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Review

# Real sample analysis on *microfluidic* devices☆

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**Abstract**

This review covers the state of the art of the analysis of real (or non-ideal) samples on *microfluidic* devices. A real sample analysis performed on *microfluidics* conceptually involves the complete integration of sample preparation, analyte separation, and detection on these platforms. Different "lab-on-a-chip" approaches have emerged in relevant application areas such as clinical, environmental, and food analysis which will be critically illustrated and discussed with respect to the strengths and weakness found. Likewise, the main challenges and perspectives will also be commented on.


*Keywords:* *Microfluidic*s; Lab-on-a-chip; Real sample analysis; Clinical; Environmental; Food samples

## Contents



## 1. Introduction

The micro total analysis system (μ-TAS) concept, later called "lab-on-a-chip," was developed from the modification of the total analysis system (TAS) approach by downsizing and integrating its multiple steps (injection, reaction, separation, and detection) onto a single device, yielding a sensor-like system with a fast response time, low sample consumption, on-site operation, and high stability [1].

Miniaturization is more than simply the scaling down of well-known systems since the relative importance of forces and processes changes with scale. One of the most relevant characteristics of analytical microsystems is the omnipresence of laminar flow (Reynold's number are typically very low), in which viscous forces dominate over inertia. This means that turbulence is often unattainable and that molecule transportation only occurs through diffusion, which has direct consequences on the designs of this type of microsystem. The other relevant feature is the possibility of handling fluidics on the nanoliter and even picoliter scale, which has widened the scope of micro-TAS to now be called *microfluidics*. *Microfluidics* is the science and technology of systems that process or manipulate small amounts of fluidics ($10^{-9}$ to $10^{-18}$ L), using channels measuring from tens to hundreds of micrometers [2,3]. For this reason,

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the term *microfluidic*s better covers the research and emphasizes the strong impact miniaturization and integration have on the fluidics and chemical engineering of analytical microsystems.

*Microfluidics* in analytical chemistry can be seen as different analytical microsystems developed according to the analytical objectives clearly based on sample complexity and selectivity criteria [4]: (i) the direct measurement of one or a few components with little or no sample preparation ((bio) sensors, probe-type sensors, flow-through sensors, and microsensors); (ii) the measurement of one or a few components which require some treatment of the sample (μFIA); (iii) the analysis of more complex samples involving the separation of their components (μ-HPLC and μ-CE).

Parallelly, and in connection with this development, it is important to point out that not all analytical steps and/or microsystems are equally incorporated and the incidence level of miniaturization is not homogeneous. In fact, the detection and separation steps are the ones most investigated, whereas sample preparation is notably the least developed [5]. From the beginning, detection has been one of the main challenges for analytical microsystems, since very sensitive techniques are needed as a consequence of the ultra-small sample volumes used in micron-sized environments. In principle, a wide variety of detection alternatives can be used in *microfluidic* systems, but laser-induced fluorescence (LIF) and electrochemical detection (ED), primarily, along with mass spectrometry (MS) are the routes most commonly used. LIF was the original detection technique and it is the most often used detection scheme in CE microchips because of its inherent sensitivity [6]. However, the high cost and the large size of the instrumental setup for LIF are sometimes incompatible with the concept of μTAS. In addition, tedious derivatization schemes are needed to use LIF with non-fluorescent compounds. The principal alternative to LIF detection is, without a doubt, ED. ED is very important because of its inherent miniaturization without loss of performance and its high compatibility with microfabrication techniques. Likewise, it possesses high sensitivity, its responses are not dependent on the optical path length or sample turbidity, and it has low power supply requirements which are additional advantages. Proof of the predominant role of ED is the publication of several reviews [7–9]. Apart from LIF and ED, there are other detection approaches for analytical microsystems, but from a realistic point of view, they have been developed to a lesser extent. These unconventional detection routes have recently been revised and include, among others, infrared, Raman, NMR, surface plasmon resonance (SPR), absorbance, thermal lens, and chemiluminescence detectors [10].

With respect to separation techniques, micro-CE was one of the earliest examples of μTAS, and it constitutes one of the most representative examples of analytical microsystems [11]. Using CE microchips, analysis times can be reduced to seconds and extremely high separation efficiencies can be achieved. The easy microfabrication of a network of channels using materials of well-known chemistry, which themselves have good electroosmotic flows, and the possibility of using the electrokinetic phenomena to move fluids are among the most important factors to understand the relevance of CE microchips to miniaturization. Since electrokinetics is easy to apply (only a pair of electrodes are needed), electroosmotic-driven flow (EOF) has been successfully implemented using different types of materials to manufacture the channels, being glass the most commonly used. Microfabrication on polymers is faster and cheaper than on glass, so these materials have great potential for mass production. However, glass chips present the best EOF and the chemical modification on the surfaces of its microchannels is better known and easier compared to polymers. Many of the benefits already discussed for CE microchips could equally be applied to downsized chromatographic techniques; however, relatively few examples of chip-based chromatographic instruments are covered in the literature compared with chip-based CE devices. This fact is not surprising since CE is almost perfectly suited to miniaturization. Also, the miniaturization of chromatographic systems involves a number of technical challenges, such as the microfabrication of valves and pumps, which are generally not encountered in CE [5].

Sample preparation steps involving emerging technology for sample preparation/pre-treatment [12] and the macroworld to microworld interfaces [13,14] have been addressed. Because of its inherent complexity, the sample preparation step is less developed than those of separation and detection. However, a very exciting future in real analysis is conceivable because of (i) the inherent possibilities of microfabrication technology to create sophisticated designs (complex layouts according to sample requirements) and microstructures (filters) in connection with sample preparation requirements; (ii) the inherent possibilities offered by *microfluidics*: the presence of laminar flow and diffusion in action (H-filter example [15]); and (iii) the ease of using electrokinetic phenomena to move fluidics into the network channels with accuracy (focusing on flow techniques).

In addition, other important general challenges still remain awaiting analytical solutions such as automation, sampling, sample introduction, and sample preparation (except derivatization schemes). Moreover, a breakthrough in the so-called *real world interface* is highly anticipated [5]. Along these lines, total integration and, especially, world-to-chip interfacing are considered the major challenges, particularly in high-throughput applications requiring frequent sample changes, such as continuous *on-line* process monitoring.

From an analytical point of view, the analysis of real samples is often the ultimate goal of a target study. Although the volume of research in this area is impressive, real sample analysis is still in its infancy and constitutes one of the major challenges of *microfluidics*. As a consequence, analyses of true real samples using analytical microsystems, *microfluidics*, or the microchip format are very scarce. This review provides in-depth coverage of the *state of the art* of *microfluidic* devices, focusing on the analysis of real samples as indicators of the grade of maturity of these microsystems. In this context, although every sample is a "real sample," by the analysis of a real sample we mean the analysis performed directly on the matrix containing the analyte (or those spiked). In addition, we understand *microfluidic* devices to be those miniaturized systems for (total) (bio) chemical analysis. These systems are interconnected networks of channels and

reservoirs containing nano- and microliter volumes. An important subset/subclass of these *microfluidic* devices, namely CE microchips, are those made on glass or polymers using electrokinetic techniques for fluidic motivation and analyte separation. Given the large number of publications in the area, this review is constructed around clinical, environmental, and food analysis while other areas, such as molecular analysis (DNA and RNA) [16], forensics [17], and biodefense [18,19], are not covered in detail and readers are encouraged to read these excellent reviews. In sum, the main challenges found and the proposed approaches for clinical, environmental, and foods areas in the sample analysis domain using *microfluidic* devices will be critically discussed.

## 2. Real clinical sample analysis on *microfluidics*

Clinical and biochemical analysis have been the most extensive fields of application for lab-on-a-chip systems and currently they continue to grow [20–23]. The measurement of relevant analytes in physiological fluids for the diagnosis and prevention of diseases is a very exciting application, especially in point-of-care testing. The advantages (cited above) of downsizing from TAS to miniaturized devices are ideal in the case of near-patient clinical analysis. Smaller patient sample volume, which is particularly important in geriatric and pediatric patients; reduced reagent consumption; shortened analysis time; integrated multiple processes in a single device (portability); and enhanced reliability and sensitivity through automation are just some of the achievable benefits which make this technology an interesting approach.

Nevertheless, as was already mentioned, not all of the processes of an analytical procedure are equally incorporated into a miniaturized device (lab-on-a-chip). In clinical analysis, many reports have been published on the detection step in *microfluidic* systems, whereas the sample preparation step tends to be accomplished *off-chip* [24,25]. Often, the analyte of interest is accommodated within an extremely complex matrix (for example blood) which requires different sample pre-treatment techniques, making it difficult to incorporate into a microchip, this being the main obstacle in processing real samples, and to achieve a true micro-TAS device.

