Microfluidics in Bioanalytical Instrumentation

by

Petr Smejkal

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of

Doctor of Philosophy

UNIVERSITY OF TASMANIA

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Declaration of Originality

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Signed Petr Smejkal
23rd May 2013

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Signed Petr Smejkal
23rd May 2013
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<tr>
<td>ACA</td>
<td>6-aminocaproic acid</td>
</tr>
<tr>
<td>ADM</td>
<td>Assay developer mode</td>
</tr>
<tr>
<td>AF647</td>
<td>Alexa Fluor 647</td>
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<tr>
<td>Ala</td>
<td>B-alanine</td>
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<tr>
<td>APTS</td>
<td>8-aminopyrene-1,3,6-trisulfonic acid</td>
</tr>
<tr>
<td>BDB</td>
<td>5-bromo-2,4-dihydrobenzoic acid</td>
</tr>
<tr>
<td>BGE</td>
<td>Background electrolyte</td>
</tr>
<tr>
<td>BLAC</td>
<td>Boronic acid-lectin affinity chromatography</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BTP</td>
<td>1,3-bis[tris(hydroxymethyl)methylamino]propane</td>
</tr>
<tr>
<td>CCD</td>
<td>Charged coupled device</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>CEC</td>
<td>Capillary electrochromatography</td>
</tr>
<tr>
<td>CGE</td>
<td>Capillary gel electrophoresis</td>
</tr>
<tr>
<td>CIEF</td>
<td>Capillary isoelectric focusation</td>
</tr>
<tr>
<td>CITP</td>
<td>Capillary isotachophoresis</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>CZE</td>
<td>Capillary zone electrophoresis</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DFR</td>
<td>Dry film resist</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EATA</td>
<td>Electrokinetic analyte transport assay</td>
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<tr>
<td>EOF</td>
<td>Electroosmotic flow</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>FFCZE</td>
<td>Free flow capillary zone electrophoresis</td>
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<td>FFITP</td>
<td>Free flow isotachophoresis</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GA</td>
<td>Glycolic acid</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GE</td>
<td>Gel electrophoresis</td>
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<tr>
<td>GG</td>
<td>glycylglycine</td>
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<tr>
<td>HEC</td>
<td>Hydroxyethyl cellulose</td>
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<tr>
<td>HEMC</td>
<td>Hydroxyethylmethyl cellulose</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>HIBA</td>
<td>α-hydroxyisobutyric acid</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>HPMC</td>
<td>Hydroxypropylmethyl cellulose</td>
</tr>
<tr>
<td>HPPE</td>
<td>High performance paper electrophoresis</td>
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<tr>
<td>HV</td>
<td>High voltage</td>
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<tr>
<td>ID</td>
<td>Inner diameter</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusation</td>
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<tr>
<td>IF</td>
<td>Indirect fluorescence</td>
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<tr>
<td>IFD</td>
<td>Indirect fluorescence detection</td>
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<tr>
<td>ITP</td>
<td>Isotachophoresis</td>
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<td>LE</td>
<td>Leading electrolyte</td>
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<tr>
<td>LED</td>
<td>Light emitting diode</td>
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<tr>
<td>LIF</td>
<td>Laser induced fluorescence</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>LOD</td>
<td>Limit of detection</td>
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<td>LOQ</td>
<td>Limit of quantification</td>
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<tr>
<td>MEKC</td>
<td>Micellar electrokinetic chromatography</td>
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<tr>
<td>MEMS</td>
<td>Micro electro-mechanical systems</td>
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<tr>
<td>MES</td>
<td>Morpholinoethane sulfonic acid</td>
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<tr>
<td>MFC</td>
<td>Microfluidic flow control</td>
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<tr>
<td>MHEC</td>
<td>Methylhydroxyethyl cellulose</td>
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<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>PC</td>
<td>Polycarbonate</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>pDMA</td>
<td>Poly(2-dimethylaminoethyl methacrylate)</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
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<tr>
<td>PEO</td>
<td>Poly(ethylene oxide)</td>
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<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
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<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
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<tr>
<td>R6G</td>
<td>Rhodamine 6G</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>TAPS</td>
<td>N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid</td>
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<tr>
<td>TE</td>
<td>Terminating electrolyte</td>
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<tr>
<td>tITP</td>
<td>Transient isotachophoresis</td>
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<tr>
<td>USB</td>
<td>Universal serial bus</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VIS</td>
<td>Visible</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
</tr>
<tr>
<td>ZE</td>
<td>Zone electrophoresis</td>
</tr>
<tr>
<td>µ-TAS</td>
<td>Micro-Total Analytical Systems</td>
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Abstract

Portable and field deployable analytical instruments are attractive in many fields, including medical diagnostics where point-of-care and on-site diagnostics systems capable of providing rapid quantitative results have the potential to improve the productivity and quality of medical care. A major limitation and impediment to the usage of portable and field deployable microfluidic chip based analytical instruments in solving real world analytical problems has been the scarcity of commercially available portable or field deployable platforms, which are fully flexible for research.

The bench-top analytical instrument, the Agilent Bioanalyzer 2100 used in this research is a microfluidic chip-based platform with fluorescence detection system, available on the market since 1999. Originally, this instrument was capable of electrophoretic analysis of deoxyribonucleic acids (DNA) and ribonucleic acids (RNA), with user-tailored application solutions including chips, reagents and pre-developed methods. More applications, specifically electrophoretic analysis of proteins and flow cytometry, were introduced later.

The commercial success of this instrument was achieved thanks to an on-a-chip platform which enables fast analysis (separation time around a minute or even less) with minimal sample consumption (microlitres). In this research we built on our long-standing research collaboration with Agilent Technologies, who provided us with access to the Bioanalyzer ‘script editor’ so that we could develop and implement our own methods, applicable in principle for any chip and analysis.

Chapter 1 offers an introductory overview of miniaturised analysis followed by a comprehensive overview focused on ITP on a microfluidic chip techniques.

Chapter 2 gives a brief overview of commercially available microfluidic chip based analytical platforms. This is followed by the first experiments aimed at developing a new
application for the commercially available microfluidic chip based electrophoretic system, the Agilent Bioanalyzer 2100. The Bioanalyzer was used in this work as fully flexible research tool, which was made possible by using an open software platform in assay developer mode. The new application was investigated for the CE separation of 8-aminopyrene-1,3,6-trisulfonic acid (APTS) fluorescently labelled oligosaccharides. Their separation was successfully implemented with the analysis completed using the commercially available Agilent DNA chip in under a minute, representing an improvement in the speed of analysis compared to CE by more than an order of magnitude. This result demonstrates that the commercial chips, specifically DNA chips, and the Bioanalyzer, have the capacity for a wider spectrum of applications. To compare with classical CE, the resulting electropherograms were obtained faster but the final resolution was poorer, a result that initiated further investigations aimed at exploring different electrophoretic methods.

In Chapter 3, isotachophoresis of carboxylic acids on a DNA chip with electrokinetic injection of a sample and indirect fluorescence detection was investigated. The indirect fluorescence detection was carried out by using fluorescent dye rhodamine 6G (R6G) as a counter ion present in leading electrolyte. Limits of detection (LOD) in millimolar range were obtained for different model analyte acids (oxalate, pyruvate, fumarate, malate, mandelate, 2-hydroxyisobutyrate, succinate, acetate). The method was later used for quantification of benzoate in a variety of soft drinks, which was the first documented use of chip ITP with indirect fluorescence detection for the separation of real samples. In this chapter we fully appreciated the properties of ITP, as no sample preparation, except degassing and sample dilution was necessary prior to the analysis.

In Chapter 4, the method developed in the previous chapter, using a hydrodynamic injection of sample, was tested for the separation of lactate in human serum but failed due
to the design of the DNA chip, which showed insufficient compatibility with ITP. A
twofold improvement of the method sensitivity for lactate in human serum was achieved
with in-house designed and microfabricated ITP glass chips. The obtained results still
showed significant room for improvement for higher sensitivity and shorter analysis time.
In Chapter 5, a twentyfold improvement of sensitivity, if compared with ITP separation of
lactate from human serum on a DNA chip, was achieved when specifically designed ITP
chips, microfabricated by dry film resist technology were used. Another advantage of the
chips introduced in this chapter was smaller resistivity of the separation channel that was
used to implement higher separation current and speed up the total analysis time. An
under-a-minute separation and quantification of lactate (calculated LOD of lactate was
42 µM) was achieved in human serum sample. Except for serum dilution, no sample
pretreatment was required prior to the analysis.
The research results presented here illustrate that the general objective of demonstrating
the feasibility of combining the positives of a commercial, field-deployable, chip analytical
platform with full research flexibility in designing new chips and methods, has been
achieved. It has been shown that from a practical viewpoint, the Bioanalyzer 2100 is
convenient to use, not just for different analytes but also for different electrophoretic
methods than those for which it was originally designed. The CE separations and analysis
of APTS labelled oligosaccharides from human plasma, ITP separations, and analysis of
benzoate in soft drinks and of lactate in human serum shown in this work are indicative of
the broader application capability and potential of the Bioanalyzer as a field deployable
research instrument.
Aims

The general aim of this research addressed the research question of the possibility of implementing of full research flexibility to a commercially available microfluidic chip based electrophoretic platform. From a practical analytical point of view, this research aimed at a significant broadening of the applicability of the commercially available desktop analytical instrument, the Agilent Bioanalyzer 2100, while implementing a number of strategies to maximally exploit research flexibility.

The specific aims of this research were to investigate and implement the above approach, step by step, as follows:

- Starting from generic chip-CE applicable to, in principle, any chip-CE separation and analysis using the commercial DNA chips,
- Progressing to ITP separations, using the commercial DNA chips,
- Following with adding further research flexibility by following onto investigations with in-house designed chips, first fabricated in glass, then using a less cost polymer chips fabricated from dry film photoresist.
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Chapter 1

General Introduction

1.1 Microfluidics in analytical chemistry

Miniaturisation of current analytical systems is a necessary way to be able to continue achieving further enhancements in analytical performance per unit of time, size and weight of the instrument, and the price per instrument or per analysis. Therefore, it attempts to reduce the required amounts of sample, solvents and reagents, increase the efficiency and shorten total analysis times with a smaller, potentially portable instrument. Since the breakthroughs in microelectronics in the early 1960s [1], miniaturisation established itself as a fresh and exciting transdisciplinary trend, impacting on all fields of science and technology, and opening countless new possibilities. The first attempt to use microfabrication technologies in analytical chemistry was miniaturised gas chromatograph made in a silicon wafer, introduced in 1979 by Terry et al. [2]. A decade later in 1990 [3], miniaturisation led to the concepts of Lab-on-a-Chip and Micro-Total Analytical Systems (µ-TAS), which proposed to integrate all analytical laboratory processes into a single chip-based analytical platform. The µTAS concept predicts that the integrated analytical systems will be faster, enable increased analytical throughput, and have the ability to perform multiple functions in an automated way. This makes it ideally suited for portable and field-deployable instrumentation for on-site analysis (in biomedical analysis often called "point-of-care") [4]. The inception and development of microfluidics analytical platforms, significantly supported by The Human Genome Project [5], started in the early 1990s. Since then a large number of research papers focused on miniaturisation in analytical chemistry has
been published. Details of microfluidics development can be found in reviews from Manz et al., periodically monitoring this rapidly growing field of analytical chemistry [6-11]. The evidence of outstanding growth in the development of microfluidic chips is documented by numerous other authors. For example, Székely and Guttman described the fabrication of microfluidic chips from glass, quartz and PDMS for electrochromatography [12]; and Becker and Gärtner wrote an interesting review describing the fabrication of microfluidic chips from different polymer materials [13]. Increased attention has been focused on soft lithography and the fabrication of PDMS chips in several other reviews [14-17]; and an interesting review by Coltro et al. described toner and paper-based fabrication of cheap and disposable microfluidic chips [18].

The success of µTAS research is significantly reflected in its commercial applications [19-24]. Currently, the market offers a wide range of commercially available handheld instruments, which use selective chemical, immunochemical or micro-electrochemical sensors often in combination with single use disposable chips. These systems offer “countless” different applications, but each application requires a specific chip and often a specific handheld instrument. More universal commercially available microfluidic analytical platforms are based on high resolution separation techniques such as high performance liquid chromatography (HPLC) or capillary electrophoresis (CE). While the HPLC microfluidic chip based systems are versatile, their dimensions are usually larger than the dimensions of their classical HPLC predecessors and we cannot expect portability with these systems. The electrophoretic platforms are typically bench-top instruments but relatively small technical modifications enable downscaling to a portable, battery operated instrument. CE is a versatile separation method with applications ranging from small molecules including drugs [25-28], preservatives [29-
30], herbicides and pesticides [31-34], glycans [35-38], carboxylic acids [39-42] to macromolecules including proteins, DNA and even whole cells and bacteria [16, 43-44]. Currently, the commercially available electrophoretic platforms target the analysis of macromolecules (DNA, RNA and proteins. The main aim of this thesis is to explore the possibilities of extending the applications of commercially available electrophoretic platforms to the analysis of small molecules.

1.2 Modes of electrophoresis

The 1940’s could be considered as the early stages of electrophoresis development [45]. Paper electrophoresis for the separation of e.g. amino acids and small organic molecules and slab of gel electrophoresis (GE) using starch and later agarose gels for separation of large molecules such as peptides, proteins and oligonucleotides, were the most powerful separation techniques.

Paper electrophoresis became more attractive with the introduction of high performance paper electrophoresis (HPPE) [46]. Experimental problems especially with Joule heating and associated electrolyte evaporation led to the technique being abandoned.

The separation of small molecules became more efficient with the invention of HPLC, but for large molecules GE remained the method of choice. In 1960’s the popularity of GE further increased with the development of isoelectric focusation (IEF) and later sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Those laborious and time-consuming methods become to be a standard in routine laboratories and have not been replaced until 1990’s.

The revolution in electrophoretic methods came in 1980’s with the introduction of narrow bore silica capillaries (20-100 μm i.d. and 375 μm o.d.) and continued in the 1990’s with the introduction of μTAS concept and microfluidic chips [3]. The high
surface-to-volume ratio of capillaries allows for very efficient Joule heat dissipation. To compare with a maximum separation voltage of 15-40 V/cm on the slab gel, the capillaries can run separation with an applied voltage of 100-1000 V/cm. Most of the methods developed on the slab gel were transferred into the capillary format and later into the microfluidic chip format, and applications extended to a range from inorganic ions and small molecules to DNA, proteins and even whole cells. In practice, the six most commonly used capillary electromigration methods are capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary electrochromatography (CEC), capillary isoelectric focusing (CIEF), capillary gel electrophoresis (CGE), and capillary isotachophoresis (CITP).

As mentioned before, the focus of this work was electrophoretic separation of small molecules on the microfluidic chip with a short separation channel (Agilent DNA chip) and with instrument using laser induces fluorescence (LIF) and light emitting diode (LED) induced fluorescence detection (Agilent Bioanalyzer 2100). This aim limited our selection to electrophoretic methods historically used for separation of small molecules to CZE, MEKC, CEC and CITP. MEKC and CEC require a (pseudo) stationary phase and whilst this could help to resolve very similar analytes, it is not expected this additional separation power would be required in this project because the electrophoretic mobility of the target analytes will be sufficiently distinct. The CZE separates ions from narrow injected zone of sample in background electrolyte (BGE) according to their electrophoretic mobilities. For a short separation channel a narrow injection zone is required to achieve a satisfactory resolution. If a narrow injection zone is introduced in the separation channel, either high concentration samples or sensitive detection system are required. The CZE method therefore was not the most appropriate for our purposes. ITP was targeted here because of its self-focusing power. The ionic
analytes are injected between leading electrolyte (LE), containing co-ion with the highest mobility in the system and counter ion, which determines the pH along the separated zones, and terminating electrolyte (TE), which contains the lowest mobility co-ion in the system. After a constant separation current or voltage is applied, a low electric field strength is created in the high mobility and relatively more conductive LE, in contrast with the high electric field strength in the low mobility, less conductive TE. In the first stage of ITP, the ions from the sample migrate according to their electrophoretic mobilities and separate one from each other. The fastest ion cannot overtake the LE as the LE co-ion has the highest mobility in the system. Therefore the fastest analyte ion forms a zone behind the LE zone at a concentration limited by its pKa and the concentration of the LE co-ion. The length of this zone increases until the rest of the sample is depleted of this ion. Once this condition has been reached, a so-called steady state has been achieved. The second fastest ion forms a zone behind the first analyte following the same principles as the first ion. Once steady state has been reached for all ions present, their zone lengths are proportional to the amount of ionic species present in the sample. The advantage of ITP over CZE is in its self-focusing effect. In CZE separated peaks broaden by diffusion, and also in the channel bends and irregularities at the channel wall [47]. In ITP with its self-focusing property, analytes will migrate back into its own zone because of the discontinuous electric field, slowing down when getting ahead into a zone with lower field strength and accelerating when falling behind in a zone with a higher field strength. The theory of ITP has been described in detail previously [48-49]. The next section provides a comprehensive overview of isotachophoresis on a chip.
1.3 Isotachophoresis on a chip

Ninety years ago, Kendall and Crittenden introduce the “Ionic Migration Method”, and while the attempt to separate the isotopes of chloride failed, they succeeded in separating rare earth metals and some simple acid anions [50]. The “Ionic Migration Method”, is fundamentally identical with the method known today as isotachophoresis (ITP).

The period of the greatest popularity for ITP was through the 1970s, when this method almost “touched the stars” in the third Skylab mission sent by NASA [51]. The success of ITP was based upon the large bore capillaries used at that time (inner diameter (ID) of hundreds of micrometers), which were not suitable for capillary zone electrophoresis (CZE) due to excessive diffusion caused by Joule heating. The self-sharpening between the boundaries counters diffusion, making ITP immune for band broadening due to Joule heating in large bore capillaries. Later ITP temporarily lost its popularity with the introduction of silica capillaries with an inner diameter of tens of micrometers, which enabled Jorgenson to introduce CZE in 1981 [52].

The comeback of ITP was initiated by the need for a powerful preconcentration technique to increase the sensitivity in CZE. This could be achieved by coupling ITP and CZE [53], later followed by the instrumentally less complicated transient ITP-CZE [54]. Advances in ITP over the last two decades has been well monitored and periodically reviewed by Gebauer and Bocek [55-59].

The renewed interest in ITP coincided with the introduction of the μTAS and Lab-on-a-Chip concepts [3]. Microfluidic devices for CZE and MEKC separations win the early 1990s, and first report of ITP on a chip was published in 1998 by Walker et al. [60]. It could be argued, however, that ITP on a chip was reported 23 years earlier, long
preceding the Lab-on-a-Chip concept. In 1975 Bocek et al. demonstrated the separation of six acids of Krebs cycle by ITP in grooves engraved in a Perspex block [61]. This block contained separation channels, detection electrodes as well as electrolyte reservoirs. The separation channel was 25 cm long, 200 µm deep and 1000 µm wide and was sealed with a polytetrafluoroethylene (PTFE) membrane. Attachment of the device to a thermostated metallic plate ensured sufficient cooling when the separation current was applied. A schematic of the Perspex ITP chip is shown in Figure 1.1. While the dimensions of this apparatus are large in comparison with modern microfluidic chips, the rationale is identical to that behind the Lab on a Chip concept and was far ahead of its time. The concept of ITP on-a-chip was revisited by Walker et al. using newer and more precise microchip fabrication techniques. Amidst many reviews on advances in chip ITP [62-69], the following text contains comprehensive overview of chip ITP techniques with deeper focus on the chips design since this technique was introduced for the first time.

Analytical microfluidic chip ITP and its applications are introduced in section 1.3.1 “Analytical isotachophoresis”. To improve detection sensitivity on microfluidic chips, ITP is often used as a preconcentration technique rather than analytically. Section 1.3.2 focuses on the use of ITP as preconcentration in CZE and other analytical techniques while transient ITP is discussed in section 1.3.3. The experimental freedom in the design of microchips inspired the development of different ITP techniques, including “Peak and plateau mode isotachophoresis” as described in section 1.3.4, “Gradient Elution isotachophoresis” and “Free Flow isotachophoresis” are discussed in section 1.3.5 and section 1.3.6, respectively. This chapter is concluded with a table listing the variety of analytes and samples analysed by microfluidic chip ITP and related (Table 1.III).
Fig. 1.1) ITP separation block made from Perspex, developed and presented by Bocek et al. in 1975. 1. and 15. platinum electrodes; 2. and 6. LE compartment; 3. glass frit; 4. connection with LE reservoir; 5. PTFE valve; 7. detection platinum electrodes; 8. thermostated metallic plate; 9. capillary groove; 10. PTFE foil; 11. septum; 12. drain tube; 13. PTFE three-way valve; 14. TE compartment; 16. connectors of the detector; 17. Perspex block. Reproduced with permission [61].
1.3.1 Analytical isotachophoresis

As mentioned before, ITP on a planar substrate was first realised in 1975 by Bocek et al. [61], preceding the Lab on a Chip concept. In 1998 Walker et al. revisited this approach by presenting a constant voltage ITP separation of herbicides (paraquat and diquat) on a single channel chip using Normal Raman Spectroscopy (2 W, 532 nm NdYVO₄ excitation laser) for detection [60]. The 40 µm wide, 75 µm deep and 210 mm long channel was fabricated on a glass microscope slide by using photolithography and chemical wet etching, sealed with a 120 µm thick glass coverslip using UV cured epoxy glue. The channel curved to fit a 210 mm long channel on a 75 mm microscope slide. The sample was electrokinetically injected into the chip channel prefilled with LE (sulphuric acid) by applying constant voltage 4 kV for 1-2 minutes. After that, the sample was replaced with TE (Tris), and separation was run under high voltage (HV) (8 kV).

The team of Prest and Baldock has made a significant number of contributions to chip-based ITP. In 1999, a mixture of sodium and potassium was separated by ITP (constant voltage) on a polydimethylsiloxane (PDMS) microfluidic chip [70-71]. Channels were cast from a copper template and sealed by adhering the top plain PDMS block. Despite the double T injection geometry design, the chip was used as a single channel device in this work. A platinum-iridium wire was integrated between the PDMS layers 5 mm from the LE reservoir for single electrode conductivity detection. Sample was mixed with TE in TE reservoir and the separation was carried out by applying constant voltage 1 kV between the reservoirs. Later, the same approach was used for ITP separation of four metal cations (lithium, lanthanum, dysprosium and ytterbium) in half the time required for the same separation in a capillary system [72]. In 2002, a new design was
introduced for bidirectional ITP [73]. The poly(methyl methacrylate) (PMMA) chip with cross injection geometry contained 300 µm wide and 400 µm deep channels. The sample was hydrodynamically injected from the middle of the separation channel and detection electrodes of a dual conductivity detector were placed on each end of the separation channel (39 mm from the point of injection). A hydrodynamic system using pressure and valves was used to introduce all electrolytes into the chip before a constant voltage of 1000 V, was applied to drive the ITP separation. Injected from the centre of the channels, anions (Cl\(^-\), NO\(_3^-\), SO\(_4^{2-}\), F\(^-\)) migrated towards the anode and cations (Cs\(^+\), NH\(_4^+\), Na\(^+\), Li\(^+\)) migrated towards the cathode.

