneumatic assistance is required to provide stable droplet formation in the classical LC [80]. Then, charged droplets undergo Coulomb fission into smaller daughter droplets: eluent solvent progressively evaporates in the heated source until reaching the Rayleigh limit where the electrostatic repulsion forces are exactly equal to the surface tension of the solvent [81]. Beyond the Rayleigh limit, droplets become unstable and divide into smaller droplets. Eq. (14) presents the relationship between droplet charge and Rayleigh radius.

$$Q^2 = 64\pi^2\varepsilon_0\gamma R_R^3 \tag{14}$$

where Q is the droplet charge , ε_0 is the vacuum permittivity and R_R is the Rayleigh radius.

The ion transfer from small droplets to the gas phase can happen following two mechanisms. The ion evaporation model described by Iribarne and Thomson is commonly admitted to describe the small ion formation [81]. According to this model, the electric field at the droplet surface becomes strong enough at an intermediate state and before reaching the Rayleigh limit to directly desorb ions from the droplet (Figure 10) [82].

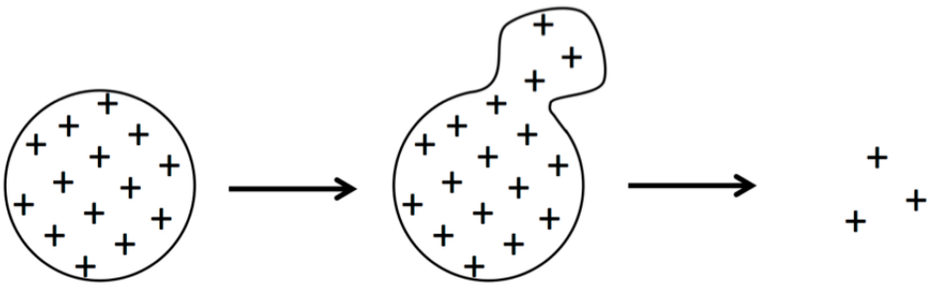

Figure 10. Ion evaporation model.

A second model proposed by Dole, or the charged residue model, could be appropriate to describe protein ionisation. This model suggests that successive Coulomb fissions occurring when the Rayleigh limit is reached, finally yielding droplets containing one single charge (Figure 11) [83].

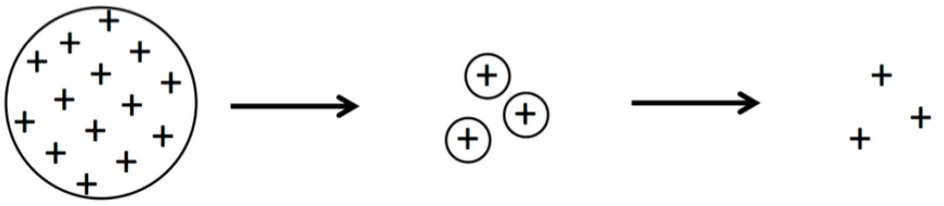

Figure 11. Charge residue model.

Nanoelectrospray (nano-ESI) source was first introduced in 1994 as a response to the development of low flow separation devices. Typical flow rates in nano-ESI are 200–1000 nl/min and the i.d. of spray emitter is about 10–20 μm. The interest of such a miniaturised ionisation source is the improvement of the overall ionisation efficiency (the number of ions recorded at the detector divided by the number of analyte molecule sprayed) [86]. Since signal intensity with ESI sources is concentration sensitive rather than mass sensitive, low analyte amount are advantageously detected at lower flow rates with higher peak concentrations thanks to the miniaturised technique, as previously explained. Lower flow rates as well as narrower emitter tip orifice produce smaller droplets (2–3 orders of magnitude reduction), and desolvation efficiency is increased: smaller initial droplet size requires less Coulomb fission and solvent evaporation to release charged compounds into gas phase, making a larger portion of ions available to detection.

4. Sample preparation

In the light of the previously described features of miniaturised separation techniques, having low volume samples with the highest concentration possible is a clear objective. On the other

hand, analysis of complex media (e.g. environmental, forensic, food, pharmaceutical or biological samples) requires preliminary purification to isolate analyte from contaminants and interferences, and to avoid column or capillary blockage, reduced separation phase lifetime and MS ion suppression. In addition, sample preparation may allow analyte concentration and analyte matrix simplification to make the sample fully compatible with separation technique and detection.