Working with real samples usually requires some degree of purification (isolation/clean-up) and preconcentration prior the measurement step. Sensitivity (only a few nanoliters reach the detector) and selectivity can pose a serious problem in the analysis of these raw samples. However, in the field of clinical miniaturized analysis, the use of enzymes, antibodies, and other biological reagents saves extensive sample pre-treatment. The sensitivity associated to the use of enzymes allows samples to be diluted to eliminate interference from other constituents of the matrix or adsorption problems of proteins to the capillary walls without lowering detection limits. Conversely, the use of antibodies, enzymes, or other affinity receptors are responsible for the selectivity required for these assays.

A wide variety of bioanalytical and clinically relevant analytes, such as peptides, amino acids, neurotransmitters, carbohydrates, and vitamins, have been determined in the microchip format. However, references describing analysis in complex matrices are few, most of them being laboratory prepared samples.

This section of the review focuses on those analytes determined in true real or spiked samples. Selected examples will be described from different approaches such as the use of capillary electrophoresis, enzymes, and antibodies to provide selectivity to the assay. The most remarkable features and the type of pre-treatment accomplished are outlined.

Table 1 lists the most relevant information about the *microfluidic* approaches used in the analysis of clinical samples.

The majority of the existing miniaturized systems require highly predictable and homogenous samples. In the case of heterogeneous samples, such as human whole blood, cell-free samples such as serum or plasma are preferable over those with problems associated with flow-path blockage and cell lysis. In this case, whole blood filtration is accomplished *on-chip* for the subsequent analysis. Thorslund et al. [26] have developed a microsystem for *on-chip* sample preparation with which testosterone is measured in diluted whole blood. This system uses a hydrophilic polypropylene membrane filter incorporated onto a PDMS device to remove blood cells with minimal adsorption of proteins.

In clinical analysis, microchip capillary electrophoresis is the most common technique used in miniaturized analytical devices due to its special suitability for miniaturization [27–29]. Relevant analytes used to diagnose different diseases have been analyzed by microchip capillary electrophoresis. Lipoprotein analysis in serum samples was demonstrated by Verpoorte's group [30,31]. In their first approach [30], low density (LDL) and high density (HDL) lipoproteins were separated in a glass microchannel using a dynamic coating (methylglucamine) to prevent lipoprotein adsorption. NBD-ceramide was used as a label for laser induced fluorescence detection. In a later study [31], they demonstrate the analysis of different forms of clinically relevant LDL lipoproteins without the need for a permanent or dynamic coating of the glass microchannels. Adding SDS below the critical micelle concentration to the sample provided sufficient sample recovery to detect the lipoprotein at low concentrations. In both cases, ultracentrifugation of the serum and its subsequent dialysis was accomplished prior microchip analysis.

Neurotransmitters and their metabolites have been determined by microchip CE in biological fluids. Simultaneous amperometric and fluorescence detection is reported by Lapos et al. for the analysis of cerebrospinal fluid samples (CSF) [32]. The use of an orthogonal detection scheme allows LIF detection of NBD-arginine and NBD-phenylalanine, and electrochemical detection of dopamine and catechol, increasing the possibilities for the detection of analytes with different physical characteristics. The use of two detectors simultaneously reduces the run-to-run migration time irreproducibility for certain samples. CSF samples from patients with multiple sclerosis were centrifuged, diluted, and spiked with electroactive species *off-chip*. Johirul et al. [33] have developed a *microfluidic* chip based on CE coupled with a cellulose-ssDNA modified screen-printed electrode for the simultaneous analysis of dopamine (DA), norepinephrine (NE),

Table 1
Microfluidic approaches used in real clinical analysis

| Sample | Analytes | Sample preparation | Microfluidic approach | Analytical characteristics | Remarks | Ref. |
|---|---|---|---|---|---|---|
| Whole blood (spiked) | Testosterona | Dilution *off-chip* | PDMS chip with membrane filter incorporated, RIA detection *off-chip* | Sample preparation microsystem | Sample preparation *On-chip*. Removing of blood cells with minimal adsorption of proteins. | [26] |
| Serum | Lipoproteins (LDL and HDL) | Ultracentrifugation and dialysis *off-chip* | Glass CE microchip, LIF detection | Separation time: 28 s | Methylglucamine as dynamic coating | [30] |
| Serum | Lipoproteins (LDL types) | Ultracentrifugation and dialysis *off-chip* | Glass CE microchip, LIF detection | Separation time: 21 s | Uncoated glass microchip | [31] |
| Cerebral spinal fluid (spiked) | Dopamine, catechol, NBD-arginine and NBD-phenylanaline | Centrifugation and dilution *off-chip* | Glass CE microchip, LIF and EC detection | LOD dopamine: 448 nM, LOD cathecol: 1.52 μM, LOD NBD-argin.: 16 μM, LOD NBD-phenyl: 28 μM | Dual fluorescence and electrochemical detection | [32] |
| Serum | Neurotransmitters (dopamine and ascorbic acid) | No treatment | Glass CE microchip, electrochemical detection | Separation time: 220 s, 1.3 (%RSD), LOD: 0.032 μM | Cellulose-ssDNA modified SP electrode | [33] |
| Mouse brain and human urine | Catecholamines and their cationic metabolites | Dilution and filtration *off-chip* | Glass CE microchip, electrochemical detection | Separation time: 150 s, LOD: subμM | Simultaneous determination. Carbon nanotubes (CNT) modified electrodes | [34] |
| Urine | Amino acids | Filtration and dilution *off-chip* | Glass CE microchip, LIF detection | Separation time: 120 s, LOD: 32.9 μM | Indirect fluorescence detection approach, 19 amino acids detected | [35] |
| Urine | Uric acid | Filtration and dilution *off-chip* | PDMS/Glass CE microchip, electrochemical detection | Separation time: 30 s, LOD: 1 μM | 50–75-Fold dilution avoid potential interferences | [36] |
| Urine | Creatine, creatinine, *p*-aminohippuric acid and uric acid | Dilution and filtration *off-chip* | Glass CE microchip, electrochemical detection | Separation time: 340 s, LOD creatine: 20 μM, LOD uric acid: 40 μM, LOD *p*-aminohippuric acid: 20 μM | Coupling of enzymatic bioassay and electrophoretic separation | [37] |
| Urine (spiked) | Creatine, creatinine and uric acid | Dilution *off-chip* | PDMS CE microchip, electrochemical detection | Separation time: 200 s, LOD creatine: 250 μM, LOD creatinine: 80 μM, LOD uric acid: 270 μM | Direct PAD detection | [38] |
| Serum and blood | Lactate and glucose | Dilution and filtration *off-chip* | Glass CE microchip, post-column enzymatic reaction, electrochemical detection | LOD glucose: 30 μM, LOD lactate: 45 μM | 100-Fold dilution avoid protein adsorption | [39] |
| Plasma | Glucose | Dilution and filtration *off-chip* | PDMS/glass CE microchip, eletrochemical detection | Separation time: 120 s, LOD: 6 μM | – | [40] |
| Serum, plasma, urine and saliva (spiked) | Glucose | Dilution | Glass microchip, colorimetric enzymatic method | – | High degree of integration, electrowetting for microdroplet actuation | [41] |
| Serum (spiked) | Lactate | Dissolved | Integrated flow cell on silicon chip, chemiluminiscent enzymatic FIA system | Analysis time: 30 s, LOD: 0.5 mM | High degree of integration | [42] |
| Serum (spiked) | $Ca^{2+}$ | No treatment | Glass/PDMS microchip, reflectance measurements | Analysis time: 30 s, LOD: 26.8 μM | No significant interferences from other ions presented | [44] |
| Blood (spiked) | Hemoglobin | Dilution | Silicon microchip, photometric detection | Concentration range: 12–17 g/dL | Optical path extensión with 45° mirrors at each end of the channel | [45] |
| Serum (spiked) | Tetanus antibody and tetanus toxin | No treatment | Glass microchip. PAGE gel CE immunoassay, LIF detection | Separation time: 3 min, LOD: 680 pM | Non-competitive and competitive immunoassay format, *off-chip* incubation | [47] |
| Saliva (spiked) | MMP-8 | Centrifuged and diluted | Glass microchip. PAGE gel CE immunoassay, LIF detection | Analysis time: <10 min, LOD: 130 ng/mL | Pretreatment on chip (filtering, enrichment and mixing) | [48] |
| Tissue fluids | Neuropeptides | Ultramicrodialyzer, immunoextraction *on-chip* | Glass microchip, immunoaffinity CE, LIF detection | Separation time: 160 s, LOD: 0.6 pg/mL | 12 neuropetides are determined simultaneously | [49,50] |
| Live cells | Insulin | Conditioning and stimulation On-chip | Glass CE microchip, immunoassay, LIF detection | Assay time: 30 s, LOD: 3 nM | Competitive immunoassay format | [51] |
| Whole blood | Histamine | No treatment | PMMA microchip, electrochemical detection | Analysis time: 2 min, LOD: 200 ng/mL | Multichanneled matrix column coated with cation-exchange resin | [52] |
| Serum (spiked) | Carcinoembryonic antigen | No treatment | PMMA microchip, immunosorbent assay, thermal lens detection | Analysis time: 35 min, LOD: 0.03 ng/mL | Sandwich format on glass beads, antibody-colloidal gold conjugate | [53] |
| Serum (spiked) | Immunosupressive acidic protein | Dilution | Quartz microchip, immunosorbent assay, chemiluminiscence detection | Analysis time: 2 min, LOD: 0.1 μM | Competitive format on glass beads, high degree of integration | [54] |
| Whole blood | Botulinum neurotoxin | Filtration *on-chip* | PDMS platform, immunosorbent assay, electrochemical detection | – | Sandwich format on agarose beads, high degree of integration | [55] |
| Serum | Human IgG | Filtration and dilution *off-chip* | Immunosorbent assay, LIF detection | Differentiation of normal from elevated IgG concentrations | Competitive format, biopassivated microchannells | [56] |
| Plasma (spiked) | Cardiac markers (CRP, Mb, cTnI and S100α) | | PDMS microchip, immunosorbent assay, LIF detection | Analysis time: 10 min, LOD: 30 ng/mL | Sandwich format, micromosaic simultaneous determination | [57] |
| Serum (spiked) | B-type natriuretic peptide | Dilution | Glass/PDMS microchip, immunosorbent assay, SPR detection | Analysis time: 30 min, LOD: 5 pg/mL | Competitive format, *off-chip* incubation | [58] |