In 2003 Baldock et al. presented fully polymeric microfluidic chips [74]. The chips, made from Zeonor 1060R and polystyrene with injection moulded electrodes (separation and detection) made from conductive polymers (8% carbon black-filled polystyrene, 40% carbon fibre-filled nylon and 40% carbon fibre-filled polystyrene). Both chips used simple cross geometry structure fitted with four reservoirs for LE, TE, sample and waste made from syringe barrels. The injection of electrolytes was realised by applying pressure (gravity) to reservoirs and by subsequent opening and closing of valves between reservoirs and chip. LE was loaded first by opening valves at LE and waste reservoirs. Then TE was loaded by opening valves at TE and waste reservoirs. Sample was injected between LE and TE by opening valves at sample and waste reservoirs. For the analysis of dyes and various inorganic ions analysis, all valves were closed and constant voltage/current was applied between polymer separation electrodes.

In 2003 Prest et al. fabricated a new PMMA microfluidic chip for ITP with conductivity detection [75]. The schematic of the chip and its operation are shown in Figure 1.2. The microchannels were direct milled in a 78 mm long, 78 mm wide and 6 mm thick PMMA and sealed with self-adhesive polyester laminate [73]. The application was the
ITP separation of a mixture containing As$^{3+}$ and As$^{5+}$ and a mixture containing As$^{3+}$, As$^{5+}$, Mo$^{6+}$, Sb$^{3+}$ and Te$^{4+}$ using conductivity detection. Similar to previous reports, the method was faster than by capillary ITP but not sensitive enough for real industrial waste stream samples. This chip design was well used, with applications ranging from inorganic ions to amino and organic acids summarised in Table 1.I.

In 2004 Baldock et al. introduced a pressure driven system for the injection of variable amounts of sample between LE and TE [76]. The separation channel was connected with a U-shaped sample-waste channel through a 1 mm long injection channel at a 45° angle. The U-shaped channel was filled with sample after filling the separation channel with LE and TE and sample was injected between LE and TE. The 45° angle reduced leaking of the sample after separation current (30 $\mu$A) was applied. The system that was equipped with a conductivity detector showed a good repeatability for analysis of a mixture of ten metal ions (Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, La$^{3+}$, Nd$^{3+}$, Gd$^{3+}$, Cu$^{2+}$).

In 2006 Prest et al. presented a new double T design (micro milled PMMA) for the separation and quantification of Cl$^-$ under 100 s with LOD 2.2 mg/mL and a throughput of 20-30 samples per hour [85]. While the LOD of 2.2 mg/mL is relatively high, the LOD was not an advantage of using a chip in this paper, but the rapid, less than 100 s analysis time was strength.
Fig.1.2) ITP chip for hydrodynamic injection controlled by pressure and valves.

LE was pumped from reservoir A to reservoir E and TE from reservoir C to E. To control the amount of injected sample, the sample injection was done in two steps. In the first step, a pressure was applied to reservoir D with open valves at D and E. In the second step, the valve at E was closed and valve at B was opened introducing larger amount of an injected sample. A separation current of 30 µA was applied between reservoirs C and A. Reproduced with permission [75].
<table>
<thead>
<tr>
<th>Analyte</th>
<th>sample</th>
<th>LE</th>
<th>TE</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ascorbic acid</td>
<td>photographic developing solution</td>
<td>5 mM GA + Tris (pH 8) + 1 g/L HEC</td>
<td>10 mM GG + Ba(OH)$_2$ (pH 8.5)</td>
<td>[77]</td>
</tr>
<tr>
<td>amino acids</td>
<td>potential applications for beverages or urine samples</td>
<td>10 mM GA + 2mM MgSO$_4$ + benzyamine (pH 9) + 1 g/L HEC</td>
<td>20 mM β-alanine + Ba(OH)$_2$ (pH 9.7)</td>
<td>[78]</td>
</tr>
<tr>
<td>Mn$^{2+}$, Cr$^{3+}$, Fe$^{2+}$, Co$^{2+}$, Zn$^{2+}$, Ni$^{2+}$</td>
<td>solutions from the metal processing industry</td>
<td>10 mM NaOH + 2.5 mM malic acid + propionic acid (pH 4.9) + 1 g/L HEC</td>
<td>10 mM tetrabutylammonium hydroxide</td>
<td>[79]</td>
</tr>
<tr>
<td>Cl, Br, I</td>
<td>spring water</td>
<td>12 mM HNO$_3$ + 0.4 mM In(NO$_3$)$_3$ + GG (pH 3) + 1 g/L HEC</td>
<td>20 mM cyanoacetic acid</td>
<td>[80-81]</td>
</tr>
<tr>
<td>As$^{5+}$, Se$^{4+}$, Se$^{6+}$</td>
<td>model sample</td>
<td>20 mM HNO$_3$ + histidine (pH 5.5) + 0.5 g/L mowiol</td>
<td>20 mM Gallic acid</td>
<td>[82]</td>
</tr>
<tr>
<td>As$^{3+}$, Te$^{4+}$, Te$^{6+}$</td>
<td>model sample</td>
<td>5 mM GA + N-methyl-D-glucamine (pH 9.8) + 0.5 g/L mowiol</td>
<td>20 mM β-alanine + Ba(OH)$_2$ (pH 10)</td>
<td></td>
</tr>
<tr>
<td>Cl, ClO$_3^-$, ClO$_4^-$</td>
<td>explosive residues</td>
<td>1.75 mM HNO$_3$ + 2.75 mM In(NO$_3$)$_3$ + GG (pH 3) + 1 g/L HEC</td>
<td>20 mM cyanoacetic acid</td>
<td>[83]</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>mineral water</td>
<td>10 mM CsOH + 2 mM malonic acid + pivalic acid (pH 5.1) + 1 g/L HEC</td>
<td>10 mM Tris</td>
<td>[84]</td>
</tr>
</tbody>
</table>

GA – glycolic acid, GG – glycyglycine, HEC – hydroxyethyl cellulose
Recently, the group of Santiago has been very active in the field of ITP research. In analytical ITP, this group has been interested in exploring indirect fluorescence detection. Fluorescent spacers were used for the detection of ITP zones of non-fluorescent analytes using a single channel of a commercially available glass device produced by Caliper (NS-95) [86]. The chip was filled with LE and sample was hydrodynamically loaded from the opposite reservoir. After injection sample was replaced with TE. The separation of two amino acids was visualised by an epifluorescent microscope using three fluorescent spacers (Oregon green carboxylic acid, fluorescein and bodipy). The isotachopherogram contained three peaks from the three fluorescent dyes with the space between these peaks related to Ser and Phe. To enable the detection of three analytes, acetic acid, aspartic acid and 3-phenyl-propionic acid, a fourth tracer in the form of fluorescein isothiocyanate (FITC) was added, illustrating a limitation of this approach. To increase the number of fluorophores, Bercovici et al. used fluorescently labelled ampholytes designed for IEF as spacers for the detection of 2-nitrophenol and 2,4,6-trichlorophenol [87].

A fundamentally different approach from the same group was introduced by Chambers et al. using a fluorescent counter ion tracer in LE (R6G) or a fluorescent coion tracer in the TE (Alexa Fluor 488) [88]. The concentration of the fluorescent dye will adjust to the local electrical field in each ITP zone, enabling the visualisation of individual zones. Indirect fluorescence detection was also used in a hand held ITP instrument (240 g, powered by laptop or computer through universal serial bus (USB) link) [89]. The instrument used glass microfluidic chips (25 x 15 x 2.2 mm) containing simple cross geometry channels fabricated by wet etching. Fluorescently labelled ampholytes were used for indirect fluorescent detection of the explosives 2, 4, 6-trinitrophenolate and 2,
4, 6-trinitrophenol and the herbicide 2, 4-dichlorophenoxyacetic acid in a river water sample.

In 2011 Bottenus et al. introduced PMMA cascade chip containing channels with different depth in different parts of the separation channel for ITP separation (constant voltage) and fluorescent detection of R-phycoerythrin and fluorescently labelled human cardiac troponin I [90]. The principle of the cascade chip fabrication is briefly explained in Figure 1.3. The detailed description is elsewhere [91]. The chip contained reservoirs for LE (cathode), sample and TE (anode) joined through a cascading separation channel decreasing in cross-section from 1 mm wide and 100 µm deep to 100 µm wide and 20 µm deep in the direction from anode to cathode. The sample reservoir was attached to the 1 mm wide and 100 µm deep section using a channel of the same dimensions. Pressure was used to fill the chip with LE before sample in LE was loaded into a sample reservoir, filling the wide channel directing towards the anode reservoir. The sample reservoir was sealed with sticky tape to prevent hydrodynamic flow while replacing the waste in anode reservoir with TE. A 10,000 fold increase of troponin and R-phycoerythrin concentration were obtained in the ITP zone.

Latter a slightly modified cascade chip was used for detection of Human cardiac troponin I in human serum [92]. The changes of chip design improved the sensitivity and minimised problems with hydrodynamic flow during sample injection and loading of TE.
Fig. 1.3) Fabrication of the cascade microfluidic channel. Glass wafer (A.) was covered with thin layer of PDMS (B.), and SU8 2025 (C.). PDMS was removed before SU8 2025 hardened which caused sloppy transition at the edge of SU8 2025 (D.). The glass wafer with hardened SU8 2025 was spincoated with layer of SU8 3010 (E.), this was hardened and UV exposed using the mask with chip structure (F.). SU8 was developed and baked. The template of cascade channel (G.) was hot embossed in PMMA slide (H.). The microchannel (I.) was covered with PMMA wafer containing access holes. PMMA wafers were bonded together (75 °C, 300 psi).
1.3.2 Preconcentration isotachophoresis

The self focusing effect makes ITP an ideal preconcentration step, for example combined with a CZE separation. A great contribution in ITP-CZE [53] comes from the group of Kaniansky, publishing a large number of articles on ITP-CE on chips. The first work using chip capable of ITP, ITP-ITP and ITP-ZE was published in 2000 [93]. The chip was made in PMMA by hot embossing, and glued together with a flat PMMA plate after sputtering electrodes [94]. The initial design was optimised by straightening the channels and to prevent trapping of bubbles formed by electrolysis, as shown in Figure 1.4, but both designs were successfully used for ITP separation of organic acids. Separations in the updated design took longer, but well developed steps were obtained. This design was applied to a range of samples as summarised in Table 1.II.

In 2002, Wainright et al. used the ACLARA LabCard chips for ITP-ZE separation of 13 eTag™ fluorescent markers and for the detection of the activity of surface protease (ADAM 17) of live THP-1 cells (human monocytic cells) [95]. The injection of electrolytes is described in Figure 1.5. The LE was injected into all channels by using pressure. Fluorescence detection was performed by using a confocal fluorescence microscope. The activity of ADAM-17 protease was analysed by ITP-ZE separation of a fluorescent peptide substrate that loses its fluorescence once cleaved by the protease. The method sensitivity was 10 cells (THP-1) in 10 µL of Hanks’ buffer and could be used for direct evaluation of surface protease activity or for indirect determination of a number of cells. Later, the same chip was used under similar conditions for ITP-ZE of dsDNA fragments (HaeIII DNA digest) [96].
The chip developed by research group of Kaniansky was made in PMMA block (30 x 70 mm). A. The bottom substrate contained channels. The volume of terminating electrolyte reservoir (TE) was 8.8 µL. Chip contained two sample loops SL1 (9.2 µL) and SL2 (0.96 µL). SC1 is pre-separation column and SC2 analytical column with volumes of 1.2 and 1.7 µL, respectively. The separation column splits in two at bifurcation point (BF). The volume of leading electrolyte reservoirs (LE1 and LE2) was 8.8 µL. D1 and D2 indicate the position of the conductivity detectors. B. The cover plate contained platinum electrodes for detector 1 (D1a/b) and detector 2 (D2a/b) as well as the HV electrodes to drive the separations (HV). Reproduced with permission [94].
### Table 1.II) Published applications of the chip shown in Figure 1.4

<table>
<thead>
<tr>
<th>Method</th>
<th>Analyte</th>
<th>Sample</th>
<th>1st LE</th>
<th>TE</th>
<th>BGE or 2nd LE</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITP</td>
<td>Organic acids</td>
<td>wine</td>
<td>10 mM HCl + Ala (pH 2.9) + 1 g/L MHEC</td>
<td>5 mM glutamate or caproate + histidine (pH 5)</td>
<td></td>
<td>[97]</td>
</tr>
<tr>
<td>ITP-ITP</td>
<td>L and D tryptophan</td>
<td>model sample</td>
<td>10 mM propionate + BTP (pH 9.1) + 1 g/L MHEC + 80 mM α-CD</td>
<td>40 M ε-aminocaproate + bis-tris propane (pH 9.8)</td>
<td>*10 mM propionate + BTP (pH 9.1) + 1 g/L MHEC + 80 mM α-CD</td>
<td>[98]</td>
</tr>
<tr>
<td>ITP</td>
<td>Cl, NO$_3^-$, SO$_4^{2-}$</td>
<td>fresh water</td>
<td>5 mM dithionate + Ala (pH 3.5) + 1.8 mM Mg$^{2+}$ + 1 g/L MHEC</td>
<td>5 mM citrate</td>
<td></td>
<td>[99]</td>
</tr>
<tr>
<td>ITP-ZE</td>
<td>NO$_2^-$, F, PO$_4^{3-}$</td>
<td>10 mM HCl + Ala (pH 3.2) + 0.5 g/L MHEC</td>
<td>10 M aspartate + Ala (pH 4.2)</td>
<td>10 mM aspartate + Ala (pH 3.35) + 0.5 g/L MHEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITP</td>
<td>glutamate</td>
<td>food</td>
<td>10 mM HCl + His (pH 6.15) + 2 g/L MHEC</td>
<td>8 mM MES + His (pH 6.2)</td>
<td></td>
<td>[100]</td>
</tr>
<tr>
<td></td>
<td>parabens</td>
<td>skin lotion</td>
<td>10 mM HCl + BTP (pH 9.5) + 2 g/L MHEC</td>
<td>10 mM Ala + BTP (pH 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITP-ZE</td>
<td>benzoate, sorbate</td>
<td>food</td>
<td>10 mM HCl + His (pH 5.7) + 2 g/L MHEC</td>
<td>8 mM MES + His (pH 6)</td>
<td>25 mM MES + His (pH 5.1) + 2 g/L MHEC</td>
<td></td>
</tr>
<tr>
<td>ITP-ZE</td>
<td>BrO₃⁻</td>
<td>drinking water</td>
<td>10 mM HCl + Ala (pH 3.2) + 0.5 g/L MHEC</td>
<td>10 M aspartate + Ala (pH 4.2) + 1 g/L MHEC</td>
<td>15 mM aspartate + Ala (pH 3.4) + 5 g/L MEHC</td>
<td>[101]</td>
</tr>
<tr>
<td>--------------</td>
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<td>--------------------------------------</td>
<td>------------------------------------------</td>
<td>------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>ITP-ZE</td>
<td>Selena-amino acids</td>
<td>model sample</td>
<td>4 mM BDB + ethanolamine (pH 9.4) + 2 mM BTP + 0.5 g/L mowiol</td>
<td>10 mM Ala + Ba(OH)₂ (pH 10.5)</td>
<td>10 mM Ala + Ba(OH)₂ (pH 10.2) + 1 mM hydroquinone</td>
<td>[102]</td>
</tr>
<tr>
<td>ITP-ZE</td>
<td>proteins</td>
<td>model mixture</td>
<td>20 mM sodium acetate (pH 5.75) + 0.5 g/L MHEC</td>
<td>10 mM acetic acid + 0.5 g/L MHEC</td>
<td>200 mM acetic acid + 1 g/L MHEC</td>
<td>[103]</td>
</tr>
<tr>
<td>&quot;ITP-ZE&quot; free SO₃²⁻</td>
<td>wines</td>
<td>10 mM HCl + Ala (pH 3) + 0.5 g/L MHEC</td>
<td>20 mM tartrate + Ala (pH 3.9) + 0.5 g/L MHEC</td>
<td>15 mM Succinate + Ala (pH 4) + 2 g/L MHEC</td>
<td>[104]</td>
<td></td>
</tr>
<tr>
<td>&quot;ITP-ZE&quot; total SO₃²⁻</td>
<td>wines</td>
<td>10 mM HCl + 4 mM bis-tris propane + Ala (pH 4) + 0.5 g/L MHEC</td>
<td>20 mM citrate + 30 mM Ala + 0.5 g/L MHEC</td>
<td>15 mM citrate + 11.8 mM Ala + 7 mM bis-tris propane + 2 g/L MHEC</td>
<td>[105]</td>
<td></td>
</tr>
</tbody>
</table>

Ala – β-alanine, BDB – 5-bromo-2,4-dihydrobenzoic acid, BTP – 1,3-bis[tris(hydroxymethyl)methy]amino]propane, His – histidine, MES – morpholinoethane sulfonic acid, MHEC – methylhydroxyethyl cellulose,

*Slightly modified chip design*
Fig. 1.5) Schematic of the chip with five reservoirs for ITP-ZE separation. Empty chip (A.) is filled with LE (B.) after all channels are filled with LE (capillary effect, pressure or vacuum) TE is load in reservoir 2 (R2) and sample in R1. Sample is injected by applying voltage between R1 and R3 (C.). TE is injected by applying voltage between R2 and R1 (D.). ITP is initiated by applying voltage between R2 and R5 (E.). When the ITP pass the junction with R4 channel the zone electrophoresis (ZE) is started by applying voltage between R4 and R5 (F.).
In 2005 Huang *et al.* described online ITP preconcentration on a glass microfluidic chip before the separation of four FITC labelled SDS denatured proteins by GE [106]. The chip architecture and the protocol for use was similar to the design shown in Figure 1.5 except it contained one additional reservoir for simultaneous vacuum injection of TE and BGE. A mixture of a sample and LE (50 mM Tris, 0.5% SDS, 2% DTT, pH adjusted with HCl to 6.8) was injected electrokinetically between TE (192 mM glycine, 25 mM Tris, pH 8.3) and BGE (100 mM Tris-NaH$_2$PO$_4$, 0.1% SDS, 10% glycerol, 10% dextran, pH 8.3). To prevent electroosmotic flow (EOF) and protein adsorption microfluidic channels were coated with linear polyacrilamide.

In 2006 Liu *et al.* used a microfluidic glass chip with five reservoirs and protocol similar to one described in Figure 1.5 for Hepatitis B virus genotyping (serum samples, polymerase chain reaction (PCR) 30 cycles) [107].

In 2006 Ma *et al.* used the UV transparent quartz to construct microfluidic chip for ITP-ZE with UV detection of the flavonoids quercetin and isorhamnetin [108]. Channels (80 µm wide and 25 µm deep) were fabricated by wet etching and sealed by low temperature bonding using a vacuum oven (120 °C). The architecture of the chip was similar to schematic in Figure 1.5. TE (R2; 50 mM H$_3$BO$_3$, 20% CH$_3$OH, pH 8.2) and BGE (R3, R4 and R5; 25 mM MOPS, 50 mM Tris, 55 mM H$_3$BO$_3$, pH 8.36) were injected simultaneously by applying vacuum to R1. BGE in R3 was replaced with mixture of sample and LE (10 mM HCl, Tris, 20% CH$_3$OH, pH 7.2) and this mixture was electrokinetically injected by applying HV between R3 and R1. Rest of the protocol was identical with Figure 1.5.

In 2008 Hirokawa *et al.* used a Shimadzu MCE 2010 microchip electrophoresis system with UV detection for the ITP-GE separation of 16 DNA fragments ranging from 50 to 800 bp using a simple cross geometry chip [109]. Before analysis, all reservoirs and
channels were filled with LE. The LE electrolyte in TE reservoir was replaced with the DNA sample. The DNA was electrokinetically injected by applying voltage between TE and LE reservoir and after this injection step sample was replaced with TE and ITP was started by applying voltage between TE and LE reservoir, while electrodes at side reservoirs filled with LE were electrokinetically floating. After the ITP stacked zones migrated beyond the cross, the separation voltage was applied between LE reservoir and both side reservoirs for transient isotachophoresis (tITP) followed by GE in HEC as sieving matrix.

Two years earlier Jung et al. demonstrated a concentration factor of $2 \times 10^6$ by the ITP-ZE separation of Alexa Fluor 488 and bodipy on a borosilicate glass microfluidic chip with simple cross geometry without replacing sample in TE reservoir [110-111]. The LE and sample mixed with TE were loaded in reservoirs of the main separation channel. One of the side reservoirs was filled with LE and vacuum was applied to the other one. Then HV was applied between reservoirs of the main separation channel and ITP was initiated. The ITP plateau zones position was monitored using charged coupled device (CCD) camera mounted to a fluorescent microscope. Later the HV was applied between reservoirs with LE. This initiated tITP followed with ZE.

ITP in combination with an immunoassay by Electrokinetic Analyte Transport Assay (EATA) was introduced by Kawabata et al. [112]. Based on the price of antibodies (especially labelled ones), this method can significantly reduce the cost of existing immunoassays techniques. A sample containing antibodies, antigen and fluorescently labelled antibodies mixed with LE, LE and TE were injected simultaneously from three independent reservoirs by negative pressure simultaneously applied to four waste reservoirs. ITP was used to mix antigen with antibodies and to concentrate the fluorescently labelled immunocomplex for subsequent separation by GE with LIF.
detection. Using a similar method, Park et al. used quartz chips from Caliper Life Sciences, the instrument Caliper42, and a modified version of LabChip90 for an immunoassay using ITP-GE [113] with a 200-fold increase in sensitivity in comparison with a conventional assays.

Four proteins (transferrin, β2-microglobulin, human serum albumin and immunoglobulin G) were detected in a human urine sample using ITP-tITP-CZE with UV/VIS detector (280 nm) [114]. The ITP was carried out inside the PMMA chip made by CO2 laser engraving to inject and preconcentrate a maximum 3.5 mm long zone containing zones of ions from sample and TE zone. Two channels joined with separation channel and both filled with LE defined this 3.5 mm long zone. The channel closer to the detector was used as a waste for ITP (300 V/cm) and unused (0 V) after tITP was started. The channel closer to terminating reservoir was unused during ITP (0 V) but was used for tITP-CZE as BGE reservoir (350 V/cm). Transient ITP followed by CZE were carried out in attached silica capillary. The PMMA plates and capillary were joined together by bonding under the pressure of 0.6 MPa at 95 °C.

Persat et al. used classical ITP for concentrating nucleic acids from a whole blood lysate for subsequent PCR [115]. The borosilicate chip with simple cross geometry chip used for this work contained 20 µm deep and 90 µm wide channels (model NS12A, Caliper). To reduce protein adsorption the channels were treated with silanizing agent Sigmacote. The LE and TE contained SYBR green to visualise separated DNA and Triton X-100 to reduce EOF and protein adsorption (in conjunction with silanization treatment). The chip was first filled with LE, then one of the side reservoirs was filled with sample (whole blood lysate treated with proteinase K) and a vacuum was applied to the reservoir intended for the TE in the main separation channel. This reservoir was then filled with TE and a voltage was applied in the main separation channel between the TE
and LE reservoirs. The process of ITP stacking of DNA was observed with an inverted epifluorescent microscope. When the DNA reached the LE reservoir, the ITP was stopped and the content of this reservoir was used for PCR. The authors highlighted the potential for online on-chip ITP-PCR when this process would be automated and multiplexed.