The combination of miniaturised sample preparation and separation techniques offers the main advantages of high throughput, high sensitivity and low costs. The most employed miniaturised sample preparation techniques are briefly described below.

Liquid-liquid extraction (LLE) is a sample preparation technique that relies on the partition of analytes between two immiscible liquid phases. The best results are obtained for compounds showing a clear preference for one liquid over the other one. Factors that influence compound partition include liquid phase polarity, pH, analyte pK_a and polarity, and mixing and contact duration. Micro liquid-liquid extraction (MLLE) is a simple downscaling of classical LLE procedure. The use of lower sample volumes has economical and ecological advantages since the apolar liquid phase is often constituted of alkanes (e.g. pentane, hexane and cyclohexane) or chlorinated solvents; moreover, reduced solvent volumes may lead to the increased analyte concentration.

Solid-phase microextraction (SPME) is a miniaturised sample preparation process involving a fused-silica rod coated with a polymeric layer employed as extraction medium. This technique is applied for the extraction of trace compounds from liquid or gas samples [94] (Figure 12). Analyte desorption is performed by heating the SPME fibre in a classical GC injector for volatile and thermally stable compounds, or by a special desorption device for non-volatile or thermally unstable compounds for subsequent LC [95, 96] or CE [97] analysis.

Dried spots are an expanding way of microsampling and purifying biological samples as blood (dried blood spots, DBS), serum (dried serum spots, DSS) or plasma (dried plasma spots, DPS). A few microliters of a biological fluid are collected on a filter paper and allowed to dry. The dried spot is then punched out and desorbed in an appropriate mixture of solvent chosen to enable maximal analyte extraction while minimising interference desorption (Figure 13). In addition to analytical advantages as small sample volume requirements and low cost, dried spots are very convenient from a sampling point of view: the collection technique is not invasive and can be performed without pain, e.g. for pharmacokinetic studies on laboratory animals or for systematic disease screening on newborns.

Finally, solid-phase extraction (SPE) follows the miniaturisation trend by reducing cartridge and solid phase bed volume (Figure 14A and B). In this technique, sample is loaded in a tube containing a few mg to a few tens mg particles maintained in the bottom of the cartridge by two frits. Sample loading solvent has to be carefully chosen to ensure analyte retention on the particles. Washing steps are then performed to remove a maximal amount of contaminants and interferences that are co-retained on the solid phase, while maintaining analyte-particle interactions. Elution is the final step of SPE to collect a sample containing the analyte for further analysis. Downscaling SPE support allows preparing sample volumes as low as 10 μl, and

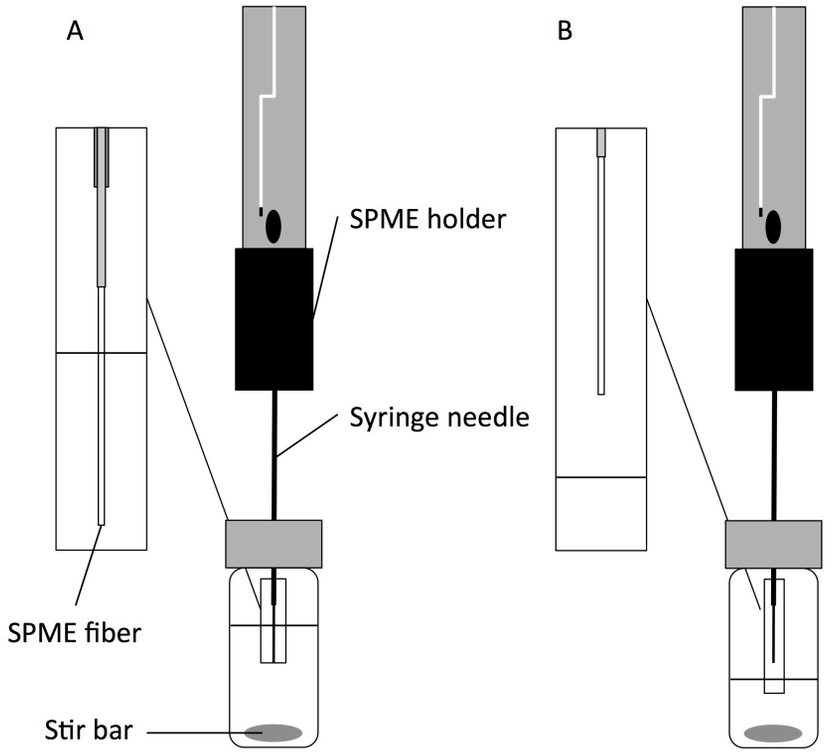

Figure 12. Extraction from aqueous sample solution by conventional SPME device. (A) Liquid phase sampling and (B) headspace sampling [99].