3,4-dihydroxy-L-phenylalanine (L-DOPA), ascorbic acid (AA), and 3,4-dihydroxyphenylacetic acid (DOPAC). The modification of the electrode with cellulose-ssDNA prevents surface passivation and amplifies electrocatalytic activity through the interaction of the analyte and ssDNA. Cellulose regulates the solubility of ssDNA in aqueous media and ssDNA acts as an efficient promoter of a direct electron transfer reaction and/or the concentration of analytes on the electrode surface by means of the electrostatic interaction. The applicability of the system to DA analysis in the presence of AA in serum samples without treatment was demonstrated.

Schwarz's group [34] has developed a sensitive and selective method for the simultaneous determination of catecholamines and their cationic metabolites, without mutual interference, in mouse brain homogenates and human urine. They used a novel combination of additives (sodium dodecyl sulphate micelles, dendrimers forming a second pseudo-stationary phase, and borate complexation) to achieve complete separation on the chip and a carbon nanotube modified electrode as a detector to enhance sensitivity. Sample pre-treatment, made *off-chip*, is necessary in both cases. For urine, sample dilution in 0.2 M $HClO_4$ containing 0.1% of $Na_2EDTA$ to prevent the oxidation of the analytes and filtration is required. The brain tissue was homogenated, centrifuged, and diluted in running buffer prior to injection.

Landers et al. [35] have demonstrated the possibility of high-throughput screening for several amino acids in urine samples using CE on a microchip format. Indirect fluorescence detection has been adapted to the electrophoretic microchip to provide a fast analysis of amino acids. Although the detection limits for indirect detection are lower than those achievable using fluorescence labeled analytes, the lack of sample preparation and simplified electrophoretic profiles make this technique attractive for many types of analysis. The analysis of urine samples required filtration and dilution of the sample to avoid the high ionic values derived from the presence of NaCl and other inorganic ions. Despite the lower signal-to-noise ratio obtained when compared to conventional CE (significantly longer capillary), the method allowed the quantitative determination of various amino acids in abnormal urine specimens.

Microchip CE-ED to measure uric acid in physiological urine samples was reported by Fanguy et al. [36]. The sample preparation for and the analysis of uric acid, with a clinically accepted enzymatic reaction, can be tedious. However, the proposed microchip system using electrochemical detection is able to evaluate single-filtered diluted urine samples in the expected range of the concentration of the analyte. Due to the low detection limit of the method, uric acid determination is accomplished in 50–75-fold dilution of the urine, without potential interference from other organic compounds with similar potential oxidation in the sample.

The groups of Wang and Henry reported the simultaneous analysis of different renal markers in urine samples using microchip CE [37,38]. In the first paper [37], creatine, creatinine, *p*-aminohippuric acid, and uric acid are determined by the coupling of an *on-chip* enzymatic assay, electrophoretic separation, and electrochemical detection. Hydrogen peroxide from the enzymatic reactions of creatinine and creatine *on-chip* is separated from *p*-aminohippuric and uric acids, and the three species are amperometrically detected at the end-column detector. Urine samples were filtered and diluted prior to analysis. Henry et al. [38] reported the separation of these renal markers (creatine, creatinine and uric acid) using 30 mM borate buffer (pH 9.4) plus 1 mM SDS and their direct detection using pulse amperometric detection. The three analytes were determined in diluted and spiked urine samples, and the creatinine results were validated using a commercially available assay kit based on the Jaffe reaction.

Heightened levels of carbohydrates in biological fluids, mainly glucose, are especially relevant in diagnosing metabolic disorders. With this in mind, different microchip systems have been developed to determine carbohydrates in urine or blood samples. Wang and his group [39] reported an electrophoretic microchip to determine lactate and glucose in blood samples. This procedure combines the selectivity and amplification features of enzymatic assays with capillary-electrophoretic separation efficiency. Specifically, this microchip integrates electrophoretic separation of lactate and glucose in the separation channel, their post column reaction with the corresponding oxidases, and amperometric detection of the hydrogen peroxide liberated in the two separated product zones, as is illustrated in Fig. 1. Electrophoretic separation avoids potential electroactive interference, enhancing the advantages of enzyme flow-injection assays. Analyzed samples (serum and blood samples) must be filtered, centrifuged, and diluted in the running buffer *off-chip*.

Du et al. reported the electrophoretic separation and direct electrochemical detection of glucose on a PDMS/glass microchip [40]. The device includes copper microelectrodes manufactured onto the electrode plate by selective electrodeless deposition. Plasma samples from healthy and diabetic patients were previously diluted and filtrated, and glucose was determined in 120 s with a detection limit of 6 μM.

Lactate and glucose were also determined using colorimetric enzyme assays from a different total microanalysis system (μTAS) approach. Srinivasan et al. [41] reported an impressive "lab-on-a-chip architecture" for the automated analysis of glucose in physiological fluids. The *microfluidic* system they used is shown in Fig. 2. This lab-on-a-chip, based on electrowetting for microdroplet actuation, integrates sample injection elements, reservoirs, droplet formation structures, fluidic pathways, mixing areas, and optical detection sites all on the same substrate. Detection based on the colorimetric enzyme-kinetic method (Trinder's reaction) allows glucose to be determined in spiked serum, plasma, urine, and saliva samples with good concurrence to the reference method. Additionally, Karube's group [42] fabricated a compactly integrated flow cell with a chemiluminescent FIA system for determining lactate concentration in serum. The cell was made by micromachining techniques and mainly consists of a reactor, a mixing cell, and a spiral groove. Lactate was determined using lactate oxidase and was catalyzed by pyruvate and hydrogen peroxide. Subsequently, the hydrogen peroxide reacted with the luminol-ferricyanide reagent and the resulting chemiluminescent product was detected. Lactate concentration

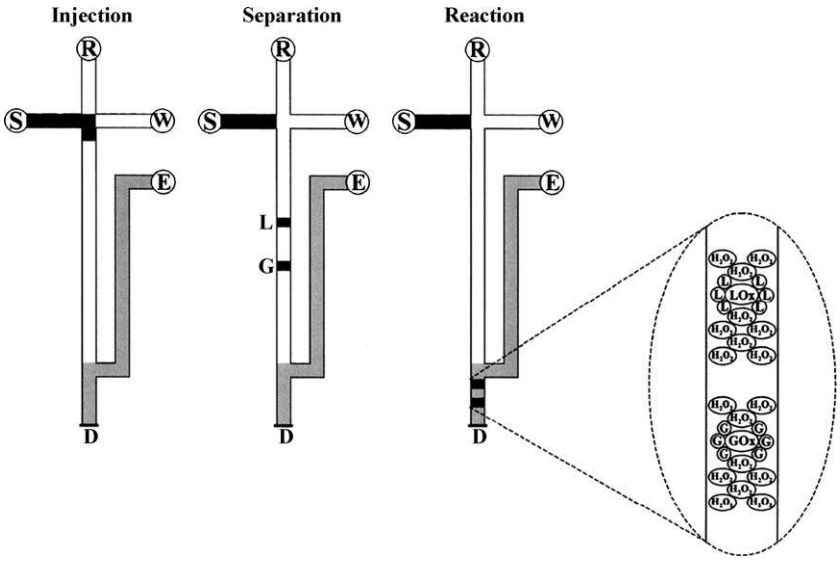



Fig. 1. Schematic of the CE-EC microchip, with the post-column enzymatic reaction, for the simultaneous measurement of glucose (G) and lactate (L). The individual analytical steps (injection, separation, reaction) are shown from left to right. Also shown (insert) is the post-column channel with the two hydrogen-peroxide zones. Reservoirs: (R) running buffer, (W) waste, (S) sample mixture, (E) 'reagent' (enzyme) solution containing glucose oxidase (GOx) and lactate oxidase (LOx); (D) the amperometric detector. The chip consisted of a sample (S), waste (W), and a running-buffer (R) reservoirs, connected through 5 mm long 'arms' to a four-way injection cross. A 77.7 mm-long separation channel followed the injection cross. An additional 77.2 mm-long channel (referred to as 'post-column') was joined the end of the separation channel, leaving a 10 mm channel for the enzymatic reaction before the detector. All channels were 50 μm wide and 20 μm deep (from reference [39], with permission).

was determined in undiluted spiked serum samples using this system.