1.3.3 Transient isotachophoresis

The capillary format of the tITP was described and used for the first time in 1992 by Foret et al. [54]. In 2003 Kurnik et al. used computer simulations to study the separation of electrokinetically injected fluorescein inside a chip with a double-T cross geometry using an electrokinetic sample injection and consequently tITP-ZE separation [116]. The main aim of this work was to investigate the effects of pinch-and-pull-back current and floating electrodes while using tITP for sample stacking. The predicted better sensitivity for a method with floating electrodes was confirmed using practical tITP experiments and could be explained by the sample loss when the pinch-and-pull-back methods was used. Practical experiments were done with fluorescein, a tyrosine kinase assay using a FITC labelled peptide substrate, and twelve small fluorescent molecules eTag™. The device was cast in acrylic resin with channels 30 µm deep and 85 µm wide. In the same year Vreeland et al. described the tITP-ZE separation of fluorescent eTag™ reporters in a commercial microfluidic PMMA chip with a double-T cross geometry [117].

In 2003 Xu et al. used the commercial instrument Shimadzu MCE-2010 with a silica glass single channel chip for separation of six SDS denatured proteins followed by UV detection [118]. Prior the analysis the channel was filled with LE and sample was loaded in TE reservoir and electrokinetically injected. The sample was then manually
replaced with TE and TE was electrokinetically injected, forming an ITP system. The voltage was switched off and the TE was manually replaced with BGE (same composition as LE without dextran), creating a tITP system when the voltages were re-applied. The analytes were separated by GE. The same method was applied for separation of a standard DNA ladder marker consisting of 16 DNA fragments in a range of 50 to 800 bp [119], and for analysing DNA fragments obtained after 30 PCR cycles [120]. In the last mentioned work, a simple cross geometry chip was used as a single channel chip by blocking non-used channels using a small pullback current. This work was later followed by Hirokawa et al. [109] as described in section 1.1.2 Preconcentration ITP.

Jeong et al. described a sensitive method for tITP-ZE of fluorescein and 2.7-dichlorofluorescein in highly saline samples (250 mM NaCl). A LOD of 3 pM was obtained for both analytes using a PDMS chip of double T cross geometry treated with fluorocarbon neutral surfactant FC-PN (EOF suppressor) [121]. The chip was filled with BGE containing N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) as a terminating anion. Depending on the geometry, a plug (12, 20 or 28 mm long) of high salinity sample was electrokinetically transported from the reservoir with sample into the waste reservoir through the spiralled part of the chip by applying constant voltage. The injection time was judged by the current as a steady current indicates steady state. The tITP separation was initiated by applying voltage between reservoirs at either end of the main separation channel, each filled with with BGE. The chloride from the high salinity sample acts as a leading anion until it dissipates, resulting in tITP.

A number of examples in the literature used tITP-GE, including for the analysis of fluorescently labelled human serum albumin and its monoclonal antibody
immunocomplex [122], and the immunocomplex of FITC labelled bovine serum albumin with specific mouse antibody [123].

In 2008 Lin et al. presented a chip for separation of dsDNA from PCR reaction [124]. Authors described the method as ITP-GE, but based on the procedure of electrolyte loading and voltage application, the method is classified here as tITP-GE. All the channels in the PMMA chip were 20 µm deep and the functionalities can be divided in three parts. First, the “injection” part contained four reservoirs (TE, sample, LE and vacuum) joined with a 500 µm wide and 10 mm long channel. Second the “stacking” part contained 140 µm wide and 20 mm long channel ending at the junction with separation channel and the first waste reservoir. Third, the GE separation channel which had the same width as stacking channel and a length of 25 mm. Prior to the separation the chip was hydrodynamically filled with LE and the sample and TE reservoirs were filled with sample and TE, respectively. Then negative pressure was applied to vacuum reservoir and zones of TE and sample were introduced between zones of LE in injection channel. The tITP separation was initiated by applying high voltage between LE reservoir and waste reservoir 1, followed by GE by switching the separation voltage from waste reservoir 1 to waste reservoir 2. Because the separation voltage was applied between reservoirs filled with LE, this work is classified as tITP not ITP.

As summarized in Table 1.III, a number of groups used tITP GE for DNA analysis using relatively simple cross or double T cross geometry devices [125-127]. In 2009 Liu et al. showed more than a hundredfold improvement in sensitivity using tITP CGE in comparison with CGE [128]. The chip they used consisted of two parallel channels joined at reservoir 2 (R2); the first one joined reservoirs R1 and R2 and the second one reservoirs R3 and R4. The fluorescent detector was placed close to R1. Reservoir 1
(R1) contained electrolyte with sieving matrix (SE1), reservoir 2 (R2) was for waste (W), reservoir 3 (R3) for electrolyte without sieving matrix (SE2) and reservoir 4 (R4) for the DNA sample. Prior the analysis R1 was filed with SE1 and negative pressure was applied to R2. Than R3 and R4 were filed with SE2 and the negative pressure was applied again to R2 to fill the channels with SE2. After filling of the channels, SE2 in R4 was replaced with the DNA sample, which was injected by applying a voltage between R2 and R4. The tITP-GE was initiated by applying a voltage between R1 and R3, applying pull back voltage to R4 during the separation. The chloride worked as a leading anion to focus the DNA in the separation electrolyte without HPMC. When the tITP zones reached the separation electrolyte with HPMC, the DNA fractions separated by GE according to their length.

1.3.4 Peak mode isotachophoresis

ITP is initiated by applying a potential difference across a channel containing a sample in a discontinuous electrolyte system, comprising a LE and TE. Minority ions from the sample form concentrating zones between LE and TE. Based on Kohlrausch regulating function, the maximum concentration of the sample zone is limited by its electrophoretic mobility and LE concentration. If this limiting concentration is reached the analyte zone forms a plateau separating the LE and TE because these have been selected to have a higher and lower mobility than the analyte, respectively. In classical ITP, the condition before the critical concentration for plateau formation has been reached is called mixed zone ITP. Recently Santiago realised the opportunities this mode of ITP offers for concentrating trace amounts of analytes and re-defined this condition as peak mode ITP [129]. The two most important differences between peak mode ITP and classical, plateau mode ITP are in detection and quantification of the
analytes. In plateau mode ITP, a universal detector, mostly conductivity detection, is used to detect the step-wise changes between zones, using the step length as a measure for analyte concentration. In peak mode ITP, an analyte specific detection technique such as fluorescence is required to selectively measure the increase in concentration of specific analyte. Here, the signal will be a peak, not a step, where the intensity is a measure for the analyte concentration.

In 2009 Khurana et al. introduced peak mode ITP with continuous sample injection (sample presented in mixture with TE) for separation of fluorescently labelled DNA and natively fluorescent proteins (green fluorescent protein and allophycocyanin) [130]. Peaks formed according to the analyte mobilities between non fluorescent plateau zones of LE, carbonate, carbamate and TE; carbonate and carbamate were present as impurities in the TE. A simple cross geometry, glass Caliper NS-95A chip was used as a single channel device, filling the separation channel and one reservoir with LE and the second reservoir with TE mixed with sample. While demonstrating the potential of peak mode ITP, the method was not really applicable for quantification due to the limited separation capacity of the described system and the unknown concentration of carbonate and carbamate impurities.

Schoch et al. described using a peak mode ITP with fluorescence detection for extraction, isolation, preconcentration and quantification of miRNAs [131]. Micro RNA (miRNA) is noncoding, 18-24 nucleotides long RNA which regulates gene expression via sequence specific interactions with mediator RNA (mRNA). Again a Caliper NS-95 was used as single channel chip. In preparation for the analysis, the chip was filled with LE (100 mM HCl, 140 mM 6-aminocaproic acid, pH 4) and placed for 10 minutes at 4 °C. Subsequently, TE reservoir was filled with LE containing 30% sieving matrix Pluronic F-127 and the separation channel was filled with sieving matrix
by applying the negative pressure to LE reservoir. After 10 minutes at ambient
temperature the viscosity of Pluronic increased and a mixture of mRNA sample in TE
was loaded in TE reservoir. The separation was started by applying the high voltage
between TE and LE reservoirs. Authors stated that this method can quantify miRNA
from ~900 cells in ~5 µL.

Persat et al. continued Schoch’s research into quantification of miRNA and introduced
so called selective ITP using three LEs in a more complex Caliper NS260 chip
(8 reservoirs) [132]. LE1/LE2/LE3 were composed of Tris HCl, pH 8 (100/20/20 mM),
PVP, MW = 1 MDa (5/55/30 g/L), urea (7/7/2 M) and 500 nM SYTO RNASelect dye.
The sample of miRNA was mixed with TE (92.5% formamide, 5 mM Tris, 2.5 mM
caproic acid). The separation channel was filled with zones of the three different LEs by
filling reservoirs 1, 2 and 3 with LE1, reservoirs 4, 5 and 6 with LE2 and the remaining
reservoirs with LE3. A negative pressure applied to reservoirs 7 and 3 enabled the
formation of three LE zones of different composition. LE1 in reservoir 1 was replaced
with mixture of sample in TE and the separation was initiated by applying a voltage
between reservoirs 1 and 8. This multi electrolyte system improved specificity and
selectivity of the peak mode ITP method for miRNA sequences shorter than
40 nucleotides and was used for quantification of miRNA in cell cultures (HeLa and
Hepa1-6). A similar method was used to visualize the separation of miRNA using
specific molecular beacons [133]. The molecular beacons were mixed with all three Les
and the mobility of miRNA and molecular beacons were slower than the mobility of LE
but faster than the mobility of TE so that the miRNAs and the molecular beacon probes
were migrating in a mixed peak zone. Hybridization of probes with miRNA could only
occur in LE3 because of the lower urea concentration. This method enabled the specific
detection and quantification of miR-122 in liver tissue.
In 2011, Bercovici et al. described the use of specially designed molecular beacons for quantification of 16S rRNA from E. coli in human urine samples using the single cross geometry Caliper NS-95 device [134]. Urine samples were centrifuged, cells from sediment were lysed, mixed with a solution of molecular beacon and subsequently with TE (50 mM tricine, 100 mM bis-tris). The sample was loaded onto the chip that was prefilled with LE and analysed. *E. Coli* could be detected in bacterial cultures as well as in urine samples in the clinically relevant range (1x10⁶-1x10⁸).

### 1.3.5 Gradient elution isotachophoresis

Gradient elution ITP (GEITP) was introduced in 2007 by Shackman *et al.* [135] in a capillary system and later applied to a microchip by Davis *et al.* [139]. The GEITP instrument was composed of two reservoirs connected by a 3 cm long capillary (30 µm i.d.). In the middle of the capillary was a 5 mm wide detection window for LIF. The larger, electrically grounded reservoir (1.4 mL) contained LE and could be pressurised. The smaller reservoir (110 µL) contained a high voltage electrode and was filled with sample in TE. The ITP separation was initiated in the smaller reservoir (TE + sample) where the pressure driven flow of LE prevented the separated ions zones from entering the silica capillary. When the hydrodynamic flow was reduced below the effective electrophoretic velocity of the analytes, the analytes could enter the capillary. The system is depicted in Figure 1.6. The device was used for the separation of fluorescent dyes (fluorescein, 6-carboxyfluorescein), fluorescein-labelled DNA, fluorescently labelled (5-carboxyfluorescein) mixture of amino acids (Asp, Gly, Ser, Val), and separation of natively fluorescent proteins (green fluorescent protein, DsRed). The separated zones could be detected as peaks thanks to the use of non-fluorescent
ampholytes to space the plateaus. Low-picomolar detection limits were reported despite the use of a mercury arc lamp and a low cost CCD camera. This instrument was also used for chiral separation of amino acids [136]. Later Mamunooru et al. incorporated a UV detector for the separation of the UV absorbing amino acids tyrosine and tryptophan [137]. This work was followed by the analysis of chromophore labelled amino acids (Asp, Glu) in cerebrospinal fluid [138]. Davis et al. extended GEITP with gradient elution CZE (GECZE) of fluorescently labelled (carboxyfluorescein) six amino acids (Asp, Glu, Gly, Ala, Ser, Val) using a 11 cm capillary with a 6 cm window [139]. GEITP was initiated as previously described but after the analytes zones entered the capillary the pressure and voltages were stopped to replace the TE in the TE reservoir with LE. When the pressure and voltages were resumed, a tITP system is formed enabling refocusing of the diffused zones. Once the ITP dissipated, the GECZE started, enabling femtomolar detection limits using LIF. This work was transferred to a chip, but unfortunately the sensitivity of the chip system was not as good as demonstrated in the capillary.
Formation of ITP zones in the sample reservoir while the positive pressure is applied to the opposite reservoir containing LE ($\mu_1$ and $\mu_4$ the highest and the lowest electrophoretic mobility, respectively). With decreasing pressure, the ITP zones can enter the capillary inlet and reach the detection point.
1.3.6 **Free-Flow isotachophoresis**

Most electrophoretic methods have been also applied in continuous, free flow format, where the electric field for the separation is applied perpendicular to the imposed hydrodynamic flow direction [140-142]. Free flow electrophoretic methods are suitable for miniaturisation and the activities in this area have been captured in a review on miniaturised free flow electrophoretic techniques was published in 2007 [65]. Free flow ITP (FFITP) combines a flow-through device with isotachophoresis and was first performed on a PDMS chip in 2006 [143]. Collapsing of the separation chamber was prevented using 30 µm wide square supporting posts in the chamber, leaving only the 10 µm wide channels between the posts for FFITP. A schematic drawing of the FFITP device (without supporting posts) is shown in Figure 1.7. The three reservoirs at the north side of the chip were filled with TE, sample and LE. The west and east reservoirs along the separation chamber contained electrodes and were filled with TE and LE, respectively. The end of the separation chamber was connected to the outlet channels where the analyte fractions are collected. In this study the outlet channels placed in the south of the chip were joined to apply a vacuum to drive the hydrodynamic flow inside the chamber. The separation of fluorescein, eosin G and acetylsalicylic acid and of myoglobin-FITC, serine and FITC were documented with images captured with a CCD colour camera.

Later Janasek et al. improved previous design and showed electrostatic induction FFITP of fluorescein in a glass chip [144]. The mask design of the chip was similar to the chip described previously, but the west and east reservoirs containing electrodes were separated from the main chamber by a 146 µm thick glass wall. In the mask, the supporting poles were 40 µm, but the wet etching to a depth 30 µm reduced the posts to
20 µm high bumps. It is unclear if the posts played any role in preventing sagging of
the glass. The most significant achievement was that by separating the electrodes from
the separation chamber the potential applied to the electrodes was transferred to the
glass walls but the current was zero. This led to the formation of ITP zones in the
separation chamber but stopped the movement of the zones towards the electrodes. This
phenomena was called electrostatic induction FFITP and was demonstrated by
concentrating fluorescein.
Fig. 1.7) Schematic of FFITP separation chamber.
Table 1.III) Summary of ITP methods used for real applications

<table>
<thead>
<tr>
<th>Method</th>
<th>Analyte</th>
<th>Sample</th>
<th>LE</th>
<th>TE</th>
<th>BGE</th>
<th>Detection</th>
<th>Substrate</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITP</td>
<td>Arsenic speciation</td>
<td>Waste water</td>
<td>8 mM HCl + 10 mM α-CD + Tris (pH 9) + 1 g/L HEC</td>
<td>10 mM glycine + Ba(OH)$_2$ (pH 9.5)</td>
<td>Single electrode conductivity</td>
<td>PMMA + polyester</td>
<td>[75]</td>
<td></td>
</tr>
<tr>
<td>ITP</td>
<td>Ascorbic acid</td>
<td>Photo developer solution</td>
<td>5 mM GA + Tris (pH 8) + 1 g/L HEC</td>
<td>10 mM GG + Ba(OH)$_2$ (pH 8.5)</td>
<td>Conductivity</td>
<td>PMMA + polyester</td>
<td>[77]</td>
<td></td>
</tr>
<tr>
<td>ITP</td>
<td>Amino acids</td>
<td>Potential for beverages or urine samples</td>
<td>10 mM GA + 2mM Mg$_2$SO$_4$ + benzylamine (pH 9) + 1 g/L HEC</td>
<td>20 mM β-alanine + Ba(OH)$_2$ (pH 9.7)</td>
<td>Conductivity</td>
<td>PMMA + polyester</td>
<td>[78]</td>
<td></td>
</tr>
<tr>
<td>ITP</td>
<td>Transition metal ions</td>
<td>Solutions from metal processing industry</td>
<td>10 mM NaOH + 2.5 mM malic acid + propionic acid (pH 4.9) + 1 g/L HEC</td>
<td>10 mM tetrabutylammonium hydroxide</td>
<td>Conductivity</td>
<td>PMMA + polyester</td>
<td>[79]</td>
<td></td>
</tr>
<tr>
<td>ITP</td>
<td>Inorganic arsenic and selenium</td>
<td>Industrial process stream</td>
<td>20 mM HNO$_3$ + histidine (pH 5.5) + 0.5 g/L mowiol</td>
<td>20 mM gallic acid</td>
<td>Conductivity</td>
<td>PMMA + polyester</td>
<td>[82]</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>5 mM GA + N-methyl-D-glucamine (pH 9.8) + 0.5 g/L mowiol</td>
<td>20 mM β-alanine + Ba(OH)$_2$ (pH 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITP</td>
<td>Chlorine containing species</td>
<td>Explosive residues</td>
<td>1.75 mM HNO$_3$ + 2.75 mM In(NO$_3$)$_3$ + GG (pH 3) + 1 g/L HEC</td>
<td>20 mM cyanoacetic acid</td>
<td>Conductivity</td>
<td>PMMA + polyester</td>
<td>[83]</td>
<td></td>
</tr>
<tr>
<td>ITP</td>
<td>Magnesium</td>
<td>Mineral water</td>
<td>10 mM CsOH + 2 mM malonic + pivalic acid (pH 5.1) + 1 g/L HEC</td>
<td>10 mM Tris</td>
<td>Conductivity</td>
<td>PMMA + polyester</td>
<td>[84]</td>
<td></td>
</tr>
<tr>
<td>ITP</td>
<td>Explosives, herbicides</td>
<td>River water</td>
<td>10 mM HCl + 20 mM bis-tris + NaOH (pH 6.9)</td>
<td>10 µM tricine mixed with 1 µM ampholytes (spacers) AF 647 labelled and sample</td>
<td>Indirect fluorescence</td>
<td>Glass</td>
<td>[89]</td>
<td></td>
</tr>
<tr>
<td>ITP</td>
<td>Human cardiac troponin I</td>
<td>Spiked human depleted serum</td>
<td>40 mM KOH + 6M urea + 0.1% triton X100 + 20 g/L PVP + acetic acid (pH 4.4)</td>
<td>20 mM acetate + 0.1% triton X100 + 6 M urea + 20 g/L PVP</td>
<td>Fluorescence</td>
<td>PMMA</td>
<td>[92]</td>
<td></td>
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<tr>
<td>ITP</td>
<td>Organic acids</td>
<td>Wines</td>
<td>10 mM HCl + Ala (pH 2.9) + 1 g/L MHEC</td>
<td>5 mM glutamate or caproate + histidine (pH 5)</td>
<td>Conductivity</td>
<td>PMMA</td>
<td>[97]</td>
<td></td>
</tr>
<tr>
<td>ITP</td>
<td>Chloride, nitrate, sulphate</td>
<td>Fresh water</td>
<td>5 mM dithionate + Ala (pH 3.5) + 1.8 mM Mg²⁺ + 1 g/L MHEC</td>
<td>5 mM citrate</td>
<td>Conductivity</td>
<td>PMMA</td>
<td>[99]</td>
<td></td>
</tr>
<tr>
<td>ITP</td>
<td>Glutamate</td>
<td>Food additives</td>
<td>10 mM HCl + His (pH 6.15) + 2 g/L MHEC</td>
<td>8 mM MES + His (pH 6.2)</td>
<td>Conductivity</td>
<td>PMMA</td>
<td>[100]</td>
<td></td>
</tr>
<tr>
<td>Parabens</td>
<td>Skin lotion</td>
<td></td>
<td>10 mM HCl + BTP (pH 9.5) + 2 g/L MHEC</td>
<td>10 mM Ala + BTP (pH 10)</td>
<td></td>
<td></td>
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<tr>
<td>ITP-ZE</td>
<td>Compound(s)</td>
<td>Sample Type</td>
<td>Conditions 1</td>
<td>Conditions 2</td>
<td>Conductivity</td>
<td>Eluent</td>
<td>Page</td>
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<tr>
<td>ITP-ZE</td>
<td>Nitrite, fluoride,</td>
<td>Fresh water</td>
<td>10 mM HCl +</td>
<td>10 M aspartate +</td>
<td>Conductivity</td>
<td>PMMA</td>
<td>[99]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>phosphate.</td>
<td></td>
<td>Ala (pH 3.2) + 0.5 g/L MHEC</td>
<td>Ala (pH 4.2) + 0.5 g/L MHEC</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>ITP-ZE</td>
<td>Benzoate, sorbate</td>
<td>Food additives</td>
<td>10 mM HCl + His (pH 5.7) + 2 g/L MHEC</td>
<td>8 mM MES + His (pH 6) +</td>
<td>Conductivity</td>
<td>PMMA</td>
<td>[100]</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25 mM MES + His (pH 5.1) + 2 g/L MHES</td>
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<td></td>
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</tr>
<tr>
<td>ITP-ZE</td>
<td>Bromate</td>
<td>Drinking water</td>
<td>10 mM HCl + Ala (pH 3.2) + 0.5 g/L MHEC</td>
<td>10 M aspartate + Ala (pH 4.2) + 1g/L MHEC</td>
<td>Conductivity</td>
<td>PMMA</td>
<td>[101]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15 mM aspartate + Ala (pH 3.4) + 5 g/L MEHC</td>
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<tr>
<td>ITP-ZE</td>
<td>Free Sulfite</td>
<td>Wines</td>
<td>10 mM HCl + Ala (pH 3) + 0.5 g/L MHEC</td>
<td>20 mM tartrate + Ala (pH 3.9) + 0.5 g/L MHEC</td>
<td>Conductivity</td>
<td>PMMA</td>
<td>[104]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15 mM Succinate + Ala (pH 4) + 2 g/L MHEC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITP-ZE</td>
<td>Total sulfite</td>
<td>Wines</td>
<td>10 mM HCl + 4 mM bis-tris propane + Ala (pH 4) + 0.5 g/L MHEC</td>
<td>20 mM citrate + 30 mM Ala + 0.5 g/L MHEC</td>
<td>Conductivity</td>
<td>PMMA</td>
<td>[105]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15 mM citrate + 11.8 mM Ala + 7 mM bis-tris propane + 2 g/L MHEC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITP-ZE</td>
<td>Surface protease activity, number of cells</td>
<td>THP-1 cell line</td>
<td>20 mM HCl + 25 mM imidazole (pH6.5) + 10 g/L PEO</td>
<td>20 mM HEPES + 10 mM imidazole (pH 6.7) or 40 mM HEPES + 160 mM imidazole (pH 7.7)</td>
<td>LE</td>
<td>Fluorescence</td>
<td>PMMA</td>
<td>[95]</td>
</tr>
<tr>
<td>-------</td>
<td>------------------------------------------</td>
<td>------------------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------------------------------------------</td>
<td>-----</td>
<td>-----------------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>ITP-GE</td>
<td>HBV genotyping</td>
<td>Serum</td>
<td>15 mM HCl + 36 mM imidazole (pH 7) + GeneFinder + 20 g/L HPMC</td>
<td>20 mM HEPES + 40 mM imidazole (pH 7.2)</td>
<td>TE over speed sample in gel matrix</td>
<td>Fluorescence</td>
<td>Glass</td>
<td>[107]</td>
</tr>
<tr>
<td>ITP-GE</td>
<td>Immunoassay</td>
<td>Suitable for biological samples</td>
<td>9 g/L pDMA + 3% glycerol + 75 mM Tris-HCl (pH 8) + 50 mM NaCl + 0.1 g/L BSA + 0.05% Tween-20</td>
<td>9 g/L pDMA + 3% glycerol + 75 mM + 0.1 g/L BSA + 0.05% Tween-20 + 125 mM HEPES</td>
<td>Same as previous</td>
<td>Fluorescence</td>
<td>Quartz</td>
<td>[112]</td>
</tr>
<tr>
<td>ITP-GE</td>
<td>Immunoassay</td>
<td>Suitable for biological samples</td>
<td>Same as previous</td>
<td>Same as previous</td>
<td>Same as previous</td>
<td>Fluorescence</td>
<td>Quartz</td>
<td>[113]</td>
</tr>
<tr>
<td>----------</td>
<td>-------------</td>
<td>---------------------------------</td>
<td>------------------</td>
<td>------------------</td>
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<td>-------</td>
</tr>
<tr>
<td>ITP-tITP-CZE</td>
<td>Proteins</td>
<td>Urine</td>
<td>25 mM Tris-HCl + 0.1 g/L PEO + 0.5 g/L HEMC (pH 3.6)</td>
<td>25 mM acetate + 0.1 g/L PEO + 0.5 g/L HEMC (pH 3)</td>
<td>LE</td>
<td>UV</td>
<td>PMMA</td>
<td>[114]</td>
</tr>
<tr>
<td>ITP-tITP-PCR</td>
<td>DNA</td>
<td>Whole blood</td>
<td>50 mM Tris + HCl (pH 8.2)</td>
<td>50 mM Tris + HEPES (pH 7.8)</td>
<td>Fluorescence</td>
<td>Glass</td>
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<td></td>
</tr>
</tbody>
</table>