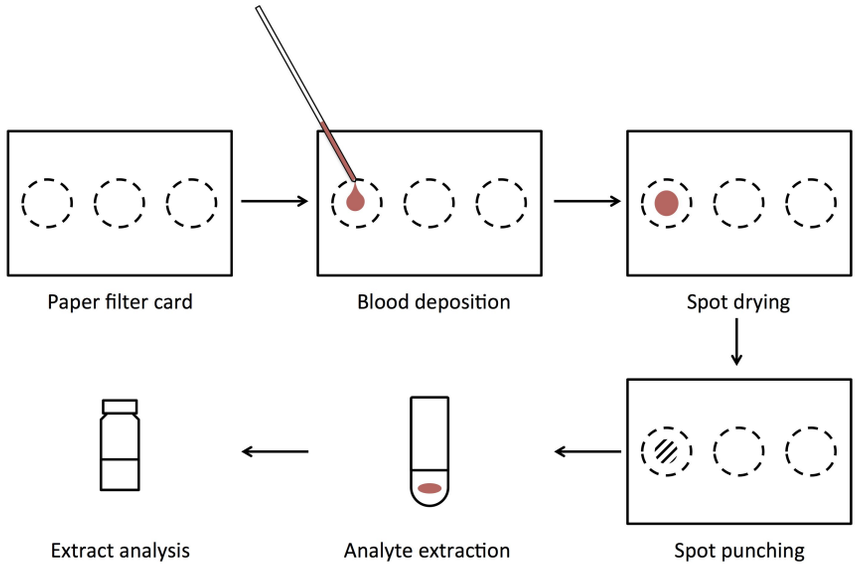

Figure 13. DBS sampling and extraction procedure.

analyte elution by similar volumes. Moreover, SPE or micro-SPE supports are increasingly available in 96-well format (Figure 14C) to provide high extraction throughput by the use of multichannel pipettes or extraction automation.

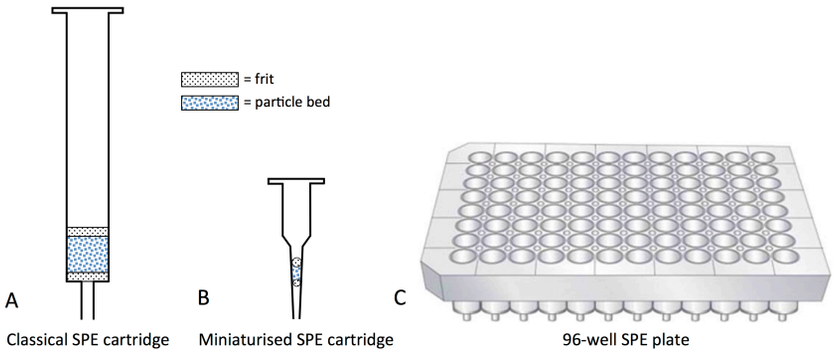

Figure 14. (A) Classical SPE cartridge. (B) Miniaturised SPE cartridge. (C) 96-well SPE plate.

5. Conclusions and perspectives

To summarise, the advantages of microfluidic devices include their small size, improved sensitivity, low sample volume requirements, rapid analysis, potential disposability, and importantly their ease of use that eliminates the need for skilled personnel to perform the assays. In the same time, ethical, analytical and sample availability considerations are a challenge faced by many (bio)analytical laboratories and have resulted in a drive to limit sample volume.

Integration of various nanotechniques through microfabrication processes and advances in detection devices and informatics drive new types of analysis facilitating on-site multicomponent analysis resulting in rapid diagnostic tools and rapid screening methods in various application fields (clinical, pharmaceutical and biopharmaceutical, environmental, food analysis, etc.).

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