In a similar fashion to ref. [41], Moon et al. [43] developed an integrated digital *microfluidic* chip for multiplexed proteomic sample preparation for MALDI-MS analysis. The system integrates the handling of solutions and reagents with electrowetting-on-dielectric (EWOD) actuation allowing the parallel processing of multiple sample droplets for high-throughput MALDI-MS. Processing four angiotensin peptide/urea samples demonstrated the suitability of the device for proteomic applications.

Spectrophotometric detection is not usually employed in *microfluidic* devices, mainly due to its low sensitivity; however, Caglar and co-workers [44] have developed a glass-PDMS microchip used to determine $Ca^{2+}$ ions in serum samples taking reflectance measurements of arsenazo III immobilized on the surface of polymer beads. The beads were used at the detection point of the *microfluidic* sensor device with a fiber optic assembly for reflectance measurements. Despite potential interference from other ions, the microchip sensor allowed $Ca^{2+}$ to be determined in untreated serum samples within the physiological range. In another approach, to overcome the usually low sensitivity associated with photometric microchips, Noda et al. [45] developed an optical path extension absorption-photometry microchip for highly sensitive hemoglobin measurements. In this device, the reflection in two 45° mirrors at each end of the

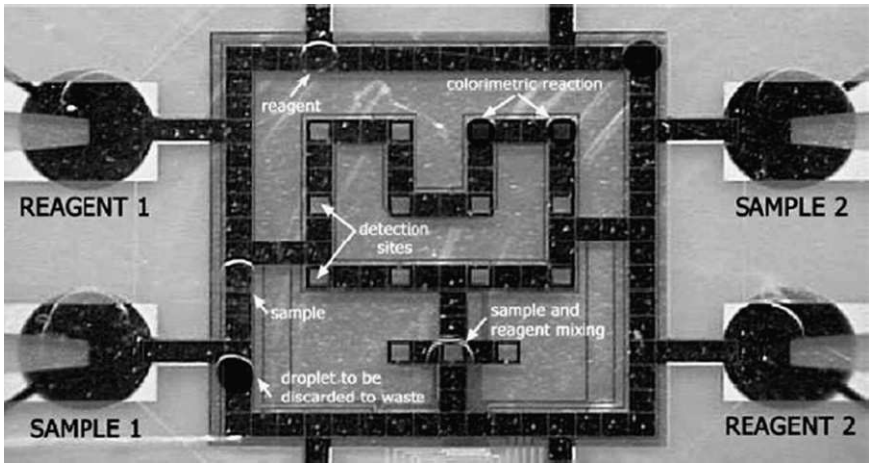



Fig. 2. Integrated digital microfluidic lab-on-a-chip for clinical diagnostics. This lab-on-chip, based on electrowetting for microdroplet actuation, integrates sample injection, on-chip reservoirs, droplet formation structures, fluidic pathways, mixing areas and optical detection sites, on the same substrate. Glucose is determined in spiked serum, plasma, urine and saliva as a proof of concept by colorimetric enzyme-kinetic method (Trinder's reaction) (from reference [41] with permission).

fluidic channel allows the propagation of light from the source to the detector along a longer optical path (5 mm). Blood samples that were previously diluted and to which the hemolysis chemical (cyanogen solution) were added were then photometrically measured allowing the determination of hemoglobin within the normal human concentration range (12–17 g/dl).

As is known, immunoanalytical systems have become one of the most widely used methods for detection and determination of a wide variety of analyte molecules in clinical diagnosis. Miniaturization of immunoassays onto microchip platforms combines the power of *microfluidic* devices with the high sensitivity and specificity of antigen–antibody interactions. The inherent reduction in diffusion distances for the species and the larger surface-area-to-volume ratio can increase the chances of the antigen and antibody of binding within the *microfluidic* channels. However, in a miniaturized immunoassay, particular points more important than in conventional systems can be considered. These include the effect of the capillary surface's chemical nature (non-specific binding), the methods of liquid transportation because of high flow resistance, special consideration for mixing procedures due to laminar flow, or specific detection principles because of the reduction in volume reaching the detector [23].

Taking into account the exceptional properties for miniaturizing CE together with the selectivity power of immunoassays, the coupling of both techniques (μCEI) constitutes one of the most widely used approaches [46]. Herr et al. have developed *microfluidic* immunoassays based on gel electrophoretic separation and the quantization of bound and unbound species. In an earlier paper [47], direct immunoassay (non-competitive) and competitive formats were used in the detection of the tetanus antibody and the tetanus toxin, respectively, in spiked serum samples. *On-chip* photopatterned native PAGE gel was used for the electrophoretic separation of the fluorescently labeled C-fragment of the tetanus toxin with the specific antibody from its complex after an *off-chip* incubation step. Recently [48], the same researches improved their system by integrating sample pre-treatment *on-chip* (filtering, enrichment, mixing) with an electrophoretic immunoassay to determine the collagen-cleaving enzyme matrix metalloproteinase-8 (MMP-8) in saliva as a diagnostic fluid. Relevant clinical concentrations (LOD 130 ng/mL) are measured in less than 10 min. Samples must be centrifuged and previously diluted to 20 μl before being loaded into the *microfluidic* system.

Exploiting the capability of CE to analyze multiple analytes in a single sample, Phillips and Wellner [49,50] designed an immunoaffinity CE to measure concentrations of up to 12 inflammatory neuropeptides and cytokines in muscle and cerebral spinal fluids. Using one reservoir of the microchip, Fab fragments from each of the specific antibodies were immobilized on their surface. After the incubation steps with sample and laser dye and the subsequent washing steps, the captured analytes were eluted and directed to the separation channel. In less than 160 s, 12 neuropeptides were resolved and determined in a 100 pL sample. Tissue fluid samples were previously passed through an ultra-micro dialyzer. In the same paper [50], an electrokinetic immunoassay was developed for the same analytes. However, 280 s were needed to resolve the immune complex of the free-labeled antibody of 1 specific analyte, and higher LOD were obtained.

Kennedy et al. demonstrated the continuous monitoring of insulin secretion from live cells (islets of Langerhans) using a capillary electrophoresis competitive immunoassay [51]. Continuous sampling, on-line reaction, and electrophoretic separation of the fluorescein isothiocyanate-labeled insulin (FITC-insulin) and FITC-insulin-Ab-complex were accomplished in 30 s. A detection limit for insulin of 3 nM and continuous monitoring up to 30 min with no intervention were achieved.

Matsunaga's group [52] reported the use of a multichanneled matrix column coated with cation-exchange resin to separate immunocomplexes after a competitive binding event. Histamine is measured in 10 μL of whole blood directly extracted from patients by an electrochemical flow immunoassay. Based on different isoelectric points of immunocomplexes, histamine-antibody and histamine-derivative-antibody, only the first is retained in the column. The conjugation of the specific antibody with ferrocene allows electrochemical detection of the immunocomplex in the eluent. Histamine concentration in the 200–2000 ng/mL range is measured in whole blood within 2 min.

The determination of cancer markers is crucial for an early diagnosis of the disease and immunoassay is considered an indispensable technique for determining small amounts of a tumor marker in serum. Different papers have reported using microchip-based immunoassays for cancer diagnosis [53,54,56]. Sometimes the electrophoretic separation of bound and unbound species is not suitable in the microchip format. An interesting alternative would be the integration of the widely used immunosorbent assay (ELISA), in which antigens or antibodies are fixed on a solid surface, onto a microchip known for its high sensitivity. Kitamori et al. [53] and Tsukagoshi et al. [54] have developed glass bead immunosorbent assays for the human carcinoembryonic antigen (CEA) and immunosuppressive acidic protein (IAP), respectively. In Kitamori's work, a sandwich immunoassay format together with thermal lens detection of colloidal gold-labeled antibodies is used. Serum samples from patients are assayed in 35 min with a determination limit of 0.03 ng/mL for CEA. Tsukagoshi uses chemiluminescence to detect unbound luminol-labeled analytes at the end of the microchannel where a microperoxidase catalyzes the hydrogen peroxide reaction. IAP spiked serum samples are determined in the relevant clinical range of concentrations within 2 min for each assay.

Also based on a sandwich ELISA format, where the specific antibody is immobilized on functionalized agarose gel beads, Moorthy et al. [55] developed a *microfluidic* platform for detecting botulinum neurotoxin directly from whole blood. Sample preparation by filtration, mixing and incubation with reagents were carried out on the device. Clinically relevant amounts of the toxin can be detected by measuring the color of an insoluble enzymatic product from the alkaline phosphatase reaction.