**Transient ITP**

<table>
<thead>
<tr>
<th>tITP-GE</th>
<th>DNA</th>
<th>PCR samples</th>
<th>Chloride ions in BGE</th>
<th>20 mM Tris-glycine (pH 8.1)</th>
<th>50 mM Tris-HCl (pH 8.1) + 20 g/L HEC</th>
<th>UV (260 nm)</th>
<th>Glass</th>
<th>[120]</th>
</tr>
</thead>
<tbody>
<tr>
<td>tITP-GE</td>
<td>DNA</td>
<td>PCR samples</td>
<td>Chloride ions in sample</td>
<td>HEPES in BGE</td>
<td>20 mM HEPES + 40 mM imidazole (pH 7.5) + Gene Finder + 20 g/L HPMC</td>
<td>Fluorescence</td>
<td>PMMA</td>
<td>[126]</td>
</tr>
<tr>
<td>tITP-GE</td>
<td>DNA</td>
<td>PCR samples</td>
<td>Same as previous</td>
<td>Same as previous</td>
<td>Same as previous</td>
<td>Fluorescence</td>
<td>Glass</td>
<td>[127]</td>
</tr>
<tr>
<td>tITP-GE</td>
<td>DNA</td>
<td>PCR samples</td>
<td>Same as previous</td>
<td>Same as previous</td>
<td>Same as previous</td>
<td>Fluorescence</td>
<td>Glass</td>
<td>[128]</td>
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</table>
### Peak and plateau mode ITP

<table>
<thead>
<tr>
<th>Peak ITP</th>
<th>miRNA</th>
<th>Cell lysate</th>
<th>Buffer Conditions</th>
<th>Fluorescence</th>
<th>Glass</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>miRNA</td>
<td>Cell lysate</td>
<td>100 mM HCl + 140 mM ACA (pH 4)</td>
<td>Fluorescence</td>
<td>Glass</td>
<td>[131]</td>
</tr>
<tr>
<td>Peak ITP</td>
<td>miRNA</td>
<td>LE1/LE2/LE3</td>
<td>100/20/20 mM Tris HCl + 0/2/2 mM MgCl₂ + 2 M urea + 5/55/30 g/L PVP</td>
<td>Fluorescence</td>
<td>Glass</td>
<td>[132]</td>
</tr>
<tr>
<td></td>
<td>miRNA (miR-122)</td>
<td>Liver tissue</td>
<td>LE1/LE2/LE3</td>
<td>Fluorescence</td>
<td>Glass</td>
<td>[133]</td>
</tr>
<tr>
<td>Peak ITP</td>
<td>16S rRNA of E.coli</td>
<td>Urine</td>
<td>0.25 M HCl + 0.5 M bistris + 5 mM MgCl₂ + 10 g/L PVP</td>
<td>Fluorescence</td>
<td>Glass</td>
<td>[134]</td>
</tr>
</tbody>
</table>
### Gradient elution ITP

<table>
<thead>
<tr>
<th>GEITP</th>
<th>Amino acids</th>
<th>Cerebrospinal fluid</th>
<th>100 mM citrate</th>
<th>25 mM borate + ethanolamine (pH 9.5)</th>
<th>UV absorbance</th>
<th>Glass</th>
<th>[138]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACA – 6-aminocaproic acid, <strong>AF647</strong> – Alexa Fluor 647, <strong>Ala</strong> – β-alanine, <strong>BDB</strong> – 5-bromo-2,4-dihydrobenzoic acid, <strong>BSA</strong> – bovine serum albumin, <strong>BTP</strong> – 1,3-bis[tris(hydroxymethyl)methylamino]propane, <strong>GA</strong> – glycolic acid, <strong>GG</strong> – glycylglycine, <strong>HEC</strong> – hydroxyethyl cellulose, <strong>HEMC</strong> – hydroxyethylmethyl cellulose, <strong>HEPES</strong> – 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, <strong>His</strong> – histidine, <strong>HPMC</strong> – hydroxypropylmethyl cellulose, <strong>MES</strong> – morpholinoethane sulfonic acid, <strong>MHEC</strong> – methylhydroxyethyl cellulose, <strong>MOPS</strong> – 3-(N-morpholino)propanesulfonic acid, <strong>pDMA</strong> – poly(2-dimethylaminoethyl methacrylate), <strong>PEO</strong> – poly(ethylene oxide), <strong>PVP</strong> – polyvinilpyrolidone,cellulose.</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
1.4 Conclusions

Reports of microfabricated devices for ITP predate the introduction of the µTAS concept, illustrating the importance of the freedom in design offered by chip-based system for ITP and ITP related techniques. This led to the revival of analytical ITP after the introduction of fused silica capillaries had almost eliminated ITP research. ITP has been applied as preconcentration technique coupled to ZE and GE and has been successfully used for the analysis of small molecules as well as large biomolecules including proteins, DNA and RNA. The use of transient systems, where the ITP system dissipates to initiate ZE, simplifies the instrumentation and chip designs required and has been widely used on chips. The recognition of peak mode ITP as a powerful way to concentrate trace analytes has changed the perspectives on detection in ITP, advocating selective detection. Using cascading channels with decreasing width and depth, concentration factors of up to 10,000 have been demonstrated using this powerful approach.

Hydrodynamic flow has been successfully used to balance electrophoretic migration to further concentrate and isolate target analytes, both in gradient elution ITP and tITP. While purpose designed devices have been used, many groups used simple cross or 2T geometry devices, sometimes even operated as a single channel device. The more complex systems requiring multiple electrolytes take advantage of multichannel devices to fill the separation channel with the appropriate electrolytes, taking advantage of experimental opportunities offered by microfabricated devices.

For the analysis of small molecules targeted in this thesis, the most significant development in analytical ITP was the introduction of indirect fluorescence detection, enabling the detection of nonfluorescent analytes.
1.5 References


Chapter 2

New applications for the Agilent Bioanalyzer: fingerprinting of 8-aminopyrene-1,3,6-trisulfonic acid derivatised glycans

2.1 Introduction

Capillary electrophoresis was the single most important analytical tool for the completion of the Human Genome Project, but it is impossible to envisage a single technology to address all the needs in current biomedical research. Complex biological samples contain proteins with a wide range of properties (e.g. different solubilities, posttranslational modifications and conjugations with non-protein compounds), polysaccharides, lipids as well as small molecule metabolites, all typically in a highly saline matrix. The detection, identification and quantification of all these compounds requires the use of sophisticated separation tools and the search for new endogenous substances suitable for disease diagnostics (biomarkers) is an example of a discipline with significant analytical interest [1]. Once the biomarker (or a set of biomarker molecules characteristic for a given disease stage) or other analyte of interest has been identified, a more specific analytical screening approach can be developed. For screening purposes, simple, economical and rapid analysis methods are required, fitting in with the general trend of miniaturisation of analytical techniques [2-3].

In the last two decades, miniaturisation in analytical chemistry has become a commercially interesting field as can be judged by the number of companies focused on this area. It would be very hard to list all the companies designing, manufacturing, distributing and selling microfluidics analytical systems, but Section 2.2 provides an overview of the variety of companies involved in this field.
2.2  Commercially available microfluidic platforms

This section gives a brief overview of the alternative chip based analytical platforms available. The commercially most successful electrophoretic and HPLC microfluidic chip based separation systems and Table 2.I with selection of the companies designing, manufacturing, distributing or selling microfluidics systems are described in following text.

2.2.1  Electrophoretic systems

Electrophoretic methods are naturally suitable for miniaturisation as analysis is driven under ambient pressure, and miniaturised separation channels are easier to cool down. The cooling down is important as Joule heating increasing dispersion of separated ions, which decreasing the separation power of electrophoretic methods. Another advantage of miniaturisation of electrophoretic system is that shorter separation channel require less powerful HV source and miniaturisation of electrophoretic systems actually leads to smaller dimensions platforms. Presently, the commercially available electrophoresis chip based instruments are DNA, RNA and SDS-proteins analysis oriented.

The first mainstream commercial instrument was the Agilent Bioanalyzer 2100, shown in Figure 2.1 [4]. This chip based instrument with LIF detection (λ_{exc} = 635 or 470 nm) is a product of cooperation between Caliper [5] and Agilent Technologies [6] and it was introduced for the first time in 1999. The very first assays targeted the nucleic acids, enabling the analysis of up to twelve samples was finished in less than one hour, making the Bioanalyzer 2100 competitive with classical GE. Nowadays the Bioanalyzer 2100 offers separation of SDS proteins and flow cytometry. The following instrument by Agilent technologies, which builds on the success of Bioanalyzer 2100 is 2200 Tape
Station [7]. This instrument offers fully automated separation of DNA, RNA and proteins samples on the credit card sized Screen Tape with sixteen independent separation channels. The 2200 Tape Station setup does require minimum manual handling with samples and reagents. The risk of samples cross contamination was eliminated as each separation runs in independent microfluidic channel.

Electrophoretic based microfluidic analytical platforms with fluorescent detection \( (\lambda_{\text{exc.}} = 635 \text{ nm}) \) for separation of nucleic acids and proteins offered by Caliper are LabChip GX [8] (DNA and RNA) and GXII [9] (DNA, RNA and proteins). The automated sample loading into the microfluidic chip used by those instruments is realised from 96 or 384-well microplates by using a sipper attached directly to a glass chip.

MCE-202 MultiNA [10], produced by Shimadzu [11] and introduced in 2007, is another example of commercial electrophoretic, microfluidic chip based, bench top analytical platform for DNA and RNA analysis. The analysis on this instrument is fully automated. The samples are injected from 96 well plate and the analysis run in parallel on four independent quartz chips. This setup decreases the total analysis time down to 75 seconds per sample. The LED excited fluorescent detector \( (470 \text{ nm}) \) is used to carry out detection of fluorescently labelled nucleic acids. The quartz microfluidic chips are automatically cleaned after each analyses. The company claims that the lifetime of their quartz chips is 3600 analyses if used correctly.

### 2.2.2 HPLC systems

The miniaturisation of HPLC systems is separation column, channels, vents and connections oriented. Any other instrumental part of those systems has the same size or is even bigger to compare with classical HPLC systems.
Fig. 2.1)  

A. Commercial chips for analysis of DNA, RNA, proteins and cells.  
B. Agilent Bioanalyzer 2100.
The smaller diameter of separation column than require high sensitive detection systems and high-pressure, stable flow mobile phase.

The 1260 Infinity HPLC-Chip/MS [12] instrument introduced by Agilent Technologies was the first system integrating the HPLC system with electrospray ionization. A credit card sized chip contains enrichment and analytical columns, injection channel, electrospray tip, and all the necessary connections. The chip is positioned in a stainless steel holder (HPLC-Chip Cube) forming an interface for connecting HPLC pumps, and positioning the chip in front of the mass spectrometer (series Agilent 6000). Recently, chips for different applications have become available, including proteomics, metabolomics, small molecules analysis, biopharmaceutical analysis, and nucleotide analysis. Moreover, Agilent offers to fill the existing chips with sorbents required by customers, which will potentially increase the number of applications. Recently, new multi-layered chip designs for multidimensional separations have also been developed. The HPLC-Chip Cube does not include integrated solvent delivery device. Instead it is connected to an external system 1260 Infinity HPLC system. Since the polyimide chip limits the maximum applicable pressure due to mechanical stability, new chips based on stainless steel, for operations at pressures over 1000 atmospheres have been under development.

A dedicated nano-HPLC system with nanoliter flow rates which does not splitting the mobile phase during gradient separation is produced by Eksigent [13]. To compare with isocratic elution the gradient elution is more complicated as changing composition of gradient mobile phase change its viscosity, which reflects at the change flow of mobile phase. The Eksigent solves this problem with system called Microfluidic Flow Control (MFC). This system is connected with syringe HPLC pumps (piston pushed with compressed gas) and monitors the flow rate of the mobile phase. If necessary the flow
driven by syringe pumps is adjust by changing the pressure supplied by compressed gas (air/nitrogen) [14]. The compressor is able to increase the pressure difference by a factor of 36. For example, if the input pressure is 10 bars the pressure inside the system can be set up to 360 bars. Two systems using MFC the „nanoLC” and „nanoLC-Ultra” were introduced. Both systems work with either capillary columns or with a system upgrade called „cHiPLC-nanoflex” [15] designed to work with microfluidic chips. Eksigent sells two different cHiPLC compatible chips either with an analytical (70 µm x 15 cm) or with a trap (200 µm x 0.5 mm) column. Both analytical and trap columns are packed with C18 particles (3 or 5 µm). The declared dead volume of the connection between capillaries and chips in cHiPLC is under 1 nL. External connection with nano-ESI MS is also possible.

Another product dedicated for nano-LC/MS applications introduced by one of the major instrument manufacturers is called TRIZAIC™ UPLC nanoTile produced by Waters [16-17]. While the dimensions of the integrated system with trap column, analytical column and ESI emitter are similar to the one produced by Agilent (analytical column has an inner diameter of 80 µm filled with 1.7 µm particles) the concept is different with the main stress on the compatibility with the popular very high pressure (>800 bar) nanoACQUITY™ UPLC system produced by Waters. To withstand the pressure, a ceramic material, known in the electronic industry as Green Tape™ [18] was selected as the chip material. The fabrication process involves laser ablation and thermal sintering. The ceramic chip is placed in a plastic frame with the spraying tip for online connection to mass spectrometer and should include also some of the related electronics.
Table 2.I) Examples of companies specialising in commercial microfluidics

<table>
<thead>
<tr>
<th>Company</th>
<th>Companies interest</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microfluidics development, design, manufacture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellix</td>
<td>Develops, manufactures microfluidic pumps and chips for cell based assays</td>
<td>[19]</td>
</tr>
<tr>
<td>Dolomite</td>
<td>Designs, manufactures and sells of microfluidic chips (glass, quartz, ceramic), pumps, valves, sensors, connectors</td>
<td>[20]</td>
</tr>
<tr>
<td>LabSmith</td>
<td>Distributor of Microfluidic ChipShop products</td>
<td>[21]</td>
</tr>
<tr>
<td>Microfluidic ChipShop</td>
<td>Designs, develops and manufactures of microfluidic chips</td>
<td>[22]</td>
</tr>
<tr>
<td>Micralyne Inc.</td>
<td>Designs and manufactures of microfluidic chip devices from different materials (borofloat and borosilicate glass, fused silica, quartz and silicon) with 30 years experience</td>
<td>[23]</td>
</tr>
<tr>
<td>Micronit Microfluidics BV</td>
<td>designs and fabricates microfluidic chips from glass</td>
<td>[24]</td>
</tr>
<tr>
<td>MicruX Technologies</td>
<td>Designs, develops, manufactures and sells miniaturised and portable analysis systems</td>
<td>[25]</td>
</tr>
<tr>
<td>MiniFAB</td>
<td>Designs, develops and manufactures microfluidic chips</td>
<td>[26]</td>
</tr>
<tr>
<td>PRECISIONmicro</td>
<td>Designs, develops and manufactures microfluidic chips</td>
<td>[27]</td>
</tr>
<tr>
<td>ThinXXS</td>
<td>designs and manufactures plastic microfluidic chips</td>
<td>[28]</td>
</tr>
<tr>
<td>Translume</td>
<td>designs and fabricates microfluidic chips from fused silica glass</td>
<td>[29]</td>
</tr>
<tr>
<td>Microfluidic separation systems</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agilent Technologies</td>
<td>Bioanalyzer 2100 and 2200 Tape station for on a chip electrophoretic separation of DNA, RNA and proteins</td>
<td>[6]</td>
</tr>
<tr>
<td></td>
<td>1260Infinity HPLC-Chip/MS system – different HPLC microfluidic chips for broad spectrum of applications</td>
<td></td>
</tr>
<tr>
<td>BioRad</td>
<td>Experion – electrophoretic separation of DNA, RNA and proteins on a chip (alternative to Agilent Bioanalyzer 2100)</td>
<td>[30]</td>
</tr>
<tr>
<td>Company</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Caliper Life Sciences</td>
<td>Caliper in a partnership with Agilent Technologies introduced 2100 Bioanalyzer in 1999. Later introduced their own microfluidic systems - LabChip GX/GXII (nucleic acids and proteins analysis)</td>
<td>[5]</td>
</tr>
<tr>
<td>Eksigent Technologies</td>
<td>Nano HPLC systems (NanoLC), cHiPLC nanoflex – upgrade for nano HPLC using chips instead of capillaries</td>
<td>[13]</td>
</tr>
<tr>
<td>Shimazdu</td>
<td>MultiNA chip electrophoresis for RNA, DNA, separations</td>
<td>[11]</td>
</tr>
<tr>
<td>Waters Corporation</td>
<td>develops and manufactures liquid chromatographs and mass spectrometers. HPLC on microfluidic chip - TRIZAICTM nano Tile technology</td>
<td>[16]</td>
</tr>
<tr>
<td>Genomics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Advanced Liquid Logic</td>
<td>LSD-100 (enzyme activity) and ModrianTM (genomics sample preparation) are instruments using Digital microfluidic technology (electrowetting) for a precise droplets on a surface manipulation</td>
<td>[31]</td>
</tr>
<tr>
<td>Affymetrix</td>
<td>GeneAtlas system - microarray for genome expression on the strips</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td>GeneChip system - microarray for genome expression on the glass wafer</td>
<td></td>
</tr>
<tr>
<td>Applied Biosystems</td>
<td>Ion Proton, Ion 314 – semiconductors chip based next-generation sequencing of genome</td>
<td>[33]</td>
</tr>
<tr>
<td>Cepheid</td>
<td>Genexpert system using specific disposable cartridges for PCR and DNA analysis</td>
<td>[34]</td>
</tr>
<tr>
<td>Fluidigm</td>
<td>BioMarkTM is a system using chips containing reaction chambers for 9,216 individual qPCR reactions</td>
<td>[35]</td>
</tr>
<tr>
<td>Helicos</td>
<td>HeliScope Single Molecule Sequencer- tSMS (true Single Molecule Sequencing) DNA sequencing</td>
<td>[36]</td>
</tr>
<tr>
<td>IntegenX</td>
<td>Apolo 100 - microfluidic DNA sequencer</td>
<td>[37]</td>
</tr>
<tr>
<td>Micronics</td>
<td>Point of care instruments for invitro diagnosis. PanNAT - molecular diagnostic system for infectious disease testing.</td>
<td>[38]</td>
</tr>
<tr>
<td>Veredus laboratories</td>
<td>Portable analysers. VereID Biosystem – screening of DNA/RNA on a</td>
<td>[39]</td>
</tr>
</tbody>
</table>
chip (VereFlu).

## Proteomics

<table>
<thead>
<tr>
<th>Company</th>
<th>Technology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biacore</td>
<td>Surface Plasmon Resonance (SPR) of protein interaction</td>
<td>[40]</td>
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</table>

## Immunoassay

<table>
<thead>
<tr>
<th>Company</th>
<th>Technology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbott</td>
<td>Immunoassay screening tests (the first HIV screening tests)</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td>i-STAT, portable and field deployable handheld blood analysis system</td>
<td></td>
</tr>
<tr>
<td>Gyros</td>
<td>Gyros immunoassay platform - ‘compact disks’ for microfluidic immunoanalyses</td>
<td>[42]</td>
</tr>
</tbody>
</table>
2.3 Aims and rationale

The long list of commercially available Lab on a Chip instrumentation illustrates great uptake of this new concept. But some feel like the economic potential of Lab on a Chip technology has not been realised, blaming this to fact that a ‘killer application’ is missing. For example, in comparison with Micro Electro-Mechanical Systems (MEMS) technology, Lab on a Chip technology is far off finding itself a place in most households in the western world. One of the reasons for this could be the high market entry barrier for the introduction of Lab on a Chip devices in everyday life. Prof Zengerle recently proposed addressing this issue by the development of microfluidic applications for existing instrumentation [43]. Currently, uptake of microfluidic technology depends on marketing a specific microfluidic application and the instrument required to operate the chip. When existing instrumentation can be used to operate the new assays, market barriers decrease because the development and therefore marketing costs will be significantly reduced and consumers are more likely to purchase the new application if they are already familiar and satisfied with the equipment.