Elevated levels of monoclonal human IgG in serum may be linked to cancer. In this sense, Thormann's group [56] reported

a heterogeneous competitive human serum IgG immunoassay. Immunoglobulin G and Cy5-IgG, used as a tracer, compete in order to bind to a specific antibody immobilized on a biopassivated microchannel. Using Cy3-mouse IgG as an internal standard allows samples from patients with normal IgG serum levels (8–16 mg/mL) to be differentiated from patients with elevated IgG concentrations (>16 mg/mL).

A sophisticated approach is reported by Delamarche's group [57] in which different cardiac markers are simultaneously detected. They combine *microfluidic* networks, in which the flow of liquids is driven by capillary forces (self-regulating), and micromosaic immunoassays based on patterning lines to capture antibodies exposed to solutions of analytes. Using secondary fluorescently labeled antibodies, a micromosaic of fluorescent zones reveals the binding event in a single imaging step. In this case, four cardiac markers – C-reactive protein (CRP), myoglobin (Mb), cardiac troponin (cTnI), and S100α – are simultaneously determined in spiked plasma samples (1 μL volume) within 10 min.

Recently, Niwa et al. [58] reported a *microfluidic* device combined with a portable surface plasmon resonance sensor system (SPR) to determine the cardiac marker B-type natriuretic peptide (BNP) in spiked serum samples. After competitive immunoreaction takes place, the thiol compound generated by the enzymatic reaction with the trapped labeled antibody was accumulated on a thin gold film located in the microchannel. The extension of the chemisorption of thiol molecules can be monitored by the SPR angle shift and related to BNP concentration. A concentration range of 5 pg/mL to 100 ng/mL covers the usual levels of BNP in blood.

## 3. Real environmental and related sample analysis on *microfluidics*

The development of portable, robust, and accurate analytical systems to monitor environmental compounds such as contaminants, explosives, chemical warfare agents, inorganic and organic ions is a demand of modern society. Portability allows analyses to be carried out outside of the laboratory, preventing or minimizing the risk of contaminating the sample and leading to a faster response time at a lower cost. The ability to use portable equipment is an intrinsic characteristic of *microfluidic* systems, which is why they are an excellent response to this analytical demand.

Sample introduction, complexity of the sample matrix, and the low concentration of the chemical species in the environment are the possible challenges of using *microfluidic* devices for environmental analysis.

There are excellent bibliographic reviews in the literature about *microfluidic* devices for environmental analysis [59,60], but not reviews are found on the analysis of real samples and their particular challenges.

In this section, the most relevant papers in the literature that use *microfluidic* devices for environmental analysis have been classified and discussed according to the kind of analytes studied: warfare agents and explosives, and pollutants and ions of environmental interest.

Table 2 summarizes the most relevant information regarding the analysis of the main compounds of environmental significance in real samples using miniaturized devices.

Wang and co-workers have developed a method for determining thiol-containing degradation products of V-type nerve agents in water samples [61]. The detection of three of these compounds was achieved in only 4 min, though only in a water sample spiked with its corresponding standards. The microsystem consists of a glass capillary electrophoresis microchip with amperometric detection (screen-printed carbon ink electrode). A derivatization of the compounds with o-phthaldialdehyde was necessary, which was developed both *on-* and *off-chip*. When the process was performed *on-chip*, the complete reaction between derivatization agent and compounds was not achieved, resulting in decreased sensibility. With the same microsystem [62], three organophosphate nerve agents were detected in 140 s from a river water sample spiked with those analytes. In both cases [61,62], the LODs were in micromolar range, but this seems to be insufficient for real applications. Better LODs were obtained by the same research group using contactless conductivity as its detection mode [63]. A detection limit within the nanomolar range was achieved for the determination of three organophosphonate nerve agent degradation products from a river water sample (spiked with standards). In this paper, a disposable PMMA microchip with integrated electrodes was used with excellent results (analysis time 130 s).

Luong et al. were able to determine trinitrotoluene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene in soil and groundwater samples by MEKC-EC on a glass microchip and using a gold wire as electrode [64]. The analysis time was 4 min and LODs were below the micromolar range. The accuracy of the method was validated by comparison with the LC procedure of the US EPA (method 8330). For the soil sample, an extraction step *off-chip* was necessary. In all papers, the water samples were filtered *off-chip*.

Wang and co-workers have also separated and detected three different groups of pollutants (nitrophenols, aromatic amines, and chlorophenols) by capillary electrophoresis on a glass microchip with amperometric detection using river and groundwater samples. In this case, the detected compounds were previously added. Five nitrophenol derivates were detected in 120 s with a glassy carbon electrode [65], three aromatic amines (4-aminophenol, 2-aminonaphthalene, and o-aminobenzoic acid) in 150 s with a boron-doped diamond thin-film detector [66], and phenol and three chlorophenols in 120 s with a carbon SPE modified with gold [67]. In these papers, the LODs were within the micromolar range and accuracy was not evaluated. The samples were filtered before the analysis.

Garcia et al. obtained good results in their analysis of environmental samples using a PDMS microchip with pulse amperometric detection (PAD). Levoglucosan (the largest single component of the water extractable organics in smoke particles) was determined in smoke particles in only 1 min [68]. The results obtained with this method are in accordance with those obtained with GC/MS. The aerosol particles were collected and extracted *off-chip* and *off-line*. The LOD achieved (17 μM) was adequate for the analysis of smoke samples but insuffi-

Table 2
Microfluidics (capillary-electrophoresis microchip) in environmental analysis

| Sample | Analytes | Sample treatment | Microfluidic system | Detection mode | LOD (μM) | Analysis time (s) | Accuracy evaluation | Ref. |
|---|---|---|---|---|---|---|---|---|
| Tap and river water (spiked) | 2-(Dimethylamino) ethanethiol, 2-(diethylamino) ethanethiol, 2-mercaptoethanol | Derivatization agent (o-phthaldialdehyde) in presence of Valina, *off-* and *on-chip* | Glass microchip, CZE | Amperometry carbon ink screen-printed electrode | 6–11 | 240 | – | [61] |
| River water (spiked) | Paraoxon methyl parathion, fenitrothion | Filtration, *off-chip* | Glass microchip, MEKC | Amperometry, carbon ink screen-printed electrode | 0.9–3.8 | 140 | – | [62] |
| River water (spiked) | Methylphosphonic acid, ethylphosphonic acid, isopropylphosphonic acid | Filtration, *off-chip* | PMMA microchip, CZE | Contacless conductivity | 0.005–0.006 | 130 | – | [63] |
| Soil and groundwater | Trinitrotoluene, 2,4-dinitrotoluene, 2,6.dinitrotoluene | Extraction, centrifugation and filtration, *off-chip* | Glass microchip, MEKC | Amperometry, gold electrode | 0.48–0.88 | 240 | Comparison with LC procedure (method 8330) US EPA | [64] |
| Groundwater (spiked) | 2-Nitrophenol, 3-nitrophenol, 4-nitrophenol, 2,4-dinitrophenol, 2-methyl-4,6 dinitrophenol | Filtration, *off-chip* | Glass microchip, MEKC | Amperometry, glassy carbon electrode | 7–30 | 120 | – | [65] |
| River water (spiked) | 4-Aminophenol, 2-aminonaphtalene, *o*-aminobenzoic acid | – | Glass microchip, CZE | Amperometry, boron-doped thin-film detector | 1.3–2 | 150 | – | [66] |
| River water (spiked) | Phenol, 2-chlorophenol, 2,4-dichlorophenol, 2,3-dichlorophenol | Filtration, *off-chip* | Glass microchip, CZE | Amperometry, carbon ink screen-printed electrode modified with gold | 1–2 | 120 | – | [67] |
| Smoke particles | Levoglucosan | Aerosol particles collection and extraction *off-chip* | PDMS microchip, CZE | Pulse amperometric detection Gold electrode | 17 | 60 | Comparison with GC/MS | [68] |
| City water (spiked), medicines | Phenol 4,6-dinitro-*o*-cresol pentachlorophenol | – | PDMS microchip, CZE | Pulse amperometric detection, gold electrode | 0.9–2.2 | 120 | Respect declared content (label medicine) | [69] |
| River water | DOC (dissolved organic carbon) | Derivatization with isothiocyanate and filtration, *off-chip* | Glass microchip, CZE | LIF | – | 120 | – | [70] |
| Electroplating sludge reference material | Pb(II) | Extraction, centrifugation and filtration, *off-chip* | PDMS microchip, sensor | LIF | 0.011 | – | Respect reference material | [71] |
| Water mimetic solution | Cr(III) | EDTA to remove interferences, *off-chip* | PDMS-glass microchip, FIA (mixer/reactor system) | Chemo luminescence (luminol-$H_2O_2$) | 0.1 | 60 | Recovery | [72] |
| River, pond and rain water | Sulfite, nitrite | Derivatization agent, *N*-(9-acridinyl) maleimide, 2,3-diamoino naphthalene, *on-chip* | PDMS-glass microchip, FIA | LIF | 0.4–1 | 900 | Recovery | [73] |
| Plexiglas surface contaminated | Cd(II), Pb(II), Co(II), Ni(II) | Chelating agent 2-(5-bromo-2-pyridylazo)-5-(*N*-propyl-*N*-sulfo) propylaminophenol, $C_{18}$ silica column, *off-chip off-line* | Glass microchip, non-aqueous CZE | Colorimetric | 0.003–0.053 | 80 | Recovery | [74] |
| River, tap, mineral water | Nitrite, fluoride, phosphate | Preconcentration and elimination of interferences, *on-chip on-line* | PMMA microchip, ITP-CZE column coupled | Conductivity | 0.5–0.7 | 360 | – | [75] |

cient for atmospheric samples. In another paper [69], three of the most important pollutants (phenol, 4,6-dinitro-*o*-cresol, and pentachlorophenol) were detected in a city water sample spiked with the corresponding standards. The LODs were within the micromolar range and accuracy was evaluated by determining phenol in two medicines with labeled content. Sample treatment was not necessary.