The aim of this research was the development of new applications for the Agilent Bioanalyzer 2100, focusing on the analysis of small molecules. In this chapter, an assay is described for the Agilent Bioanalyzer 2100 to identify prostate cancer based on the glycan profile following the separation of the glycans by capillary electrophoresis. The remaining chapters of this thesis focus on the development of methods for the isotachophoretic methods for the Agilent Bioanalyzer to taking advantage of the self-focusing effect.
2.4 Separation of 8-aminopyrene-1,3,6-trisulfonic acid labelled glycans by the Agilent Bioanalyzer 2100

DNA/RNA analysis and SDS-protein separations are the main application areas of commercial microfluidic chip based electrophoresis stations. These applications are performed routinely in thousands of laboratories around the world; however, there are many additional types of analyses, which might benefit from the speed and simplicity of microchip electrophoresis. An area of strong interest has been identified in the analysis of glycans released from serum or plasma proteins with a potential future application in medical diagnostics [44-46]. Proteins, especially their posttranslational modifications, produced by aberrant cells are changed when compared with normal cells. These changes may concern altered glycosylation of the proteins of interest. Identifying differences between glycans released form glycoproteins released, e.g., from plasma of cancer negative person and cancer diagnosed patient could help in early detection of pathological states.

2.4.1 Chemicals and materials

Water was purified using a Millipore (Bedford, MA, USA) MilliQ water purification system. Pipette tips (1200-μL) containing 10 μL normal-phase polyamide resin and 200-μL pipette tips containing 10 μL boronic acid (boronic acid-lectin affinity chromatography) and wheat germ agglutinin resins (BLAC/WGA) were obtained from PhyNexus (San Jose, CA, USA), and are referred to as PhyTips. TRIS was obtained from Fluka (Buchs, Switzerland); 8-aminopyrene-1,3,6-trisulfonic acid (APTS), acetic acid, NaBH$_3$CN (1 mol/L in tetrahydrofuran), trifluoroacetic acid, taurine, sodium chloride, magnesium chloride, manganese chloride tetrahydrate, sodium azide, calcium
chloride, peptide-N-glycosidase F (PNGaseF), fetuin glycoprotein from fetal calf serum and ethanol were purchased from Sigma–Aldrich (St Louis, MO, USA). Microcon ultrafiltration devices (0.5 mL with 10 kDa cut-off) were from Millipore. The Glycoprotein Denaturing Buffer, G7 and NP40 solutions were part of the Endoglycosidase buffer kit from New England BioLabs (Ipswich, MA, USA). Hydrochloric acid solution (1 mol/L) was from Merck (Haar, Germany). The pooled human plasma sample was kindly provided by the Central Laboratory of the Semmelweis University (Budapest, Hungary). Signed informed consent was obtained from all participants and the research protocol was approved by the local Research Ethics Committee (Medical Research Council, Budapest, Hungary).

The background electrolyte used for all experiments was prepared of ammonium acetate and acetic acid purchased from Sigma–Aldrich (St Louis, MO, USA) mixed with commercial DNA analysis gel provided by Agilent Technologies (Santa Clara, CA, USA), 50 mM acetate buffer (pH 4.75) mixed with Agilent DNA gel in ratio 4:1.

2.4.2 Instrumentation

The Agilent Bioanalyzer2100 (Agilent Technologies, Santa Clara, CA, USA) and commercial DNA chips (separation channel $l_{tot} = 40$ mm, $l_{cf} = 14$ mm) were used for all chip-CE experiments.

The sequences programmed in the software for the control of the Bioanalyzer are predefined for a small number of assays. The standard version of the instrument software is locked, allowing only dedicated DNA/RNA and SDS-protein analysis protocols. The unit used in these experiments was accessible using the development software provided by Agilent Technologies. The developer software is described in detail in the Appendix.
The first step of all protocols in the Bioanalyzer is focusing the LIF system because the position of the separation channel will vary slightly between chips. For the DNA assays, focusing is facilitated by the intercalating dye in BGE. In this work, the BGE did not contain any dye. The middle of the separation channel was focused on a 10 µM fluorescein solution prior to analysis. After focusation, the chip was flushed with water and dried using pressurised air.

For the analysis of APTS labelled oligosaccharides the DNA chip was used as a simple cross geometry chip as is shown in Figure 2.2A. Prior to analysis the reservoir (2) in the DNA chip was filled with 12 µL of BGE. The pressure was applied to this reservoir and BGE filled all the channels. Reservoirs (3) and (4) were filled with 12 µL of BGE. Reservoirs at the unused channels (the grey channels in Figure 2.2A) were filled with 6 µL of BGE. Reservoir (1) was flushed and filled with 12 µL of a sample. A chip was placed in the Bioanalyzer and the specifically designed protocol started running a separation program. The HV (ΔU = 1060 V, 65 s) applied between reservoirs (1) and (2) electrokinetically injected the sample of APTS labelled oligosaccharides (Figure 2.2C). The CE separation (Figure 2.2D) was initiated by applying HV (ΔU = 1060 V) between reservoirs (3) and (4). The light source used for LIF detection of APTS labelled glycans was blue LED inbuilt in the Bioanalyzer (2 mW, λ<sub>ex</sub> = 470±25, λ<sub>em</sub> = 525±30 nm).
Loading, electrokinetic injection, and CE separation of APTS labelled oligosaccharides. A. DNA chip was used in this experiment as a simple cross geometry chip. B. Reservoirs (2), (3), (4) and all channels were filled with BGE. Reservoir (1) was filled with sample. C. Sample was electrokinetically injected in the Bioanalyzer by applying HV between reservoirs (1) and (2) ($\Delta U = 1060$ V). D. CE separation was initiated by applying HV between reservoirs (3) and (4) ($\Delta U = 1060$ V).
The CE experiments were carried out using an in-house built CE system equipped with fluorimetric detector (ZetaLIF, Picometrics, Toulouse, France). A fiber-coupled light emitting diode was used as a light source in this detection system. LUXEON LED (3 W, 700 mA, 460 nm) was coupled into the optical fiber (1 mm in diameter) providing 8 mW optical power output. A high voltage power supply (Unimicro Technologies, USA) was used to run the separation. Separations took place in a fused silica capillary (375 μm OD, 75 μm ID) with 64 cm total length and 55 cm effective length. Prior the first use, the capillary was flushed with 0.1 M NaOH for 10 minutes followed by flushing with 0.1 M HCl for 10 minutes and the background electrolyte for 15 minutes. Samples were injected electrokinetically for 20 s at -10 kV.

2.4.3 Sample preparation

APTS labelled glycans samples were prepared in Horváth Laboratory of Bioseparation Sciences, University of Debrecen, Hungary. The sample preparation protocol used five steps described below:

1. **BLAC/WGA affinity micropartitioning:** The mixture of 5 μL agarose-bound wheat germ agglutinin and 5 μL m-aminophenyl boronic acid agarose beads (BLAC/WGA) was packed into 1200-μL pipette tips (PhyNexus) and used with a semi-automated multichannel micropipettor unit from Rainin (Oakland, CA, USA). The intake and expel flow rates of the pipettor were both set to 250 μL/min, controlled by the PhyNexus Controller Software. The BLAC/WGA pipette tips were kept at 4 °C in glycerol and used only once to avoid any sample carryover. Before glycoprotein enrichment, all BLAC/WGA tips were washed with 100 μL water to eliminate the glycerol storage solvent. This was followed by a second washing step with 100 μL 20 mM potassium phosphate buffer (pH 2.85) and 100 μL deionised water. All of these
steps were done by using ten intake–expel cycles. Then the BLAC/WGA pipette tips were conditioned by $3 \times 10$ intake–expel cycles with a semi-automated multichannel micropipettor using 100 μL fresh binding buffer (50 mM taurine, 1 mM CaCl$_2$, 1 mM MnCl$_2$, 20 mM MgCl$_2$, 0.05% NaN$_3$, pH 8.7). After the washing and conditioning steps, the tips were used for glycoprotein affinity capture from 100 μL human plasma diluted with 100 μL binding buffer (50 intake–expel cycles). After the affinity capture, the unbound proteins were washed off with excess binding buffer (20 intake–expel cycles, 100 μL each). The captured proteins were eluted by addition of 100 μL 0.1 M HCl solution (20 intake–expel cycles) and stored at −20 °C until use.

2. **Ultrafiltration of the glycoprotein fraction:** The low-molecular-mass components of the human plasma, in particular the glucose content, were removed by means of 10 kDa Microcon centrifugal ultra-filtration at 13,000 g for 20 min (Labofuge 400R Centrifuge; Heraeus, Osterode, Germany) prior to affinity micropartitioning. The cellulose membrane filters were washed with 500 μL 0.1 M NaOH solution and flushed with 500 μL HPLC water. Three washing steps were used with 150 μL MilliQ water. The glycoproteins were subsequently recovered by inverting the cartridge, followed by another centrifugation step at 1,000 g for 3 min. To increase sample recovery, the membrane was additionally washed four times with 10 μL MilliQ water.

3. **PNGase F digestion:** Glycoproteins were denatured at 98 °C for 10 min after the addition of 1 μL of denaturing buffer. This step was followed by peptide-N-glycosidase F digestion using 2 U of enzyme at 37°C for 120 min in 25 μL of total reaction volume (2.5 μL of G7 and 2.5 μL of NP40 buffer solutions from the Endoglycosidase buffer pack of New England BioLabs). The deglycosylated proteins were then precipitated by the addition of three times volume of ice-cold ethanol, followed by centrifugation at 11,000 g for 10 min.
4. **APTS labelling:** The glycan-containing supernatants were dried in 0.2 mL microfuge vials in a centrifugal vacuum evaporator (Eppendorf AG, Hamburg, Germany) and labelled through reductive amination by the addition of 1 μL of 0.2 M APTS in 15% acetic acid and 1 μL of 1 M NaBH₃CN in tetrahydrofuran. The labelling reaction was incubated at 37 °C overnight to assure no decomposition of sialic acid containing structures. The reaction was stopped by the addition of 100 μL of water to the reaction mixture.

5. **Sample purification:** In this instance, 10 μL of DPA-6S normal phase polyamide resin containing PhyTips (PhyNexus) were used (1200-μL pipette tips with multichannel pipettor). The 100 μL sample was diluted with 900 μL of acetonitrile and 5 μL of 25% NH₄ was added to the mixture prior to the purification process. Acetonitrile (95%) was used in the washing and 20% acetonitrile in the elution process, respectively. Eight intake/expel cycles were applied followed by 24 washing cycles using four aliquots of 1 mL of 95% acetonitrile. The captured molecules were then eluted with 250 μL of 20% acetonitrile.

### 2.4.4 Results and discussion

APTS labelled glycans can be analysed by capillary electrophoresis with excellent efficiency and resolution. On the other hand, microfluidic chips with short separation distances does not offer as good resolution but can provide high separation speed [47]. This is demonstrated in the electropherograms of APTS labelled glycans released from fetuin glycoprotein - Figure 2.3. The peak of free APTS appeared after 15 seconds on the DNA chip while CE needed 220 seconds. Both methods show similar profiles of the glycan structures in the sample. The major components are resolved in both cases;
however, the analysis time is more than 14 times shorter in case of microchip electrophoresis.

The possible applicability of this method as a screening test was shown on the sample of APTS labelled glycans released from the human plasma sample. The results of these experiments are shown in Figure 2.4 (DNA chip electropherograms of samples from healthy and prostate cancer patients) and in Figure 2.5 (comparison of electropherogram of prostate cancer patient obtained from DNA chip and from CE). The difference between shown electropherograms indicates that the Bioanalyzer has the potential for the proposed method. Nevertheless, while the efficiency and peak capacity was superior for a 40/35 cm (total/effective length) capillary in a CE system using 10 kV compared to the chip with a 40/14 mm (total/effective length) channel at 1.5 kV, this was expected, as the achieved efficiency in CE is proportional to the voltage. However, for fingerprint-type analysis it is not necessary to have knowledge of the identity of the peaks. To implement the on-chip method in routine screening tests it would be necessary to conduct a proper medical diagnostic study involving building a database containing various electropherograms from a statistically large group of patients with different health conditions.

2.5 Conclusions

This work aimed applying chip-based CE with fluorescence detection for glycan fingerprint analysis of human plasma samples using commercially available instrument the Agilent Bioanalyzer 2100. The short length of the separation channel in the electrophoresis microchip enabled rapid analysis in less than one minute. Although, the resolution of the separation was apparently lower than that of in regular capillary electrophoresis, it seemed to be adequate for rapid glycan fingerprint screening.
Additional improvements in both resolution and sensitivity would require a new chip design with longer separation path and different separation enhancement additives to minimize or optimize the electroosmotic flow. If the analysis was based on identification of the peaks, not on fingerprint comparison, this could be achieved by connecting the chip with a mass spectrometer.
Electropherograms of glycans released from fetuin glycoprotein using **A.** microchip electrophoresis ($l_{\text{tot}} = 40$ mm, $l_{\text{ef}} = 14$ mm) and **B.** standard CE equipment with $l_{\text{tot}} = 64$ cm, $l_{\text{ef}} = 55$ cm separation length.

Conditions: $\Delta U = -1060$ V and -17 kV for chip CE and capillary CE respectively (265 V/cm), for all other conditions see experimental.
Fig. 2.4) Comparison of chip CE electropherograms obtained from plasma samples obtained from A. healthy and B. prostate cancer patients.

Conditions: $\Delta U = -1500$ V (375 V/cm), for all other conditions see experimental.
Electroferograms of glycans released from plasma samples obtained from prostate cancer patients; A. chip CE (conditions as in Figure 2.4); B. standard CE equipment with $l_{tot} = 40$ cm, $l_{ef} = 35$ cm separation length. Conditions of CE separation: hydrostatic sample injection (15 s at level difference 7 cm), separation at $\Delta U = -10$ kV (250 V/cm).
2.6 References


Chapter 3

Isotachophoresis on a chip with indirect fluorescence detection as a field deployable system for analysis of carboxylic acids

3.1 Introduction

As was previously mentioned the main aim of this work was to investigate applicability of the Agilent Bioanalyzer 2100 for separation and detection of low molecular weight analytes. In the previous chapter was shown that the Bioanalyzer 2100 is capable to quickly profile APTS labelled oligosaccharides, but the resolution is inferior to that obtained in a capillary-based system. To improve performance of the Bioanalyzer to detection of small molecules our investigation here focused to develop a method, which would be suitable for short separation channel and more importantly will not require fluorescent labelling.

Despite its high sensitivity, fluorescence detection required a labelling reaction for non-fluorescent analytes. This introduces an additional step in the analysis, but also significantly reduces the differences in size to charge ration when bulky fluorescent tags are coupled to small molecules. In CZE, indirect detection methods are typically less sensitive than direct detection methods because of the difficulties in measuring a small signal on a large background. In 1984 Reijenga developed and published an ITP method using a fluorescent counterion for indirect fluorescence (IF) detection in ITP [1]. Because sensitivity in ITP is determined by the zone length, this indirect detection approach did not suffer from the sensitivity issues known from indirect detection in
CZE. More recently, Chambers and Santiago revisited IF detection in ITP using Alexa Fluor 488 as fluorescent overspeeding coion or R6G as fluorescent counterion for ITP with IF of model sample containing MOPS and HEPES [2]. The aim of this work was to demonstrate the versatility of the Bioanalyzer and its existing microchips to perform ITP separations of carboxylic acids. Protocols for ITP with indirect fluorescence detection were developed and validated on the Bioanalyzer and the method was applied to the analysis of benzoate in soft drinks. Benzoate is a preservative agent in beverages, which can form the carcinogenic benzene in in combination with ascorbic acid [3]. The allowable levels limits of benzoate in food and beverages differ in different countries, but the most stringent limit is in the USA at 0.1 % (ca. 7 mM.) [4].

3.2 Experimental section

3.2.1 Instrumentation

All experiments were performed on an Agilent Technologies Bioanalyzer 2100 (Palo Alto, CA, U.S.A.) with commercial DNA chips, using blue LED for LIF detection. The CZE method for determination of benzoate in soft drinks published by Costa et al [5] was used for verification of results obtained by the Bioanalyzer. The CZE was carried out using Agilent Technologies HP3D CE capillary analyser (Palo Alto, CA, USA). Separation polyimide uncoated fused silica capillary (50 µm I.D. x 375 µm O.D.) was 33 cm long with effective separation length 24.5 cm. The BGE was 25 mM Tris with α-hydroxyisobutyric acid (HIBA) (pH 8.1). Sample was injected by pressure (50 mbar, 3 s), separation voltage was 30 kV applied to the injection side, temperature was 25 °C and direct UV detection of benzoate was carry out at 200 nm. Prior the first use the
capillary was preconditioned with 1 mM NaOH (4 bar, 5 min), with water (4 bar, 3 min) and with BGE (4 bar, 5 min). The capillary was flushed with BGE (3 bar, 1.5 min) between runs.

3.2.2 Leading electrolyte (LE), terminating electrolyte (TE) and samples

Conditions used for ITP of carboxylic acids were adopted from the work by Dolnik and Bocek [6] and Chambers and Santiago [2].

The LE contained 20 mM HCl and 1% (w/v) PVP adjusted to pH 3.3 with β-alanine and 50 μM R6G. The LE was prepared daily by mixing stock solutions of (1) 100 μM R6G and (2) 2% (w/v) PVP in 40 mM hydrochloric acid adjusted to pH 3.3 with β-alanine in a ratio of 1:1 (v/v). The TE was 50 mM propionic acid.

A sample containing oxalate, pyruvate, fumarate, malate, mandelate, 2-hydroxyisobutyrate, succinate and acetate (2 mM each) was prepared in Milli-Q water from 64 mM stock solutions of each acid.

Benzoate was quantified in soft drinks with the standard addition of 2 mM of sodium benzoate. Three different diet soft drinks (Kirks Lemonade, Kirks Club Soda Lemon Squash and Waterfords Apple Berry) were purchased from the local supermarket and sonicated for fifteen minutes to release the carbon dioxide before use.
3.2.3 Protocol for running isotachophoresis on a DNA chip

The protocol developed for injection and subsequent ITP separation on Bioanalyzer is illustrated in Figure 3.1. To fill the chip, 12 µL of LE was loaded in the reservoir W2 and positive pressure was applied to this reservoir by the plastic syringe with rubber seal provided with the Bioanalyzer kits. LE was loaded in reservoir W1 and all unmarked reservoirs (the first three rows from left 6 µL, the row on the right 12 µL). Reservoir T was filled with 6 µL of the TE and reservoir S with 6 µL of the sample. The chip was then placed inside the Bioanalyzer.

The sample was loaded for 200 s by applying a constant negative current to reservoirs T (-0.15 µA) and S (-0.45 µA) to simultaneously load TE and sample (Figure 3.1B). A positive current was applied to reservoir W1 (+0.6 µA) while all other reservoirs were set to 0 µA. After sample loading, a current was set between reservoirs T (-0.3 µA) and W2 (+0.3 µA) for ITP separation while all other reservoirs were set to 0 µA. The blue LED inbuilt in Bioanalyzer was used for IF detection. Absorption and emission spectra of R6G are shown in Figure 3.2

3.2.4 Fluorescent microscopy for the method visualisation

Fluorescence microscopy was used to visualise the step formed between LE and TE shown in Figure 3.3 in section 3.3.1 and to visualise electrokinetic injection and ITP separation with and without pinch and pullback current discussed in section 3.3.3. The fluorescence microscopy system used for the experiments presented here and in all following chapters, is described in “Appendix” (A.2.2.1).
Schematic illustration of the way the DNA chip was used for ITP. A. Channel design with the channels used for ITP analysis (highlighted). Total length of channel used for ITP separation was 28.9 mm (a distance between reservoirs T and W2), the effective length 16.8 mm (the red zone in Figure 3.1A). Reservoir T was filled with TE and reservoir S with sample. All other reservoirs were filled with LE. B. TE (orange) and sample (blue) were electrophoretically injected by applying current between reservoirs T, S and W1 for 200 s. C. The ITP separation was started by applying a current between reservoirs T and W2.
Fig.3.2) Absorption and emission spectra of R6G are suitable for indirect fluorescence detection using blue LED inbuilt in Bioanalyzer 2100.
3.3 Results and discussion

3.3.1 Visualisation of isotachophoretic zones; detection system sensitivity

Reijenga et al. [1] published IF detection in ITP by using quinine as fluorescent counterion, however this is incompatible with the excitation systems in the Bioanalyzer. More recent work by Chambers and Santiago [2] used R6G for IF detection. The principle of this approach was that a small concentration of fluorescent counter-ion was present in LE at a concentration lower than the concentration of any other ions in the ITP system, and therefore it does not significantly affect the ITP zones. The fluorescent counterion migrates through all ITP zones where its concentration adjusts according to Kohlrausch’s law.

In an ITP electrolyte system, there is often a change of pH between zones. Numerical simulations conducted with Simul [7] for the targeted electrolyte system showed that the pH changed from 3.3 to 4.3 when moving from the LE to the TE. Based on excitation and emission spectra, R6G was selected as tracer ion for this work, and with pKₐ values estimated as 3.73 and 6.13 by software Marvin Sketch [8], a change in ionization of R6G when moving from LE to TE may compromise its functionality as a tracer ion. The change in charge will change the concentration of R6G to satisfy the Kohlrausch regulating function and the fluorescence intensity may also be affected by the net charge of R6G. We nevertheless demonstrated R6G could be used for IF under these conditions, as illustrated with the abrupt change in fluorescence between zones of LE and TE in Figure 3.3.
Fluorescence microscopy image visualizing the use of R6G as tracer ion illustrating the transition from LE to TE. The isotachopherogram shows response of the fluorescence detection system of the Bioanalyzer to this transition. The expanded baseline section shows the detection system noise. Conditions: LE = 20 mM HCl + 1% PVP + 50 μM R6G + β-alanine; TE = 50 mM propionic acid; ITP on a DNA chip with $I_{\text{const.}} = -0.3 \ \mu\text{A}$. 

Baseline noise = 0.2
Step height = 22.5
The isotachopherogram in Figure 3.3 was used to evaluate the theoretical capacity of the proposed ITP – indirect fluorescence detection (IFD) system. Based on the assumption a step can be distinguished if the difference in fluorescence intensity is three times larger than the noise (0.2 x 3 = 0.6), yields a theoretical capacity 37 analytes (22.5/0.6 = 37.5).

### 3.3.2 Analysis of carboxylic acids

To evaluate the performance of the Bioanalyzer for the analysis of small molecules, a series of organic acids were selected according to their electrophoretic mobilities [9]. The first experiment contained only LE and TE (one step) and acids were added sequentially. When nine acids were injected, the step corresponding to the slowest acid disappeared, hence the capacity of the system was the separation of eight acids (Figure 3.4). This is significantly below the theoretical capacity of 37 analytes determined based on the fluorescence intensity, indicating the separation system, not the detection system limits the capacity of this protocol. Limitations in the separation system can be contributed to the limited microchannel length, the low voltages, and the presence of residual EOF, deteriorating the sharpness of the zone boundaries, and thus increasing the minimum zone length which can be detected.

Nevertheless, this is the first time the Agilent Bioanalyzer using chips designed for GE of large molecules was successfully applied for the separation of small molecules without any modifications of the instrument or commercial chips. The LOD values were based on a shortest step length of 0.3 s with LODs for the tested carboxylic acids ranging from 0.12 to 0.4 mM.
Fig. 3.4)  

A. Isotachopherograms of the separation of 8 organic acids with IF detection; Sample: 1=oxalate, 2=pyruvate, 3=fumarate, 4=malate, 5=mandelate, 6=2-hydroxyisobutyrate, 7=succinate, 8=acetate (2 mM each). B. Length of the ITP step (L_{st}) was measured as a distance between peaks from the first derivation of the detector signal (noise filtering using 15-point moving average). Conditions: LE = 20 mM HCl + 1% PVP + 50 μM R6G + β-alanine; TE = 50 mM propionic acid; ITP on a DNA chip with I_{const.} = -0.3 μA.
3.3.3 Method repeatability

The repeatability of the ITP system in the Bioanalyzer was evaluated using a mixture containing 2 mM pyruvate, lactate and 3-hydroxybutyrate, resulting in a 8.25% RSD for the length of lactate step. To provide some insight into the reasons for suboptimal performance, the processes inside the microchannels during ITP were visualised using a fluorescence microscope and Cy-5 as fluorescent sample. With the current set to 0 µA to all channels not required for loading of the sample, Cy-5 leaked from the main channel into the unused channels. When performing the separation, the Cy-5 slowly leaked back out into the main separation channel, potentially causing the poor repeatability.

3.3.3.1 Pinch and pullback current applied to block a leak in and out of the unused channels

From the literature [10, 11], in a microfluidic chip, channels where the sample should not enter, are often blocked by using pinch and pullback current. Pinch current is applied while sample is injected. This blocks leaking of sample inside the channels where the sample should not enter. Later, when separation is started the pullback current is applied to the unused channels to ensure that electrolyte from these channels will not leak into the separation channel. In this work pinch and pull back currents of ±0.05 µA were applied to the unused microchannels as illustrated in Figure 3.5. The pinch and pullback currents improved the RSD of the length of the lactate step from 8.25% to 1.93%, however, shorter steps (3.45 seconds without to 2.49 seconds with pinching and pullback) were obtained due to the smaller volume injected. For better understanding of this problem a video of the ITP analysis of Cy5 (sample) on a DNA
chip with and without pinch/pullback current is available at attached CD with supporting information.

3.3.4 Multiple sample analysis with electrokinetic injection

The DNA chips for the Bioanalyzer enable the analysis of up to twelve samples per chip. With the discontinuous electrolyte system in ITP, a maximum of eight samples can theoretically be analysed by ITP on the DNA chips. The sequence of events required for analysis of eight samples is illustrated in Figure 3.6. To keep the protocol simple pinch and pullback current was not used in this experiment.

Multiple sample analysis – sequence of events:

1) Chip preparation (Figure 3.6A):
   - Leading electrolyte was loaded into the waste reservoir C4 (12 μL),
   - Pressure by plastic syringe (0.5 mL of air for 10s) was applied to C4, LE filled all channels, the rest of LE in the reservoir C4 was removed.
   - LE was loaded into the reservoirs A4, B4, C4, D4 (12 μL) and A3, D3 (6 μL),
   - Reservoirs A2 and D2 were filled with terminating electrolyte,
   - All other reservoirs (A1, B1, C1, D1, B2, C2, B3, C3) were filled with a sample.

2) Electrokinetic injection (200 s) of the first sample and TE is shown in Figure 3.6B:
   - Chip was placed inside the bioanalyzer and analysis protocol was started,
   - Current was applied for 200 s between reservoirs A1 (sample; -0.45 μA), A2 (TE; -0.15 μA) and waste reservoir D4 (LE; +0.6 μA),

Channel between reservoir A1 and the first channels cross-section was filled with anions from the sample. Channel between reservoir A2 and the first channels cross-section was filled with anions from TE. Channel between the first cross-section and waste reservoir D4 was filled with the mixture of ions from the sample and TE.
Fig. 3.5) Schematic explanation of pinching (A.) and pullback current (B.) implemented to prevent sample from leaking into and out of the unused channels. The video documenting the effect of pinch and pullback current is available at attached CD with supporting information.
Fig.3.6) Multiple sample analysis; A. loading of LE, TE and sample into the reservoirs, B. sample and TE electrokinetic injection, C. ITP separation, D. channels flushing, E. the next sample and TE electrokinetic injection. The detailed figure explanation is in text below.
3) ITP separation (Figure 3.6C):
   - Constant current was applied between reservoir A2 (TE; -0.3 μA) and waste reservoir C4 (LE; +0.3 μA) for 200 s.

4) Electrokinetic flushing of the separation channel (Figure 3.6D):
   - Constant voltage was applied between reservoirs C4 (1600 V), A1 (100 V) and A3 (100 V) for 200 s. Chlorides started to migrate from the A1 (LE) and A3 (LE) toward to the C4 (LE).
   - This step was used to fill the separation channel with LEs anion and restore the initial conditions before the next analysis.

5) Repeat 2nd, 3rd and 4th step with the next sample (Figure 3.6E)
   - Next analysed sample was loaded from reservoir B1, then from B2 and then from B3.
   - The samples from D1, C1, C2 and C3 were injected from the opposite side of the chip, therefore the protocol used opposite reservoirs (the TE was injected from D2 instead of A2, the waste reservoir for loading the sample and TE was in D4 instead of B4 and the flushing step was driven from D3 instead of A3)

This sequential injection process followed for all eight reservoirs containing a 2 mM sample of pyruvate, lactate and 3-hydroxybutyrate. The resulting isotachopherograms are shown in Figure 3.7. The three steps for the three carboxylic acids can only be distinguished in the first isotachopherogram, each following isotachopherogram is missing the step for 3-hydroxybutyrate, the slowest ion in the sample. The fluorescence intensity decreased with each successive injection, with the most significant drop between the first and the second sample.
Isotachopherograms of eight sequential injections of the 2 mM pyruvate, lactate and 3-hydroxybutyrate from different reservoirs. Sequence of injection was described in Figure 3.6 all other conditions as in Figure 3.4. **A.** Raw data. **B.** For easier detection of differences, isotachopherograms were stretched vertically and aligned.

Numbers indicate the order of separation.
To understand these results, the chip ITP system was modelled using Simul 5.0 [7]. The model consisted of a 5 mm long, 50 µm ID capillary divided into two compartments; a 1 mm long injection compartment and a 4 mm long separation compartment. The detector was placed 4 mm from the start of the capillary. A schematic illustration of the model used in Simul is given in Figure 3.8. The consequent analysis of three samples in six simulations is described in the following text:

1. The first simulation = injection of the first sample: The first compartment was filled with 50 mM propionic acid (pKa 4.874; \( \mu = 37.1 \text{m}^2\text{V}^{-1}\text{s}^{-1} \)), and the second compartment was filled with 20 mM hydrochloric acid (pKa -2; \( \mu = 79.1 \text{m}^2\text{V}^{-1}\text{s}^{-1} \)), 0.0001 mM dye (pKa 9.25; \( \mu = 20 \text{m}^2\text{V}^{-1}\text{s}^{-1} \)) and 201.5 mM alanine (pKa 9.857/2.33; \( \mu = 32.2/34 \text{m}^2\text{V}^{-1}\text{s}^{-1} \)). The simulation program runs for 200 s. When the first simulation ended the second compartment contained no hydrochloric acid and 14.437 mM propionate.

2. The second simulation = electrokinetic flushing of the second compartment: The first compartment was filled with 20 mM hydrochloric acid. The data obtained from the first simulation were used for the second compartment (14.437 mM propionate). The main aim of this simulation was to find if we are able to replace propionate with chloride by electrokinetic flushing. The simulation run for 200 s. When the second simulation ended the concentration of hydrochloric acid was just 4.798 mM instead of required 20 mM, and the concentration of propionate decreased to 10.987 mM.

3. The next simulations: The third simulation run under the same conditions as the first one, except the second compartment in the capillary contained data obtained from
the second simulation (4.798 mM HCl and 10.987 mM propionic acid). The fourth simulation copied the second, except the second compartment contained data obtained from the third simulation. The analogue process was applied to the fifth and sixth simulations. The results of the simulation with respect to the electrolyte levels are summarised in Table 3.I.

From Table 3.I the most obvious is the increase in propionate concentration in the separation compartment with each consecutive run (0, 10.99 and 11.09 mM for the first, second and third sample, respectively). This demonstrated that the electrokinetic flushing did reduce, but not remove propionate from the separation channel. The most significant change in propionate was found between the first and the second analysis. In contrast with the increase in propionate in the LE, the chloride, β-Ala and R6G concentrations all decreased for each subsequent analyses. The changing isotachopherograms from the consecutive samples can therefore be explained by the significant changes to the composition of the LE while the decreasing concentration of the R6G resulted in the lower fluorescence intensity. The changes in LE composition make the proposed electrokinetic flushing unsuitable for automated ITP analysis of multiple samples on the DNA chip.
The conditions for simulation
Table 3.I. Simulation of multiple sample analysis by Simul 5.0

<table>
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<th>2&lt;sup&gt;nd&lt;/sup&gt; compartment</th>
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3.3.5 Quantitative analysis of benzoate in soft drinks

The ITP method was used for the quantitative analysis of benzoate in soft drinks. When using pinching and pullback currents citrate was the only identifiable step in the isotachopherogram, as illustrated in Figure 3.9. The broad transitions suggest the presence of other analytes below the detection limit. As mentioned before, the use of pinching and pullback currents increased the reproducibility but decreased the amount of sample injected. The larger volume injected when not applying pinching and pullback currents enabled the identification of multiple steps, as illustrated in Figure 3.9.
Isotachopherograms from the analysis of Waterfords Apple Berry diet soft drink. With (A.) and without (B.) the use of pinching and pullback currents. Steps 3 and 5 were identified as citrate and benzoate, respectively. Conditions as in Figure 3.4.
To correct for the poor repeatability of injection without pinching and pullback, the length of the benzoate step was standardised to the length of the first step in the isotachopherogram. Benzoate was then quantified by standard addition using 2 mM benzoate. The results obtained from chip ITP were validated with a CZE method for the analysis of benzoate and sorbate in soft drinks (Figure 3.10). The two methods were in good agreement, with the ITP method providing a consistently lower concentration than the CZE method. The linear range for benzoate in the model mixture by ITP-IFD was between 0.5 and 40 mM benzoate ($R^2 = 0.9969$) using 2 mM citrate as an internal reference for step normalization (all experiments were done in triplicate).

The citrate in soft drinks was found to have a non-linear effect on the electrokinetic injection, increasing the LOD more than LOQ (limit of quantification). Using aqueous standards, the calculated LOD and LOQ for benzoate were 0.3 mM and 0.35 mM, respectively. In the tested soft drinks (5-10 mM citrate) LOD and LOQ values for benzoate were increased to 1.15 mM, and 2.6 mM, respectively. Even in presence of these elevated citrate levels, the LOD and LOQ of the developed chip ITP method are still below the regulatory limits of 0.1% or approximately 7 mM benzoate. The benzoate level for all tested soft drinks was within the approved limit.
Benzoate in diet soft drinks was quantified by the method ITP – IFD on the DNA chip. Results were compared with CZE method. Bars indicate confidence interval ($\alpha=0.95$) based on three replicates.
3.4 Conclusions

The Agilent Bioanalyzer 2100 was demonstrated as an ITP on a chip platform for the separation of eight carboxylic acids by ITP-IFD using the commercially available DNA chips. This was the first time that a commercial CE-chip based platform originally developed and used for specific application (DNA) was demonstrated to be applicable to a very different method, namely ITP-IFD.

The method developed here was used for quantification of benzoate in three different diet soft drinks, validated using a capillary-based CZE method. The results shown here demonstrate an alternative separation method on an existing commercially available platform, opening a wide range of new applications.
3.5 References


Chapter 4

Separation of carboxylic acids in human serum by isotachophoresis using a commercial field-deployable analytical platform combined with in-house glass microfluidic chips

4.1 Introduction

In the previous chapters it was demonstrated that the Agilent Bioanalyzer 2100 has potential for quantification of small, nonfluorescent molecules without labelling. ITP–IFD on a Agilent DNA chip using the Bioanalyzer was applied for quantification of benzoate in soft drinks.

While successful, soft drinks represent a relatively simple sample, and it is necessary to determine whether the method and platform are suitable for the separation of ions in samples with a more complex matrix as serum or plasma. The rationale behind using ITP for the separation of this type of complex samples is in the potential simplicity of the analytical method. Sample pre-treatment is typically the most time consuming part of the analytical procedure, and avoiding sample pretreatment will significantly reduce the total analysis time. This is particularly important for the development of point of care and on-site diagnostics systems. Almost three decades ago, ITP was used as an analytical method capable to preconcentrate and separate analytes of interest from samples such as serum or plasma without any pre-treatment [1-6].

In this chapter, the analysis of organic acids in serum by ITP with indirect fluorescence detection using the Agilent Bioanalyzer 2100 is described. We developed a new glass
microchip for ITP compatible with the Bioanalyzer 2100 to improve analytical performance over the standard commercially available DNA chips. The microchip design included four different channel lengths, 25.7, 34.5, 62.8, and 125.8 mm, for comparison of pressure, negative pressure and two ways of electrokinetic injection. The optimal design used the 25.7 mm ITP channel with pressure injection, where the ITP steps for lactate from human serum were twice as long as those obtained in the standard DNA microchip.

4.2 Experimental section

4.2.1 In-house fabrication of glass ITP chip

The ITP chip was designed by using the free software Draft Sight (Dassault Systemes, Velizy Villacoublay, France). The microfluidic chips were made from soda-lime glass slides obtained as 76 x 26 x 1 mm microscopes slides (Menzel Gläser, Braunschweig, Germany) or 75 x 50 x 1 mm microscopes slides (Corning, NY, USA). ITP chip fabrication was accomplished in the Department of Bioanalytical Instrumentation, Institute of Analytical Chemistry of the Academy of Science of the Czech Republic, v.v.i., Veveří 97, 602 00 Brno, Czech Republic (ASCR). The fabrication process shown in Figure 4.1 is further described in the following sections.

4.2.1.1 Sputtering of the layer of chrome

Before sputtering of chrome, the sodium lime glass microscopic slides were cleaned in a piranha solution (H₂SO₄:H₂O₂ in ratio 3:1 (v/v)) for 10 min, then rinsed in distilled water and acetone.
Fig. 4.1  Microfluidic chip fabrication. A. Glass wafer (3) protected by a layer of chrome (2) and positive tone photoresist (1). Photoresist was exposed by using direct laser pattern generator. B. The layer of chrome protects the glass during the wet etching process. C. Channels are enclosed with the second glass wafer. D. Chip is finished after the glass wafers are bonded together at 590 °C.
Cleaning and drying were accomplished in the clean room in a laminar flow box BIO60 (Faster, Milano, Italy) to avoid contamination of cleaned glass with dust. The Sputter Coater SCD500 (Bal-tec, Danhill, UK) was used to sputter the chrome on the surface of glass wafer (100 nm, Figure 4.1A3).

4.2.1.2 Spin coating of the thin layer of a positive photoresist

Positive Tone Photoresist ma-P 1225 (Micro resist technology GmbH, Berlin, Germany) was coated on chromium layer to protect chrome. The thin and uniform layer of photoresist (Figure 4.1A2) was applied onto the glass wafer covered with chrome by the Spin coater WS-400B-6NPP-Lite (Laurell Technologies Corporation, North Wales, USA). The three-stage program was used for photoresist spin coating. In the first stage, 1 mL of photoresist was transferred on the wafer. The spin coater began to rotate at 1000 rpm for 5 s. In the second stage, the spin coater rotated with speed 3000 rpm for 30 s. At the last stage spincoater rotated at 6000 rpm for 5 s. A wafer covered with photoresist was placed onto the hot plate (assembled in the laboratory of ASCR), which was set to a 100 °C, for 6 min.

4.2.1.3 Maskless photolithography by Laser Pattern Generator

A bench top Laser Pattern Generator µPG 101 (Heidelberg ins., Heidelberg, Germany) equipped with a UV laser (375 nm, 18 mW) was used for photoresist ‘maskless’ exposition. The layout printed by laser pattern generator is shown in Figure 4.2.

4.2.1.4 Photoresist developing, metal etching, glass etching

Wafer with exposed photoresist was placed onto the hot plate (100 °C) for 5 min, and subsequently developed for 1 min in the Developer for Photoresist ma-D 331 (Micro resist technology GmbH, Berlin, Germany).
Fig. 4.2) The layout printed by the Laser Pattern Generator. The layout was uploaded as a bitmap with the resolution 1 pixel = 1 \( \mu \text{m} \).

A. ITP microchannels; B. black dots mark the spots for drilling the holes. The red-circled lay-out is a scale for determining the depth of the etched channel (Figure 4.3).
Chrome that was not protected by the photoresist was dissolved after 30 s in solution of chrome etch. This solution was prepared by dissolving 16.5 g of (NH$_4$)$_2$Ce(NO$_3$)$_6$ (p.a., min. 98.0%, Fluka, Austria) and 4.2 g of HClO$_4$ (p.a., 70-72%, Sigma Aldrich, Czech Republic) in distilled water. The total volume of this solution was adjusted to 100 mL with distilled water.

Unprotected glass was etched in a solution containing 7.5 mL of HF buffer, 82.5 mL of distilled water and 10 mL of 35% HCl (p.a., min. 35%, Onex, Czech Republic). The HF buffer was prepared by dissolving of 40 g of NH$_4$F (p.a., min. 97%, Lach-ner, Czech Republic) and 15 ml of HF (p.a., 38-40%, Lach-ner, Czech Republic) in 55 mL of distilled water.

To obtain the appropriate channels depth, the depth of the wet etched microchannels (anisotropic etching) was examined under the microscope by using the scale mentioned earlier in Figure 4.2. This scale contained 22 lines which each of them was 10 μm wide. The distance between the first and the second line was 20 μm, between the second and third line it was 22 μm, and between the twenty-first and twenty-second line was 60 μm. The etching scale is shown in Figure 4.3A. When the etching was finished, the channels depth was examined by using the scale under a CCD microscope SWM 340 (LabSmith, CA, USA) and was estimated as the half distance between two channels which were just touching at their tops as shown in Figure 4.3B.

The depth of in-house ITP chips was kept at 13 μm. The time necessary to achieve this depth was estimated to 25 min under the wet etching conditions described above. After etching, the rest of the photoresist was removed with acetone and the rest of chrome was removed with the chrome etching solution.
A. The scale was printed onto the glass wafer together with the lay-out of the ITP chip. (Figure 4.2); B. The depth of etched channel was equal to the half distance between two channels with just touching tops.
4.2.1.5 Drilling the holes

The top glass wafer was glued onto the graphite block (70x70x20 mm) by a wax and immersed under the surface of cooling water. Holes were drilled by a high speed rotary tool (10,000 rpm) using a 2 mm wide, spherical diamond drill bit.

4.2.1.6 Glass wafer bonding

Glass wafers were subsequently washed in a soap detergent, acetone, and distilled water. The bottom and top glass wafers were connected under the filtered distilled water to avoid any dust pollution between them. After the water between wafers dried, it was put between two silicon wafers (Electronics and Materials, Ashiya, Japan) and high temperature resisting glass-ceramic wafers Neoceram (Nippon Electric Glass, Otsu, Japan). This was placed in laboratory furnace 5013L (Clasic, Řevnice, CZE) under a 700 g weight as it is shown in Figure 4.4.

The furnace was closed and the five-stage temperature program began. In the first step, the temperature increased from room temperature to 100 °C (at a speed 10 °C / 1 minute). Then the furnace temperature was maintained at 100 °C for one hour. For the next step, the temperature was increased to 590 °C (at a speed 10 °C / 1 minute). This temperature was maintained in the furnace for 15 hours. In the last step, the furnace cooled down to room temperature.
Thermal bonding of the chip was accomplished in the electric furnace at 590 °C. Chip was placed between two silicon wafers, two glass wafers and the weight (700 g) was placed on the top of it.
4.2.1.7 Glass chip sawing

The reservoirs in glass chips were sealed with electrical tape (obtained from the local hardware store) and the bottom part of the chip was glued onto the graphite block (70x70x20 mm) by wax, and cut with a Dicing Saw EC-400 (MTI corp., Richmond, USA) to a 17.5 mm square. The diamond cutting blade rotated at 3000 rpm, and it was cooled by an anti-corrosion coolant Koring 551 (Korchem, Blučina, CZE), mixed with distilled water in the ratio 1:5. The cutting speed was set to 2 mm per a min.

4.2.1.8 Gluing chip into a plastic chip frame

The glass chips were glued into the black PMMA plastic frame also used for the commercial chips (Agilent Technologies) by two minute epoxy glue (obtained from a local hardware store) deposited as a layer to ensure that all reservoirs entrances in glass chip were well separated and that the in-house chip was fitted well in the plastic caddy. This last step of chip fabrication ensured that the chips would be placed in the Bioanalyzer as required.

4.2.2 Isotachophoresis of human serum

4.2.2.1 Leading electrolyte, terminating electrolyte and samples

Conditions for ITP of carboxylic acids in human serum were adopted from the previous chapter. Briefly, the LE was prepared daily by mixing two stock solutions in a ratio of 1:1 (v/v). The first stock solution contained 100 μM R6G dissolved in water, and the second stock solution was prepared by dissolving 2%(w/v) PVP in 40 mM hydrochloric acid, adjusted to pH 3.3 with β-alanine.

The TE was prepared by diluting propionic acid to a concentration of 50 mM.
A model sample was prepared by dissolving pyruvate, lactate and 3-hydroxybutyrate in water. Human serum was collected from a healthy volunteer by staff at Hobart Pathology and was pipetted into plastic vials and stored in the freezer at -20 °C.

4.3 Results and Discussion

4.3.1 DNA chip: electrokinetic and pressure injection for isotachophoresis of serum sample

The ITP system for the analysis of benzoate in soft drinks on the Bioanalyzer 2100 using the commercial Agilent DNA chip was shown in previous chapter (Figure 3.8). Figure 4.5A shows that this method failed for the analysis of carboxylic acids in human serum with no steps observed with a 200 s injection and only one minor step after injection times of 500 and 1000 s, respectively. The electrokinetic injection used to inject sample into the microchips was suitable for the low conductivity samples used before, but not for high conductivity samples like serum, containing an abundance of chloride, approximately 100 mM, which is ~100 times higher than the concentration of the target analytes. Further, as chloride has a much higher mobility than the organic acids present in serum, chloride ions are preferentially injected. Thus under electrokinetic injection conditions, the majority of ions injected into the microchannel are chloride ions because of its higher mobility and naturally high abundance, making electrokinetic injection unsuitable for this sample type.
Fig. 4.5) ITP of human serum on a commercial DNA chip by Bioanalyzer 2100 using A. electrokinetic and B. negative pressure injection. Conditions: LE = 20 mM HCl + β-alanine (pH 3.3) + 1% PVP + 50 μM R6G; TE = 50 mM propionic acid; Sample = human serum; injection methods A. electrokinetic (serum/TE -0.45/-0.15 μA, waste +0.6 μA, details in previous chapter) and B. hydrodynamic by negative pressure (30 s by 1 mL plastic syringe); current used for ITP separation was -0.3 μA.
Hydrodynamic injection was conducted on a DNA chip by applying a negative pressure as illustrated in Figure 4.6. In the first step the LE was loaded into the reservoir (+). Pressure was applied to this reservoir by a 1 mL plastic syringe with rubber sealing (0.5 mL for 10 s), and LE filled all the channels in the DNA chip. All reservoirs except those reserved for TE and sample were filled with LE. Three reservoirs were filled with TE (blue) and three with sample (red). Negative pressure was applied to the reservoir marked as vacuum for 30 s by using a 1 mL plastic syringe with rubber sealing (see Figure A.8 in Appendix) and after that, the reservoir was filled with LE to prevent hydrodynamic flow. The filled chip was placed inside the bioanalyzer and ITP separation was started by applying separation current between the reservoirs (-) (-0.3 μA) and (+) (+0.3 μA). All other electrodes were set to current of 0 μA. This hydrodynamic (non discriminative) injection method introduced a serum sample into a DNA chip and resulted in an isotachopherogram as shown in Figure 4.5B. However, the sloping transitions between the first steps suggest that the steps were not fully developed and steady-state had not been reached when the zones passed the detector. This shows the need for a purpose designed ITP chip.