An original method was developed for evaluating the environmental index DOC (dissolved organic carbon) [70]. The microsystem consists of a glass capillary electrophoresis microchip with LIF as its detection mode. Derivatization with a labeling agent, fluorescein isothiocyanate, was needed (overnight). A sample of river water was analyzed, but the results were not compared with other methods.

Anions and cations of environmental interest have also been explored. Chang et al. fabricated a sensor to monitor $Pb^{2+}$ in environmental samples [71]. This microdevice was made on PDMS and contained a network of *microfluidic* channels that were fluidically coupled via a nanocapillary array interconnect (NAI) and used LIF as its detection mode. This sensor's selectivity was achieved using a lead-specific DNAzyme constructed by the authors which possessed a fluorophore fragment. In the presence of $Pb^{2+}$, the substrate DNA was cleaved, resulting in the release of fragments and a concomitant increase in fluorescence, as is illustrated in Fig. 3. Accuracy was evaluated with an electroplating sludge reference material, but no further samples were analyzed. An excellent LOD was achieved (11 nM).

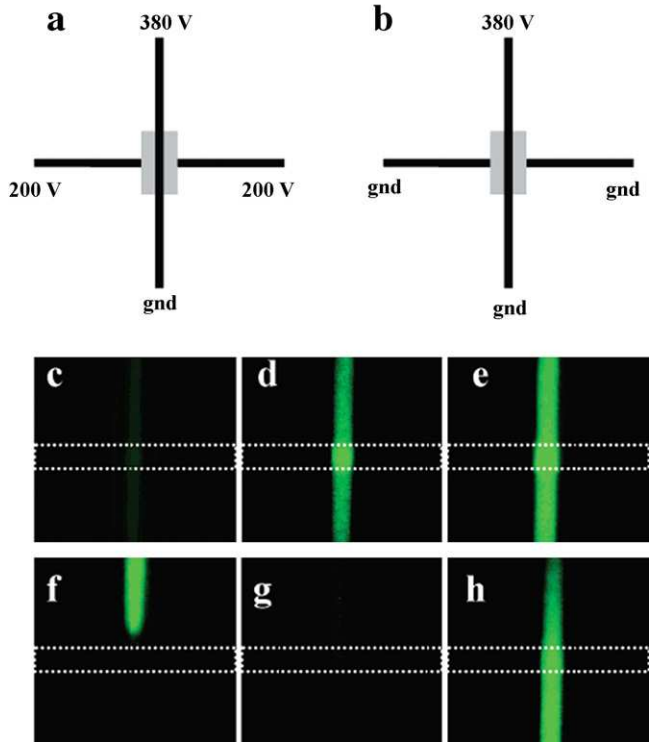



Fig. 3. Electrical bias configurations for fluidic control of lead and DNAzyme within an NAI/microfluidic device: (a) on state and (b) off state. Temporal sequence of fluorescence images at the intersection of the crossed microchannels (c–h) (from reference [71] with permission).

Two different research groups have used capillary electrophoresis microchips as FIA systems. In the first paper [72], Cr (III) was detected by chemiluminescence in a water mimetic solution in 1 min. The catalytic property of Cr (III), the result of the reaction between luminol and $H_2O_2$, was taken advantage of to determine this cation. All parameters of the mixer/reactor microsystem were optimized and *off-chip* sample treatment with EDTA was needed to remove interferences (other metal cations). In the second paper [73], sulfite and nitrite were detected by FIA-LIF in different water samples, but the use of two derivatization agents was needed (*on-chip*). LODs within the micromolar range and a huge analysis time (15 min) were obtained. The accuracy of both methods was evaluated by recovering the standards added in the studied samples. Deng and Collins separated and detected four metallic cations ($Cd^{2+}$, $Pb^{2+}$, $Co^{2+}$, $Ni^{2+}$) using a non-aqueous capillary electrophoresis microchip with colorimetric detection on a contaminated Plexiglas surface prepared by the authors [74]. Sample treatment was carried out *off-chip* and *off-line* and consisted of extraction with a chelating agent. The extract was then preconcentrated in a $C_{18}$ silica microcolumn, and, finally, the sample was introduced onto the microchip achieving LODs within the nanomolar range.

Bodor et al. have determined three anions ($NO_2^-$, $F^-$, $PO_4^{3-}$) in river, tap, and mineral water on a PMMA column-coupling microchip with conductivity detection [75]. The illustration of the microdevice used is shown in Fig. 4. The microchip consists of two coupled columns: The first is used as an isotachophoresis (ITP) column and the second as a CZE column. In the ITP stage, the sample was preconcentrated and cleaned up and, in the CZE stage, the anions were separated. Minimal sample treatment *off-chip* was needed (filtration and degassing). LODs were within the micromolar range and the accuracy of the method was not evaluated.

## 4. Real food sample analysis on *microfluidics*

The state of the art of food analysis on CE microchip platforms has recently been revised [76]. In food analysis, sensitivity and selectivity are also the major drawbacks. In contrast to biochemical analysis where the very specific reagents (immunoassay/enzymes) are frequently used and dramatically improve the selectivity in the analysis, in food analysis, the use of bio-reagents is obviously less developed and sample preparation is always needed involving complex sample preparation protocols and analyte separation with advanced detection schemes.

In food analysis, *microfluidics* are also now emerging since, as was mentioned before, sample preparation integration is less developed due to its inherent complexity. The *microfluidics* used are mainly CE microchips applied to both liquid and solid samples. The first approaches focused on exploring fast separation and suitable detection routes for prominent analytes with food significance. In all cases, the sample preparation step is carried out *off-chip* and microchips are merely used as fast analytical separation systems. In consequence, the first "real samples" analyzed are still *easy*, such as nutraceuticals and dietary supplements.