4.3.2 In-house ITP chip design

The results using the commercial DNA chips for the experiments above indicated a number of deficiencies in the chip design when used for ITP of serum. The separation of ions by ITP on a chip can be improved by using specifically designed chips [7-11], an approach undertaken here to improve the performance capability of ITP in the Bioanalyzer.
A. A manual negative pressure injection for 30 s was used to fill the DNA chip with sample and TE (darts marking flow direction of the electrolytes). After applying negative pressure, the vacuum reservoir was filled with LE. The filled chip was placed in the Bioanalyzer 2100 and ITP separation was started by applying current (-0.3 µA) between reservoirs (+) and (-). B. Image captured with a fluorescence microscope shows the negative pressure injection.
To ensure compatibility of the ITP chip with the Bioanalyzer 2100 instrument and its detection system, the glass microchip needed to be bonded to the plastic chip holder used for the DNA chips to position electrodes in the fluid reservoirs in the instrument. Furthermore, the separation channel needed to be in the same position as that used in the commercially produced chips such that optical alignment could be performed. The positions of the non-flexible components of the microchip are shown in Figure 4.7A and were obtained from the literature [12] and measurements using a microscope. Figure 4.7B shows the layout of the in-house ITP chips which can be divided into four quadrants, each containing a single ITP channel. Each ITP channel was composed of a wide and a narrow section. The wide section was the same for all four channels (5 mm long and 154 µm wide) and allowed different methods for loading samples into a chip. The narrow channel was 10 µm wide and its length was different for each four channels. The shortest channel was 20.7 mm long, the second was 29.5 mm, the third was 57.8 mm, and the longest channel was 120.8 mm. The total length of separation channels was 25.7, 34.5, 62.8 and 125.8 mm.

Soda-lime glass used for chips fabrication is a non-standardized material and its properties can be dramatically different not just between producers, but also from batch to batch. As shown in Figure 4.8, there was a significant difference in ITP separations of organic acids obtained for the two different glass manufacturers. Isotachopherograms obtained by using chips made from Menzel Glaser soda-lime glass microscope slides had longer, sloped ITP steps. This was most likely caused by residual EOF even at the electrolyte pH used (pH 3.3) and with a content of 1% PVP in LE. Shorter and sharper ITP zones were formed using a chip made from Corning soda-lime glass microscope slides. Because of their superior separation performance, the chips made from Corning glass were subsequently employed for all other experiments.
Fig. 4.7) Chip design; A. dimensions (in millimetres) which are important for the compatibility of in-house chips with the Agilent Bioanalyzer 2100; B. master pattern used for chip fabrication. Reservoirs at the end of each channel are marked: S - sample, T - terminating electrolyte and W represent waste (leading electrolyte). The vertical numbers represent the length of each ITP channel (in millimetres) from the injection point (junction S-T). Dimensions of the wide and narrow channels are 154 and 10 µm.
Fig. 4.8) ITP separation of model acid mixture, performed in chips made from soda-lime glass obtained from two different suppliers. Sample, comprising 1 mM pyruvate, 1 mM lactate and 1 mM 3-hydroxybutyrate, was injected into the shortest channel (25.7 mm in length) by applying pressure (1 bar for 1 s). ITP separation performed with a constant current of -0.2 µA.
4.3.3 Sample injection

The in-house designed chips were designed to test different injection methods, which is documented in the following text. Four different approaches to injection were possible, two based on electrokinetic injection (section 4.3.3.1 and 4.3.3.2), and two based on hydrodynamic injection (section 4.3.3.3 and 4.3.3.4). These differ not only in the way in which the sample is injected into the chip, but in the volume injected and whether or not the solutions within the chip need to be changed between injection and separation. For better orientation, the schema of the injection part of the chip is shown in Figure 4.9.

Each reservoir in the schema shown in Figure 4. is numbered. In the further text the “reservoir number” abbreviated R. For example, “reservoir number 1” is abbreviated as R1. The real in-house chip is more complicated than the schematic in Figure 4.9 shows, therefore the further text uses abbreviation RX for all other reservoirs.

All the reservoirs in the chip are designed to hold 10 µL of electrolytes/samples. This volume was used for loading of all electrolytes into the chip reservoirs.

LE used for all experiments contained 20 mM HCl, β-alanine (pH 3.3), 1% PVP and 50 µM R6G. TE contained 50 mM propionic acid in Milli-Q water. A sample used for ITP experiments was containing 2 mM pyruvate, lactate and 3-hydroxybutyrate.

Injections were visualized using a fluorescence microscope by directly monitoring the fluorescence of R6G in LE. To visualize the sample, a dilute solution of Cy5 dissolved in Milli-Q water, was used. The TE did not contain any fluorescent dye, but its zone was slightly visible due to migration of R6G through the ITP system as a counterion from the LE.
Fig.4.9) Schematic of the injection part of the in-house chip. The numbers of reservoirs are used in the following text to address the position of electrolytes used for testing different injection methods. Reservoir RX represents all unused reservoirs in the in-house chip.
4.3.3.1 On-line electrokinetic injection

LE was loaded inside the R4 (reservoir 4 in Figure 4.9), pressure was applied to R4 (with a syringe), and the LE filled all the channels. Sample mixed with TE (1/1) was loaded inside R3 and TE inside R1. R2 and RX were filled with LE.

The filled chip was placed inside the Agilent Bioanalyzer 2100 and the electrokinetic injection was started by applying a constant current (R1 = 0 µA, R3 = -0.3 µA, R2 = 0.3 µA and RX = 0 µA). During the injection mode the optics of Bioanalyzer focused the separation channel, and sample and TE filled the channel between R1, R2 and R3. After 200 s constant current was switched from the injection mode to the separation mode (R1 = -0.3 µA, R4 = 0.3 µA and RX = 0 µA).

A schematic of the on-line electrokinetic injection and ITP separation, and photos obtained by fluorescence microscopy are shown in Figure 4.10.
Fig. 4.10) Schema (A., C.) and microscope visualisation (B., D.) of the on-line electrokinetic injection. A. and B. injection, C. and D. ITP separation.
4.3.3.2 Off-line electrokinetic injection

LE was loaded as described previously (4.3.3.1). Sample mixed with TE (1/1) was loaded inside R1, and RX were filled with the LE.

The filled chip was placed inside the Agilent Bioanalyzer 2100 and electrokinetic injection was started by applying a constant current (R1 = -0.3 μA, R2 = 0.3 μA, RX = 0 μA). During the injection mode the optics of Bioanalyzer focused the separation channel, and sample filled the channel between reservoirs 1 and 2. After 200 s constant current was switched off, and the chip was taken out off the Bioanalyzer.

Sample was removed from R1 with a pipette. R1 was subsequently flushed with TE three times by using a pipette and then filled with TE. The chip was placed back in the Bioanalyzer and the analysis was started by applying constant separation current (R1 = -0.3 μA, R4 = 0.3 μA and RX = 0 μA).

A schematic of the off-line electrokinetic injection and ITP separation, and photos obtained by fluorescence microscopy are shown in Figure 4.11.
Fig. 4.11) Schema (A., C.) and microscope visualisation (B., D.) of the off-line electrokinetic injection. A. and B. injection, C. and D. ITP separation.
4.3.3.3 Hydrodynamic injection – Negative pressure

LE was loaded as described previously (4.3.3.1). Sample mixed with TE (1/1) was loaded inside R1 and R3. RX were filled with the LE. Negative pressure was applied to the R2 for 20 s by using a 1 mL plastic syringe with seal.

Sample was replaced with TE as described previously (4.3.3.2). The chip was placed in the Bioanalyzer and the protocol driving the analysis was started. In the first 50 s the constant current applied to all reservoirs was 0 µA. In those 50 s detection optic focused the middle of separation channel. The rest of the protocol was ITP separation (R1 = -0.3 µA, R4 = 0.3 µA and RX = 0 µA).

A schematic of the negative pressure injection and subsequent ITP separation, and photos obtained by fluorescence microscopy are shown in Figure 4.12.
Fig. 4.12) Schema (A., C.) and microscope visualisation (B., D.) of the negative pressure injection (V). A. and B. injection, C. and D. ITP separation.
LE was loaded as described previously (4.3.3.1). Sample mixed with TE (1/1) was loaded inside R1. RX were filled with the LE. Pressure was applied to the R1 by using a 1 mL plastic syringe with a seal. The pressure and time needed to inject approximately the same amount of a sample into the different channels in the in-house chips were examined by using fluorescence microscopy. The volume of air in the syringe and time used for pressure injection was strictly specific for each channel. The injection pressure and time were different not just between channels dimensions but even between the same channels on two different in-house chips.

Sample was replaced with TE as described previously (4.3.3.2). The chip was then placed in the Bioanalyzer and the protocol driving the analysis was started. In the first 50 s the constant current applied to all reservoirs was 0 µA. In those 50 s detection optic focused the middle of separation channel. The rest of the protocol was focused to ITP separation (R1 = -0.3 µA, R4 = 0.3 µA and RX = 0 µA).

A schematic of the pressure injection and ITP separation, and photos obtained by fluorescence microscopy are shown in Figure 4.13.
Fig. 4.13) Schema (A., C.) and microscope visualisation (B., D.) of the pressure injection. A. and B. injection, C. and D. ITP separation.
All the above mentioned injection methods are more clearly shown in video obtained from fluorescent microscope. The video including narration is accessible at attached CD supporting information.

The only method evaluated that could be implemented without refilling the chip was the on-line mode electrokinetic injection. This used electric fields to define the injection volume, as depicted in Figure 4.10A. All of the other 3 methods required removal of the sample from the injection well and replacement with TE. While the simplest method for injection is on-line mode electrokinetic injection as it is performed without refilling the chip, it failed to introduce a sufficient amount of serum sample of the organic acids due to the high abundance and mobility of chloride, as previously discussed for the DNA chip. Electrokinetic and negative pressure injections were highly irreproducible because of the loss of the sample when flushing the injecting reservoir with TE (as can be seen in video mentioned above). The most promising results were obtained with the pressure injection (using 1 mL plastic syringe with rubber seal). This injection introduced the largest amount of sample, which increased the system sensitivity. The pressure and time needed to inject approximately the same amount of a sample into the different channels in the in-house chips were examined by using fluorescence microscopy. The volume of air in the syringe and time used for pressure injection was strictly specific for each channel. For example, to fill the wide part of the shortest channel the pressure (20 µL in the syringe) was applied for 1 s, the second shortest channel needed to apply the same pressure but for 3 s. The third channel was filled by applying pressure (100 µL in the syringe) for 3 s, and the longest channel used the same pressure but applied for 5 s. The injection pressure and time were different not just between channels dimensions but even between the same channels on two different in-house chips.
In the Figure 4.14 can be seen that the pressure injection introduced the largest amount of sample between the LE and TE, and the resulting isotachophoregrams demonstrated the longest steps from all tested methods. This ensured that minor loss when flushing the injecting reservoir with TE was inconsequential when compared to the volume that had been injected. Despite the relatively largest amount of introduced sample in the chip with the pressure driven injection, it exhibited relatively poor reproducibility. The calculated RSD based on ten replicates was 15.54% for pressure driven injection.

4.3.5 Microchannel length

As mentioned above, the narrow part of each ITP channel in the chip had different lengths in order to study the length required for the ITP system to reach steady state. In accordance with the ITP principles [13] the results from the isotachophoregrams in Figure 4.15 showed that the length of the separation channel only influenced the total analysis time and did not affect the length of the steps. The shortest channel (25.7 mm) and pressure injection were chosen for ITP analysis of the human serum sample.
Fig. 4.14) Different length of pyruvate step obtained by using different injection methods on an in-house ITP chip. The bars indicate confidence interval ($\alpha=0.95$) based on three replicates.
Fig. 4.15) Effect of different lengths of separation channel. Conditions: LE contained 100 mM HCl, 1% PVP, 50 µM R6G and β-alanine (pH 3.3), TE contained 100 mM propionic acid. The sample contained 10 mM pyruvate, 10 mM lactate and 10 mM 3-hydroxybutyrate. The time and pressure used for the sample injection were tested in every channel separately before commencing the experiments. A constant current of -0.5 µA was used for all experiments. A. Isotachopherograms show time delay between different separation channels, B. Aligned isotachopherograms show that similar data were obtained from all four ITP channels.


**4.3.6 Isotachophoresis of human serum**

The process of analysis of the human serum started with filling the chip with LE, TE and untreated serum, as described above. The filled chip was placed in the Bioanalyzer, and the protocol running the ITP separation was started. A constant current of -0.3 µA was applied between reservoir with TE- T1 and reservoir with LE- W5 (see Figure 4.7B), the first ITP step appeared after 830 s with the entire separation completed within 900 s, as can be seen in Figure 4.16. In contrast, the separation shown in Figure 4.5B, using the commercial DNA chip with negative pressure injection, the first step appeared after 350 s and was complete in 425 s.

Comparison of isotachopherograms from DNA chip from in-house ITP chip is shown in Figure 4.16. On the DNA chip, the first ITP step was longer. The second step, later identified as lactate (standard addition), was longer in the isotachopherogram obtained from the in-house ITP chip. The step of lactate was 34.2 s long in the in-house chip and 17.95 s with the DNA chip, indicating that pressure injection on the custom designed ITP chip introduced double the amount of sample into the channel when using the DNA chip. More importantly, as was mentioned before, the sloppy transitions between steps on the DNA chip suggested that the steps were not fully developed and steady-state had not been fully reached. It is likely that this was the reason why the first step was longer on DNA chip. While this indicates the improvements from the in-house designed chip, the large difference between the injected amounts resulted in a doubling of the total analysis time for the in-house chip. This time difference could theoretically be reduced by applying a higher constant current, however, this was not possible due to the dimensions and high resistivity of the narrow channels, and the limited high voltage that can be supplied by the Bioanalyzer (1500 V max.).
Fig.4.16) ITP of human serum using the shortest channel (25.7 mm) of the in-house chip and pressure injection. Conditions: LE = 20 mM HCl + β-alanine (pH 3.3) + 1% PVP + 50 µM R6G; TE = 50 mM propionic acid; Sample = human serum; A constant current of -0.3 µA was used for separation, unused channels 0 µA.
From Figure 4.16, the additional step behind the lactate step was regarded as a mixed zone. This would suggest using the longer separation channel on the in-house chip.

The attempt to repeat the same condition analysis on a longer channel failed due to inability to keep required constant separation current -0.3uA.

While the use of in-house ITP chip successfully demonstrates the increased detection limits by a factor of two, the repeatability was poor due to an inability to accurately control pressure injection. Due to problems with injection and repeatability in this generation of chip design, we were not able to clearly determine the LOD and LOQ values. Nevertheless, estimated LOD values for this method and used chip design was in the mM range. The problems associated with the in this paper described 1st generation of in-house ITP chip design were latter resolved by using next generation of purpose designed DFR chip. The DFR chip design and its application for ITP separation are described in Chapter 5.

4.4 Conclusions

In this chapter it was demonstrated ability to successfully use in-house designed and fabricated glass ITP chips fitted in the Agilent Bioanalyzer 2100 combines the strength of research chip designs with the advantages of a commercial instrumental platform with in-built LED induced fluorescence detection. The in-house chips allowed testing of different methods of sample injection. The longest ITP steps were obtained for pressure injection, which also introduced the largest quantity of sample into the system. Testing of soda-lime glass chips from different producers established that the results depend strongly on the properties of the glass wafers used. The different length of separation channels were also tested, with all channels showing similar results. For all subsequent experiments we used the shortest channel to ensure shortest analysis time.
The in-house ITP chip was employed to separate acids in human serum, which was injected into the chip without any previous sample treatment. Better results were obtained with the in-house chip than the commercial DNA chip due to a bigger amount of injected sample entering the channels, and higher separation capacity. The benefit of this work includes a better understanding of the chip-ITP with indirect fluorescence detection implemented on a commercial field deployable analytical platform in terms of future directions, especially in the design of new in-house ITP-chips.
4.5 References


Chapter 5

Analytical isotachophoresis of lactate in human serum using a commercial field-deployable analytical platform combined with in-house dry film photoresist microfluidic chips

5.1 Introduction

In Chapter 4 it was shown that the Agilent Bioanalyzer 2100 can be used for ITP analysis of carboxylic acids in a human serum. The results from the commercial DNA chip were slightly improved upon when the in-house made chip was used, but the in-house chip still showed room for improvement.

In this chapter a redesigned ITP microchip was made to improve the repeatability and significantly reduce the analysis time. Despite glass having good optical transparency and chemical stability, Lab on a Chip research has moved to the use of polymer devices because their lower manufacturing costs. The material used for fabrication of newly designed microchips compatible with the Agilent Bioanalyzer 2100 was dry film photoresist (DFR). The Ordyl SY300 series DFR was previously described for fabrication of microfluidics chips at low cost using budget infrastructure, such as a LED light source and office laminator [1].

The repeatability in the previous glass in-house ITP microchip was poor due to lack of accurate control during pressure injection. To address this, the new chip design allowed a negative pressure injection. The new chips' channels dimensions were varied to reduce the hydrodynamic flow of electrolytes from different reservoirs, to improve detection sensitivity [2-4] and also to allow the use of a higher current, and therefore faster ITP
separation. The DFR-ITP chips were used for the detection of lactate in serum using indirect fluorescence detection. The results were confirmed using a capillary-based CE method [5].

5.2 Experimental section

5.2.1 In-house Dry-film resist (DFR) chip fabrication

The ITP chip was designed by using the free CAD software Draft Sight (Dassault Systemes, Velizy Villacoublay, France). The DFR chips were fabricated using the same instruments and a slightly modified procedure, as described previously by Guijt et al. [1]. A chip base layer used in this work was 70x50x1 mm polycarbonate (PC) sheet (Polytech Plastics, WA, Australia). A layer of 30 µm thick DFR (Ordyl 330, Elga Europe, Italy) was laminated using an office laminator (Peach 3500, Lamination Systems, Australia) onto the PC substrate and exposed using a UV shark series high-flux LED array (OHTL-0480-UV, Opto Technology, Wheeling, IL, USA) with an output intensity 0.8 mW/m². After UV exposure the wafers were baked on a hot plate (ECHOtherm™ MODEL HS40, Torrey Pines Scientific, CA, USA) fitted with a 5 mm thick piece of polished aluminium plate. A second layer of DFR was laminated and exposed using the same lithography source for 1 min with a chrome on sodium-lime glass mask (Figure 5.1) manufactured by Bandwidth Foundry (NSW, Australia). Channels were developed in a BMR developer (Elga Europe) and rinsed in a BMR rinse (Elga Europe). The profile and dimensions of the channels in the Ordyl DFR were examined by using a Wyko NT 9100 optical profiler (Veeco, NY, USA). Chips were cut by using a high-speed rotary tool (Dremel 400 series, Dremel Europe, Breda, Netherlands) obtained from local hardware store.
The final design of the mask used for fabrication of six ITP-dry film chips per one polycarbonate slide (75x50x1 mm). The in-house designed mask was made from chrome covered glass wafer by Bandwidth Foundry (NSW, Australia).
Holes for injecting electrolytes into the chip were drilled by using a drill press (Ryobi EDP 2521L, TTI, Hong Kong, China) and 2 mm drill bits obtained from a local hardware store and laminated with a final layer of DFR to seal the microchannels. The finished chips were glued into the plastic frames (a gift from Agilent technologies, Waldbronn, Germany) used for positioning chips in the Bioanalyzer 2100 by 5 minute epoxide obtained from a local hardware store.

5.2.2 Isotachophoresis of human serum

5.2.2.1 Leading electrolyte, terminating electrolyte and samples

Conditions for ITP of carboxylic acids in human serum were adopted and slightly modified from previous chapter. The LE was prepared daily by mixing three stock solutions. 200 µL of the first stock solution (100 µM R6G dissolved in Milli-Q water) was mixed with 40.7 µL of the second stock solution (98.28 mM hydrochloric acid with pH adjusted to pH 3.3 with β-alanine) and with 120 µL of the third stock solution (2% HPMC in Milli Q water). The total volume of LE was adjusted to 400 µL by adding 39.3 µL of Milli-Q water.

The TE was prepared daily by mixing stock solutions of 20 µL of 100 mM nicotinic acid with 120 µL of 2% HPMC. The volume of TE was adjusted to 400 µL by adding 260 µL of Milli-Q water. All stock solutions for preparing LE and TE were stored in the fridge at 6 °C.

The artificial samples were prepared by diluting a 100 mM stock solution of lactate to a required concentration.

Human serum samples were collected from a healthy volunteer by staff at Hobart Pathology and were pipetted into the plastic vials and stored in the freezer at -20 C.
Before analysis, 45 µL of defrosted serum was mixed with 40 µL of water, 38.6 µL of 2% HPMC, and 5 µL of standard lactate addition (0, 5, 10 and 20 mM lactate).

5.2.2.2 Negative pressure loading of electrolytes into a DFR-ITP chip

The in-house DFR-ITP chip was filled with electrolytes as shown in Figure 5.2A. First the reservoirs were filled with LE, TE and sample (S). Negative pressure was then applied to the reservoir (V – vacuum) by using a 1 mL plastic syringe with a rubber seal previously described in Figure 5.7 (-300 µL, 5 s) which caused electrolytes from reservoirs to fill the separation channel in the chip. To prevent hydrodynamic flow and to provide a conductive connection with an ITP system, the reservoir V was filled with LE after applying negative pressure. The injection and ITP separation was visualised by using a fluorescence microscopy. The visualisation was possible thanks to the R6G in LE. As sample was used LE due to incompatibility of Cy5 solution with DFR. After the current was applied between LE and TE reservoir, the zone of TE was visible as low fluorescence zone. Video obtained from this experiment is included on supporting information CD, which is attached to this thesis.