Table 3
Microfluidics (capillary-electrophoresis microchip) in food analysis

| Sample | Analytes detected in food sample | Sample preparation | Microfluidic approach | Analytical characteristics | Remarks | Ref. |
|---|---|---|---|---|---|---|
| Red wine (one) | Chlorogenic, vanillic | Dilution and filtration, *off-chip* | Glass CE microchip, electrochemical detection (SPE electrode) | Separation time (<300 s), good precision, less than 7% in peak areas, external calibration ($r > 0.99$), LOD = 10 μM | Use of red wine (high polyphenolic content), use of different oxidation potentials during wine analysis | [77] |
| Beverages | Inorganic cations (water, juices, beer and milk): ammonium, potassium, calcium, sodium, magnesium. Inorganic anions (water): chloride, nitrate, sulfate | Dilution and filtration, *off-chip* | PMMA microchip, contactlees conductivity | Separation time (<40 s), LODs (90–150 μg/L) | High degree of integration, quantitative analysis is given for all analytes | [78] |
| Vitamin C tablets | Vitamin C | Power and filtration | PMMA | Separation time (<50 s) | Quantitative analysis is given for all analytes | [79] |
| Soft drinks | Preservatives, benzoate, sorbate | *Off-chip* | Simple cross, capacitively coupled contactless conductivity | Good precision (less than 4%), external calibration ($r > 0.99$), LODs 3–10 mg/L | Comparison with CE conventional | |
| Wine, beer | Tryptamine, tryptophan, tyramine | Filtration, *off-chip* | Miniaturized CE-ED, electrochemical detection (Cu electrode) | Separation time (<360 s), good precision (RSD 3.6%), LODs 0.6–1.5 μM | Home-made miniaturised CE system | [80] |
| Green tea extract (nutraceutical) | (+) Catechin, epigallocatechin gallate, (−)epicatechin, epicatechin gallate | Solid–liquid extraction and filtration, *off chip* | PDMS CE, microchip, electrochemical detection | Separation time (<300 s), good precision, less than 7% in peak areas, external calibration ($R^2 > 0.99$), LODs 8–10 μM | Use of suited real sample (high concentration), quantitative analysis is given for all analytes | [81] |
| Pear pulps and commercial juices | Arbutin, ascorbic acid | Juice filtration or solid–liquid extraction and filtration, *off-chip* | Glass CE microchip, electrochemical detection | Separation time (<200 s), good precision, less than 7% in peak areas, external calibration ($R^2 > 0.99$) | Separation of target antioxidant couples | [82] |
| Formulations | Water-soluble vitamins: pyridoxine B6, vitamin C, folic acid | Solution and filtration, *off-chip* | Glass CE microchip, electrochemical detection | Separation time (<130 s), high accuracy (errors less than 7%) | Calibration and determination in less than 350 s, quantitative analysis is given for all analytes | [83] |
| Vanilla pod and sugars | Vanillin, ethyl vanillin | Extraction and maceration, *off-chip* | Glass CE microchip, electrochemical detection | Separation time (<200 s), good precision less than 7% in peak heights | Detection of frauds | [84] |
| Green tea | Arg, The, Gly | Extraction and filtration and derivatization of aminoacids, *off-chip* | PMMA microchip with LIF detection | Separation time (120 s), precision chip-to-chip 3% | Derivatization is not selective to aminoacids, selectivity is improved by removing the high content of catechins in tea samples, validation of the method using HPLC | [85] |
| Natural extract | Total isoflavones | Extraction and filtration | Valveless microsystem | Analysis time (60 s) | Calibration integrated. | [86] |
| Dietary supplements | | *Off-chip* | | | Accuracy is evaluated using a secondary standard (error less than 7%) | |
| Vegetables, fruits and meats | Nitrite | Extraction and 732-cation resin column and, filtration, *off-chip* | PMMA microchip (μFIA), chemiluminiscence detection | Separation time (<120 s), good precision less than 4.1%, external calibration ($r > 0.99$), LOD = 4 μg/Lhydrodynamic flow | Validation respect AOAC official method (Griess method), interferents ($Fe^{2+}$, $Ni^{2+}$, V (V), $Cu^{2+}$, uric acid and vitamin C) | [87] |
| Milk (spiked) | Staphylococcus enterotoxin B | Filtration, *off-chip* | PDMS microchip, fluorescence detection | Sandwich-type immuno assay, fluorescently labelled antibody, LOD = 0.5 ng/ml, hydrodynamic flow | – | [88] |
| Baby powder, milk, sucrose (spiked) | Botulinum neurotoxin A | Filtration, *off-chip* | Polycarbonate ELISA-chip, colorimetric detection | Sandwich-type immuno assay, cross-flow immuno-chromatography, horseradish peroxidase, LOD = 2 ng/ml | Five-fold enhancing of LOD respect the kit commercially established | [89] |
| Infant formula | Folic acid | Filtration, *off-chip* | Commercial polyimide immuchip for ELISA, electrochemical detection (Gold electrode) | Competitive immuno assay, hydrodynamic flow, analysis time = 5 min, R.S.D. 7% alkaline phosphatase | Validation respect reference method (microbiological assay) | [90] |

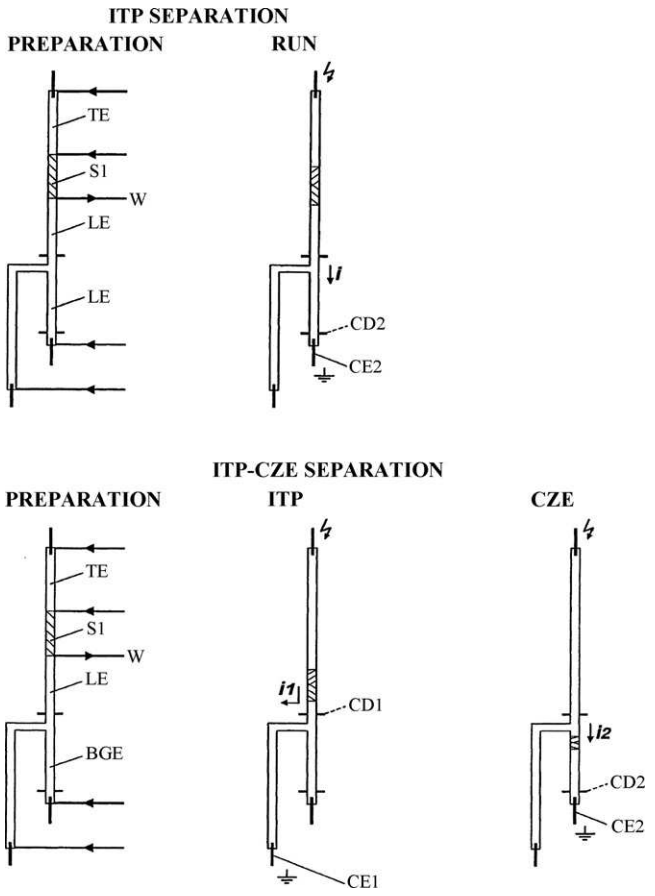



Fig. 4. Schemes of the ITP (the tandem-coupled separation channels) and ITP-CZE separation modes as employed in the separations of inorganic anions on the CC chip (from reference [75], with permission).

Table 3 lists the relevant information about food analysis on, primarily, CE-microchips and other *microfluidic* systems. Next, we are going to discuss the relevant keys to understanding the present role of *microfluidics* in food analysis.

Liquid samples, as opposed to solid samples, require minor sample preparation, and they have been studied by direct injection using both amperometry and conductimetry. This way, two single phenolic acids, vanillic and chlorogenic acid, were detected in red wine samples in less than 300 s using a CE microchip with amperometry detection [77]. The overall protocol involves simple sample preparation *off-chip* (filtration and dilution in an electrophoretic buffer), and the wine sample was directly injected onto the microchip. Selectivity and sensitivity was checked using different oxidation potentials as a strategy to obtain relevant information using the wine sample. As expected, lower potentials provided greater selectivity while higher ones led to increased sensitivity. Red wines are very complex and a lot of polyphenols are present since red wine is one of their most important sources. Probably, the detection of just acid structures was due to the fact that these compounds are prominent, while flavonoids did not show any interference because of the dilution performed.

The elegant approach, based on the use of a universal detector (*contactless* conductivity) coupled to a microchip in the analysis of liquid samples where selectivity is achieved by microchip separation, is illustrated in the papers of Hauser's group [78]. These papers strategically combine the minor sample preparation requirements of this kind of samples (just dilution and filtration) with the inherent characteristics and advantages of (*contactless*) conductivity. Indeed, water, wine, beer, and fruit juice samples were simply diluted in electrolyte solution. Separations involving inorganic cations (ammonium, potassium, calcium, sodium, and magnesium), inorganic anions (chloride, nitrate, sulphate), and the simultaneous analysis of up to 12 inorganic and organic anions (oxalate, tartrate, malate, citrate, succinate, acetate, lactate) in less than 100 s were obtained. In addition, a detailed evaluation of the methods including reliable quantitative data was given indicating the power of these microdevices. The inherent advantages of miniaturization for food analysis using both liquid (beverages) and solid samples (vitamin C tablets) were also demonstrated illustrating the separation of commonly used preservatives such as benzoate and sorbate, and vitamin C using a PMMA microchip coupled with contactless conductivity [79].

Ye et al. [80] fabricated a miniaturized CE-ED system cutting a conventionally fused silica capillary and gluing it onto a Plexiglas plate. This system was used in the determination of three bioactive amines (tryptamine, tryptophan, and tyramine) in wine and beer. Good reproducibility (3.6%), analysis time (6 min), and LOD (μM range) were obtained.

Solid samples are very complex, and tedious *off-chip* extraction processes are always required before analysis. An analysis of five flavan-3-ols (catechins and derivatives) in commercially available green tea extract was performed using a PDMS microchip with MEKC and pulsed amperometric detection by Henry's group [81]. The detection of (+)-catechin, epigallocatechin gallate, (−)-epicatechin, and epicatechin gallate was achieved in less than 3 min in tea extracts (functional food). In addition, two more unidentified peaks were detected during analysis (probably attributable to other polyphenols). A quantitative analysis was performed and good accuracy was demonstrated. Sample preparation was still complex and *off-chip* (methanol–water, stirred extraction, and filtration), and the sample matrix was still "easy" since a nutraceutical was assayed containing high analyte concentrations; however, this paper was relevant since it clearly demonstrated the excellent analytical performance of these microsystems during sample quantitation.

Escarpa's group has proposed different strategies in the analysis of food samples involving natural antioxidants (polyphenols and vitamins) using single channel microchip electrochemistry platforms with electrochemical detection [82,83]. Polyphenolic analysis is very complex because many compounds could contribute to the complexity of the electropherogram obtained (as was also illustrated in Henry's papers). Although fast detection of prominent natural antioxidants including ascorbic acid and polyphenols was obtained in less than 200 s, the analysis of pear samples was very complex and only arbutin in some pulps and ascorbic acid in juice (where up to four peaks were observed) was really detected [82]. Recovery studies using blank samples were carefully performed for both analytes in order to confirm their provisional identification. The detection of phlorizdin (the *fingerprint* analyte of apples and

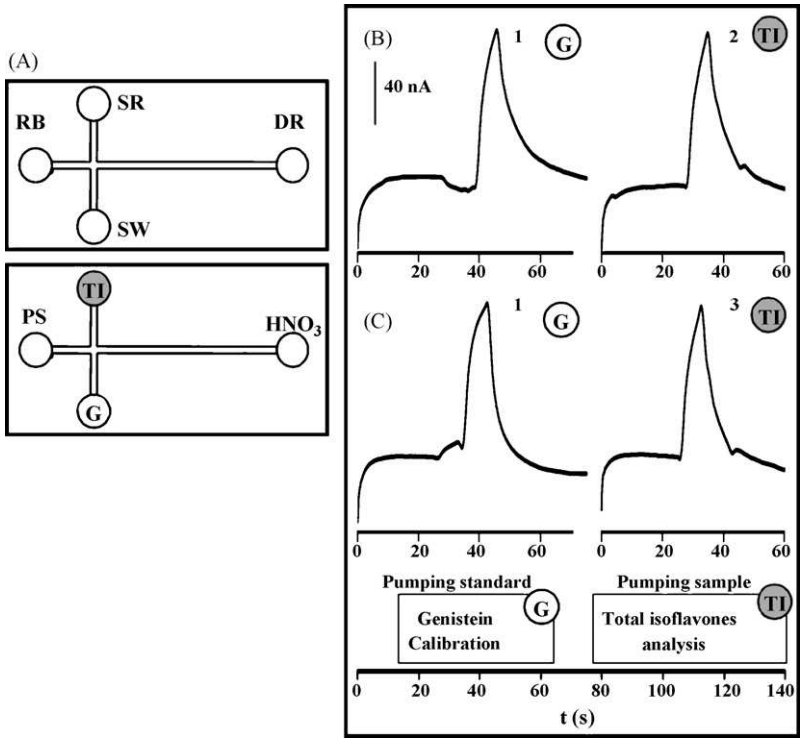



Fig. 5. Microchip layout used with classic and proposed terminology (A): genistein reservoir (G), total isoflavones reservoir (TI), pumping solution reservoir (PS). Calibration (using genistein G) and analysis signals obtained for total isoflavones (TI) in the reference material (B) and soy supplements (C) using the proposed integrated strategy. Conditions: 100 mM genistein peak (G, peak 1), total isoflavones peak [TI, peak 2 (reference material), peak 3 (dietary supplement)] (from reference [86], with permission).

unexpected in pears) in the presence of arbutin (the *fingerprint* of pears) was also noticed, demonstrating the possibility of adulterations. Real detection and the quantitative analysis of water-soluble vitamins has been reported in several formulations containing one or more of the analyzed vitamins at different concentration levels in less than 130 s [83]. The amount of each vitamin declared by the manufacturer (per capsule) on the label closely matched the values obtained using the microchip protocol (relative error ranging from 2 to 9%). The well-known problems associated with the inherent features of electrokinetic injection (modification of ionic strength, compatibility between buffers and solvents/matrices) made quantitation more difficult than expected in those samples with high mineral salt content.

Very recently, an approach for detecting fraud in vanilla samples based on the analysis of ethyl vanillin and vanillin was proposed [84]. Vanillin was really detected in vanilla beans (*Vanilla planifolia* and *Vanilla tahitensis*), being this compound the prominent compound in vanilla extracts and which confirms their authenticity. In addition, ethyl vanillin was really detected in certain vanilla sugars indicating the non-natural origin of the vanilla flavor used since the presence of ethyl vanillin is unambiguous proof of fraud. On the contrary, vanillin was really detected in the other sugar samples which did not necessarily indicate a natural vanilla origin because, in and of itself, the presence of vanillin is not unambiguous proof of the natural origin. A detailed quantitative analysis is given and important differences in the quantitative levels of vanillin were found between natural and adulterated samples revealing the possibilities of the method in control analysis.

Three prominent green tea amino acids (theonine (The), arginine (Arg), and glycine (Gly)), previously extracted and derivatized *off-chip*, were separated onto a PMMA microchip format using LIF detection in less than 2 min [85]. While sensitivity was not a challenge because of the detection route used, the selectivity of the analysis was improved by removing catechins, since the derivatization scheme involved both kind of compounds, amino acids and catechins.

Recently, methodological calibration and analysis on board a planar *microfluidic* device to determine *total* isoflavones in soy samples was proposed [86]. The strategy, in connection with microchip layout, is shown in Fig. 5. Accuracy (systematic error below 6%) is demonstrated for the first time using these microsystems with a secondary standard from the Drug Master File (SW/1211/03) as *reference* material. Ultra-fast calibration and analysis of total isoflavones in soy samples was successfully integrated, taking only 60 s each, notably enhancing the analytical performance of these microdevices with an important decrease in overall analysis times (less than 120 s) and with an increase in accuracy by a factor of 3. In addition, the method was applied to the analysis of soy tablets with acceptable accuracy.

He et al. [87] have developed a method to determine nitrite in different food samples (vegetables, fruits, and meats) using a PMMA chip as a μFIA system with chemiluminescence detection (luminol-ferricyanide system). Exhaustive extraction and sample treatment (732-cation resin column) was needed per ana-

lyzed solid sample. Good analytical performance was achieved with a reproducibility of 4.1%, a detection limit of 4 μg/l, and an analysis time under 2 min. An interference study was performed discovering that $Fe^{2+}$, $Ni^{2+}$, V (V), $Cu^{2+}$, uric acid, and vitamin C interfered with the analysis. Nevertheless, the method was compared with the AOAC official method (Griess method) with excellent results.

To improve selectivity and sensitivity, some immunoassays have been developed on *microfluidic* devices for food analysis as an alternative strategy. Cheng et al. [88] determined staphylococcal enterotoxin B in spiked dry milk using a PDMS chip with fluorescence detection. Sandwich-type immunoassays were developed utilizing the chip as a chamber reaction. In the first step, the chip surface was functionalized with biotinylated antibodies. In the second step, the sample was hydrodynamically introduced onto the chip and, then, the fluorescently labeled secondary antibodies were added. Finally, the chip was imaged and quantified on a fluorescence scanner. An excellent LOD was achieved with this method (0.5 ng/ml).

Botulinum neurotoxin A [89] was detected in spiked food samples (infant formula, milk, sucrose) on a polycarbonate ELISA-chip with colorimetric detection. A cross-flow immunochromatographic assay was developed in which a sandwich-type immunoassay with an enzyme-linked antibody was performed vertically, and a flow of the enzyme substrate revealed the presence of Botulinum neurotoxin A horizontally. An excellent LOD (2.0 ng/ml) was achieved, enhancing five-fold the LOD established by the commercial kit.

Another interesting ELISA on *microfluidic* devices was performed to determine folic acid in infant formula [90]. A commercial polyimide Immuchip ELISA with eight channels and hydrodynamic flow was used. In this case, a competitive assay between free antigens (sample analyte) and fixed antigens on the surface of the chip was performed and the product of the enzyme reaction was monitored by electrochemical detection ($E = 250$ mV). Excellent analytical performance (reproducibility 7%, LOQ 2 ng/ml, and analysis time 5 min) was obtained. The method was validated with the reference method (microbiological assay).

## 5. Conclusion and future prospects

Nowadays, and strictly speaking, there are few papers that deal with the analysis of real samples using *microfluidic* devices regardless of the application area. It clearly shows the enormous difficulties that the analysis of real samples presents on a microscale. Sensitivity and selectivity are the main challenges to developing a true "lab-on-a-chip," with their improvement in terms of the use of ultra-sensitive detectors and the integration of sample treatments on the *microfluidic* devices being the main requirements. Moreover, additional efforts have to be made towards the validation of the methods to demonstrate the reliability of *microfluidic* systems. In spite of these drawbacks, incredible analysis times, low sample and reagent consumptions, and interesting applications involving clinical, environmental, and food analysis have been published.

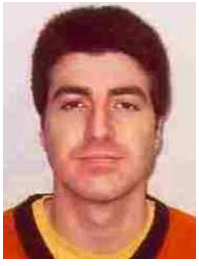
No doubt exists that performing sample preparation *on-chip*, discovering novel ultra-sensitive detection routes, and systematically using *smart* bioreactives and nanotechnology are the keys to defining the success and incorporation of *microfluidic* devices in our laboratories.

## Congratulations

I had the privilege of knowing Dr. Wang in 2001 during my *post-doc* position at NMSU and I was highly impressed by the quality of this person. I soon realized that not only was he an extraordinary scientist but a unique, talented human being. I firmly believe that we have before us a scientist with enormous creativity and a novel vision. I will never find the right words to say thanks!

Thank you very much, Dr. Wang!

*What can we expect from words?*
*At times, they hardly sing.*
*But today they are here to tell us*
*That in your intelligence you are great*
*That you belong to that group of great men,*
*Different men, who live awake because they have a dream.*
*Mentor of mine,*
*My heart sings something about you*
*I find you in my memory*
*Like a friend that I have loved.*
(Poem written by Alberto Escarpa for Dr. Wang in his 60th birthday)



Agustín González

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