5.2.3 CZE of human serum

The CE method validated for determination of lactate in serum, published by Jager and Tavares [5], was used for verification of results obtained with the Bioanalyzer. The CZE was carry out using Agilent Technologies HP3D CE capillary analyser (Palo Alto, CA, USA). Separation polyimide uncoated fused silica capillary (75 µm I.D. x 375 µm O.D.) was 48.5 cm long with effective separation length 40 cm. The BGE was 5 mM 3,5-dinitrobenzoate, 0,1 mM cetyltrimethylammonium bromide (CTAB) (pH 3.5). Sample was injected by pressure (35 mbar, 2 s), separation voltage was -15 kV applied to the injection side, temperature was 25 °C, indirect UV detection of lactate was carry
out at 254 nm. Prior the first use the capillary was preconditioned with 1 mM NaOH (2 bar, 5 min), with water (2 bar, 5 min) and with BGE (2 bar, 30 min). The capillary was flushed with BGE (2 bar, 2 min) between runs. Serum samples were prepared by mixing 45 µL of human serum with 5 µL water, 10 mM lactate (1 mM standard addition) or 20 mM lactate (2 mM standard addition).

5.3 Results and Discussion

5.3.1 DFR-ITP chips design – electrolytes injection, dimensions

The most important aspect of ITP chip design is the injection method. ITP requires inject three different electrolytes (LE, TE, and sample) and the chip design has to allow it. Electrokinetic and hydrodynamic injections can both be used for sample introduction in ITP separations. Nevertheless, as was discussed in previous chapter using the discriminative electrokinetic injection of serum samples failed due to the high concentration of chloride, and the expected ITP results were obtained with hydrodynamic injection.

From the literature, Kaniansky’s and Prest’s research groups provided the most significant contributions in the areas of design, fabrication and use of in-house microfluidic chips for ITP with hydrodynamic injection of electrolytes. The hydrodynamic injection by positive pressure is difficult to realise in a simple, controlled and reproducible manner, and both research groups were using a system of valves and pumps [6-9]. In the work by Prest the microfluidic chip fitted in a square 78x78 mm [9]. Kaniansky was working with a chip 30x70 mm [6]. In this work, the designed microfluidic chip had to fit in a square 17.5x17.5 millimetres. With respect to these dimensions, the designing of a positive pressure injection system was a difficult task.
beyond our instrumental facility and time schedule. Negative pressure injection used here is another manner of hydrodynamic injection. A negative pressure can be applied to just one reservoir, enabling fluidic control of the flow of the different electrolytes injected from the other reservoirs using the microchannel design.

One of the insights gained from our previous experiments with in-house glass ITP chips was that wider channels increase the amount of injected sample and increase the conductivity of the ITP system. This is beneficial in ITP because the increased sample injection increases ITP system sensitivity, and the increased conductivity in wider channels allows to increase separation current, which decreases the analysis time. Here, we opted to use separation channel composed from a wide channel (300 µm width, 7.65 mm length), which was connected to a narrow (30 µm width, 3.65 mm length) microchannel to improve detection sensitivity [3-4]. The transition between wide and narrow part of separation channel was 1 mm long (Figure 5.2C).

A schematic of chip filing is depicted in Figure 5.2A. 10 µL of each electrolyte was loaded into the corresponding reservoir (LE, TE, S). The electrolytes were injected into the 300 µm wide separation channel by applying negative pressure to the negative pressure reservoir (V). To prevent hydrodynamic flow due to the different level of electrolytes in the reservoirs the negative pressure reservoir was filled with 10 µL of LE after the injection was finished. Another way how to restrict unwanted hydrodynamic flow was realised by designing 30 µm wide connections between the reservoirs and the wide separation channel. To avoid losing conductivity in those connections their length was 100 µm for reservoirs TE, S and V. Previously was mentioned that the length of narrow channel between the wide separation channel and the LE reservoir was 3.65 mm long. This length was purposely longer than was necessary to ensure that the detector optics would focus in the narrow channel of manually produced chips (Figure 5.2C).
In the design of the ITP chip, restrictions due to the size, location of reservoirs on the Agilent DNA chip frame and the position of the LIF detector in the instrument, are summarized in Figure 5.2B. Within these restrictions, the final microchannel designed is depicted in Figure 5.2C.

Using the schematic shown in Figure 5.2C, the variable in the design is the position at which the negative pressure injection microchannel interests the main separation channel. This defines the volume of sample injection, as well as the length of microchannel available for ITP separation prior to narrowing and detection. Using Simul 5.0 [10] three designs with different dimensions of negative pressure connection were evaluated (Figure 5.3) to determine whether steady state ITP could be reached prior to detection. The depth of microchannels chosen for simulations was 30 µm, which is the thickness of dry film further used for chip fabrication. The results are shown in Figure 5.3 by the colour coding on the microchannel: the change from black to red indicates the position at which a steady state system is first reached. Design A, which introduced the smallest amount of sample into the separation channel, showed that steady state was reached well before narrowing. Design B which introduced the largest amount of sample into a separation channel indicated that there was insufficient space in the separation channel and steady state was reached behind the detection point. The third design, design C, reached a steady state just before the transition from the wide to narrow channel. The third design was chosen for fabrication.
Fig. 5.2)  

A. The sketch of the chip design (LE = leading electrolyte, TE = terminating electrolyte, S = sample, V = reservoir for applying the negative pressure)  

B. Dimensions (mm) taken into account for compatibility with the Agilent Bioanalyzer 2100.  

C. Chip design with the dimensions (mm, unless otherwise stated) of the separation channel. In this stage of chip designing, the dimension of where the negative pressure injection microchannel will connect the separation channel was unknown, and additional simulations were required.
Three designs with varying length of the injection zone (indicated in mm) tested in Simul 5.0. The change from black to red indicates the position in the channel where a steady state is achieved. Design C was selected for further fabrication because steady state was reached just before the channels narrowed.
5.3.2 Chip fabrication

The material used for chip fabrication was Ordyl DFR previously described as suitable for fast and cheap microfluidic chip fabrication by Guijt et al. [1]. Ordyl is a thin plastic foil and needs a support. To address this Guijt et al. used PMMA wafers. The commercial DNA chips are made from glass wafers 1 mm thick. The substrate for the DFR chip fabrication had to have the same dimension to maintain the compatibility of the DFR chips with the detection optics in the Bioanalyzer. Because we could not access 1 mm thick PMMA, PC with a thickness of 1 mm was selected for the fabrication of the microchip. Unfortunately, PC is incompatible with the Ordyl BMR developing and rinsing solutions. During development and rinse, the exposed sides of the PC substrate were covered with sticky tape. Another issue with PC was that this material bends when placed on 110 °C hot plate. The resulting deflection caused an uneven distribution of temperature in different parts of the PC sheet, resulting in irregular dimensions in the microchip channels. This issue was resolved by placing a glass sheet (200x100x5 mm) on the top of PC sheet while baking on a hot plate. Six microchips were fabricated on each 50 x 75 mm PC substrate. An optical profiler was used to select the chips with the correct channel dimensions. As shown in Figure 5.4 approximate dimensions of narrow and wide channels after process of DFR fabrication were 49/28/27 µm and 330/308/28 µm (width<sub>top</sub>/width<sub>bottom</sub>/height), respectively. Reservoir holes were drilled using a 2 mm drill bit and the channels were sealed using a layer of Ordyl dry film. The chips were glued with 5 min epoxide glue inside the PMMA caddies provided by Agilent Technologies, which are specifically designed for use in the Bioanalyzer 2100. The finished chip is shown in Figure 5.5. After optimising the manual fabrication process, the fabrication yield was ~50%.
Ordyl chip channels were inspected using Wyko NT 9100 profilometer.

The magnification used for channels examination was 11x (lens 20x combined with Wyko inner optic 0.55x).
The finished DFR-ITP chips were glued inside the plastic frame, which positioned the chip for use inside the Bioanalyzer.
5.3.3 **Performance of DFR-ITP chips versus commercial DNA chips**

The first experiments with the DFR chips were to compare their performance with the commercial DNA chips. The conditions used for this experiment were similar to the conditions used in previous chapter. The sample was injected by applying a negative pressure (negative pressure injection on the DNA chip was described in previous chapter). The LE contained 20 mM HCl with β-alanine (pH 3.3), 1% PVP and 50 µM R6G. The TE contained 10 mM nicotinic acid, and the sample was composed from 2 mM each of pyruvate, lactate and 3-hydroxybutyrate. ITP separation was performed with a constant current of -0.3 µA. The pullback current applied to all unused channels during the ITP separation was 0.05 µA. The resulting isotachopherograms are compared in Figure 5.6A.

From Figure 5.6A, the fluorescence signal was approximately 4 times higher for the DFR-ITP chips in comparison to the DNA chips, due to the native fluorescence of the Ordyl DFR. While this would increase detection limits in conventional CE, it does not have a significant adverse affect on ITP separations, as the length of the step, not the height of a peak, defines detection limit. From the Figure 5.6A, it is very obvious that the ITP steps are much longer in the DFR-ITP chip than the DNA chip. The length of the lactate step by ITP on a DNA chip was 2.2 s and 42.2 s in the DFR-ITP chip, an increase of a factor 19.2. While this was a significant improvement, the isotachopherogram obtained from the DFR-ITP chip was missing the butyrate step. This step reappeared in the isotachopherogram after the separation was performed at a higher constant current of -3 µA (Figure 5.6B). The reason why the step of butyrate was missing, when the low constant current (-0.3 µA) was applied, was traced to a small difference between the separation and pull back currents (0.05 µA).
Fig.5.6)  

A. Comparison of DNA and DFR-ITP chips. LE = 20 mM HCl + β-alanine (pH 3.3) + 1% PVP + 50 μM R6G, TE = 10 mM nicotinic acid, Sample = 2 mM pyruvate, lactate, 3-hydroxybutyrate. Negative pressure injection, separation $I_{\text{const.}} = -0.3 \, \mu\text{A}$, pull back $I_{\text{const.}} = -0.05 \, \mu\text{A}$ (pull back current was used at all unused channels).  

B. The same separation on a DFR-ITP chip by using separation $I_{\text{const.}} = -3 \, \mu\text{A}$. 
Presumably, this difference caused migration of butyrate into the unused channels where the pull back current was applied. Another advantage of increasing the separation current in the DFR-ITP chip was shortening of the total analysis time from 175 s to 35 s. The effect of the used current is discussed further in section 5.3.4.

For the DFR-ITP chips, hydroxypropylmethyl cellulose (HPMC) was chosen as an EOF suppressant instead of PVP. This polymer is more viscous than PVP, and DFR-ITP chips showed better reproducibility with HPMC (lactate step ~5.16 s long, RSD = 2.5%) in comparison to PVP (lactate step ~5.2 s long, RSD = 19.5%).

### 5.3.4 Influence of separation current on the isotachopherogram

The new microchip design allows the use of higher separation current, which significantly broadens the scope of possibilities for ITP on the Bioanalyzer. To explore the capabilities of the DFR-ITP chip design, we investigated the dependence of the applied separation current on the isotachophoretic separation. Conditions used for this experiment were slightly different from those used for previous experiments, with the main difference being the addition of 0.6% HPMC to the LE, TE and sample. HPMC was added to the TE and sample to maintain similar viscosity with the LE, facilitating a predictable negative pressure injection of electrolytes. Figure 5.7A shows a plot of the relationship between the time necessary for the migration of chloride (LE) from the separation channel to the point of detection and the separation current; the relationship between the length of the lactate step and the applied separation current is illustrated in Figure 5.7B.
Fig. 5.7) The effect of the applied current on A. the step length of chloride from the LE and B. step length of lactate from the sample. Conditions: LE = 10 mM HCl + β-alanine + 0.6% HPMC + 50 μM R6G; TE = 5 mM nicotinic acid + 0.6% HPMC; sample = 2.5 mM lactate + 0.6% HPMC; applied separation currents were -0.3, -0.5, -1, -2, -3, -4, -5 and -6 μA. The pull back current applied to all unused channels was 0.05 μA. Bars indicate the confidence interval (α=0.95) based on three replicates.
As can be seen from Figure 5.7A, as the current is increased, the length of the chloride step decreases. For example, increasing the current from -1 to -2 µA, decreased the step length from 37.3 ± 0.1 s to 19 ± 0.3 s. From Figure 5.7B, a corresponding decrease in the step length of the lactate zone, reducing from 6.7 ± 0.15 s long to 3.3 ± 0.05 s can be observed when changing the separation current from -1 to -2 µA. However, at higher currents (-4, -5 and -6 µA) the length of the lactate step did not become shorter. This is thought to be due to the maximum high voltage of 1500 V that can be applied using the Bioanalyzer. In order to achieve the higher separation currents, the high voltages required were beyond the limits of the instrument. Once the highly conductive chloride zone had exited the microchannel, the voltage increase required to maintain the constant separation current with each successively less conductive ITP zone could not be achieved by the instrument.

However, the higher current (-6 µA) can be used at the start of analysis when the separation channel is still filled with high conductivity ions. For the analysis of high salinity samples as serum, the higher separation current can be used to remove the high concentration of chloride ions, as these will be quickly transferred from the separation channel into the waste reservoir. Once most of the chloride has left the separation channel, the current should be changed to a lower value to keep within the limitations of the Bioanalyzer. This reduced current, however, also reduces the speed of the ITP zones migrating through the detection point hereby increasing the system detection sensitivity. This approach of switching currents has previously been used to significantly reduce the total analysis time of high salinity samples and increase sensitivity [11]. Using this principle the on DFR chip ITP analysis of lactate in human serum could be performed in less than a minute.
5.3.5 Lactate quantification: method linearity, limit of detection and limit of quantification

To determine the linearity of the described method in the DFR chips, aqueous lactate solutions were tested in the range 0.3125 to 5 mM (0.3125, 0.625, 1.25, 2.5, 5 mM lactate). The constant separation current used for the experiment was -1 µA. As it is shown in Figure 5.8 the response was linear across the tested calibration range ($R^2 = 0.9989$) and limit of detection (LOD) and limit of quantification (LOQ) were calculated from the linear calibration at 42 µM and 0.23 mM, respectively. Similar to Chapter 4, the ITP zone length of 0.3 s was chosen as the LOD and the three times longer ITP zone (0.9 s) as the method LOQ.

5.3.6 Quantification of lactate in human serum

The main aim of this chip design was to develop a method capable of the analysis of complex samples, such as human serum. Serum has high salinity and is rich in proteins content. Most of the advanced separation methods will require pretreatment of serum samples before injection. In contrast, ITP is able to handle high salinity samples, which makes ITP an ideal choice for the analysis of serum and other high salinity samples. To mix the serum sample with aqueous solution of HPMC was the only pretreatment required for the method developed here.

Lactate is present in serum in millimolar concentration level, which is perfectly suitable with analytical ITP. The normal range of lactate in serum is approximately from 0.5 to 2.2 mM (the levels can vary significantly, for example as a result of physical exercise). Concentration of chloride in serum is approximately 100 mM, and therefore the step of lactate was expected at different time to compare with the aqueous lactate samples.
Fig. 5.8) Calibration curve for lactate. Bars indicate the confidence interval ($\alpha=0.95$) based on four replicates. LOD and LOQ of the method were 0.042 and 0.23 mM, respectively. Separation current was -1 µA, all other conditions were the same as in Figure 5.7.

$y = 3.1741x + 0.166$

$R^2 = 0.9989$

LOD = 0.042 mM

LOQ = 0.23 mM
Indeed, the time required for chloride to exit the separation channel was ~200 s for a separation current of -1 µA. To speed up the total analysis time for the serum, the current switching protocol was used by initially applying -6 µA to quickly remove the chloride (time reduced from ~200 s to ~34 s). The second part of ITP separation was driven by current -2 µA enabling the quantification of lactate within a minute. An isotachopherogram for the determination of lactate in human serum is shown in Figure 5.9A.

The large amount of manual handling in the microchips fabrication process made each chip unique. Therefore, external calibration was inconvenient and lactate was quantified in serum samples by standard addition. The quantitative results obtained from the DFR-ITP chip were compared with results obtained by traditional capillary zone electrophoresis using a method developed by Jager and Tavares [5]. The results obtained from three different serum samples by ITP on the DFR chips and CZE are summarized in Figure 5.10. The total analysis time for lactate in serum by CZE was just under 8 minutes. Eightfold improvement of this analysis time was achieved with DFR-ITP chips (less than one minute). However, it is important to note that while analysis on the chip was rapid, only one sample could be processed per a chip and the chips required manual conditioning between uses, while the CE method could be fully automated.
The isotachopherograms (A. and B.) of the human serum obtained from DFR-ITP chips by Agilent Bioanalyzer 2100 and from the literature (C.) [12]. Conditions: the constant current used for separation was -6 µA in the first stage and -2 µA (A.) and -1 µA (B.) in the second stage. The pullback current applied to unused channels was 0.05 µA during the analysis. The sample was prepared by mixing 45 µL of human serum with 45 µL of water and 38.6 µL of 2% HPMC. Figure A. and figure B. were obtained from two different chips and from two different samples. All other conditions were the same as those in Figure 5.7. Conditions for figure C.: LE = 10 mM HCl + 0.3% polyethylene glycol + β-alanine (pH 3.3), TE = 10 mM propionic acid, sample = human serum (for more details [12]). Figure 5.9C reproduced with permission [12].
Fig. 5.10) Lactate in three different serum samples quantified by CZE with indirect UV detection at 254 nm (yellow) and ITP with indirect fluorescence detection on the DFR-ITP chips using Agilent Bioanalyzer 2100 (red). Both methods showed comparable results. Bars indicate confidence intervals (α=0.95) based on three replicates.
5.4 Conclusions

A new ITP chip design convenient for serum samples was fabricated in Ordyl SY-300 series dry-film photoresist. The proposed chip’s design and materials used for the fabrication were fully compatible with the Agilent Bioanalyzer 2100. Using 300 μm wide channels, twenty times more sample could be injected than it was possible with the commercial DNA chips available for use with the Bioanalyzer, which led to twentyfold incensement in sensitivity with DFR-ITP chips.

The wider channels enabled a twentyfold increase in applied separation current when used with high conductivity samples. To optimise performance between sensitivity and time, a current switching method was used where high currents were used to quickly remove high ionic strength ions (chloride) while a lower current was used to increase the steplength and therefore the sensitivity of the method. Using this approach, a fast analysis was obtained without affecting the precision of the analysis.

Lactate could be quantified in human serum by using ITP on the DFR chips, with sample pretreatment restricted to dilution in water and the addition of HPMC to maintain equal viscosity across all electrolytes in the chip. The results from the DFR-ITP chips were in good agreement with results from the same samples analysed by CZE in a capillary electrophoresis instrument, with the ITP chips more than eight times faster than CZE.
5.5 References


Chapter 6

General Conclusions and Future Directions

6.1 General Conclusions

The Agilent Bioanalyzer 2100 chip based analytical electrophoretic platform with fluorescent detection, originally designed for chip-CE separations of fluorescently labelled large molecules (DNA, RNA, proteins), has been successfully integrated in this research with a number of strategies to allow full research flexibility. Thus it was shown that it represents a flexible platform merging the benefits of both a commercial instrumental platform and full research flexibility to separate in principle any analytes. Fulfilment of this general aim was then demonstrated specifically in several areas as presented in the corresponding chapters.

In Chapter 2, the results show that an implementation of the fully flexible assay developer mode of chip-CE on the Bioanalyzer platform is possible. Moreover, the versatility of the Bioanalyzer 2100 was demonstrated with already existing commercial DNA chips. Using the “Assay developer mode” accessed the script editor and opened new possibilities for developing new applications for the Bioanalyzer. Specifically investigated for the electrophoretic separation of APTS fluorescently labelled oligosaccharides demonstrates an improvement in speed of analysis compared to CE by more than an order of magnitude, and the fingerprint analysis highlights the potential for medical diagnostics using this platform.

In Chapter 3, in our following research was shown that the Agilent Bioanalyzer 2100 can be used as a separation station applying different electrophoretic technique than it was originally designed for. The ITP implemented on a DNA chip and Agilent
Bioanalyzer 2100 was demonstrated for quantification of benzoate in three different diet soft drinks. The soft drinks samples, except sample degassing did not require any demanding sample pretreatment. This work shown for the first time research where a commercial CE-chip based platform originally developed and used for specific application (DNA) was demonstrated to be applicable to a very different method, namely isotachophoresis with indirect fluorescence detection (ITP-IFD). Moreover the utility of ITP-IFD on a chip was demonstrated here for the first time as this method has so far been demonstrated for separation of model samples only [1].

The results of ITP separation of benzoate in soft drinks led to a deeper research of the possibilities of the newly presented method. The separation of human serum was shown in both commercial DNA chips and in-house fabricated ITP glass chips. The twofold improvement of sensitivity of ITP-IFD on the Bioanalyzer was achieved with in-house ITP glass chips. The in-house chip design allowed us to test different injection methods and to test different length of separation channels. While the only influence of investigated longer separation channels was the extension of total analysis time, the results from different injections methods show that the highest sensitivity is achieved by using hydrodynamic pressure injection. The highest sensitivity of the pressure injection was attributed to its ability to inject the largest amount of a sample into the in-house ITP glass chip. This result proved to be particularly important later, when the new ITP chips were designed.

The designing of the new ITP chips started with two main purposes: (i) to improve the sensitivity of the ITP-IFD on the Bioanalyzer, (ii) and to design and fabricate a chip, which would be applicable to serum samples. The designing process included scientific reasoning on optimal channels shapes and numerous computer simulations carried out with simulation software Simul 5.0 [2]. A new ITP chip design suitable for serum
samples was fabricated in Ordyl SY-300 series dry-film photoresist. The proposed chip
design and Ordyl DFR were designed to be fully compatible with the Agilent
Bioanalyzer 2100. The obtained results proved that the new design DFR-ITP chips
allowed a twentyfold improvement of sensitivity of ITP-IFD compared to the
commercial DNA chips. This improvement was achieved by using a 300 µm wide
separation channel prior the a narrow detection channel (30 µm). With the twentyfold
improvement in sensitivity, the total separation time also increased twenty times. The
total analysis time could be shortened by applying higher current but with losing some
of the sensitivity. To optimise the performance while balancing sensitivity and time, a
current switching method was used where high currents were used to quickly remove
high ionic strength ions (chloride) while a lower current was then used to increase the
step length and therefore the sensitivity of the method. Using this approach, lactate was
quantified in human serum by using ITP on the DFR chips within one minute. The
sample pretreatment could be restricted to a mere dilution with water and an addition of
HPMC to maintain equal viscosity across all electrolytes in the chip. The obtained
results show good agreement with results obtained from CZE but with eightfold
improvement in total analysis time.

6.2 Future Directions

This work showed that the commercial analytical platform Agilent Bioanalyzer 2100
has a far greater potential in research then currently utilised. Nevertheless, the results
presented in this work still show space for further improvements. Those improvements
can be reached in both system sensitivity and performance.
The electrophoretic cartridge of Agilent Bioanalyzer 2100 contains 16 electrodes and
each of them can be driven independently. In this research all introduced applications
were using Bioanalyzer for analysis of a single sample on a single chip and most of the electrodes were unused or used in sense of blocking the unused channels. The future research should focus on the possibility of efficient usage of all present electrodes to carry out analysis of more than one sample per chip.

A further widening of the Bioanalyzer applicability could be achieved with a pressure pump used in the Bioanalyzer so far for flow cytometry of cells. By combining electrodes and pressure pump, the Bioanalyzer could become an example of a flexible analytical platform with automated injection of electrolytes and samples. This would also improve instrument repeatability and decrease the amount of manual work needed. Moreover the combination of electrophoretic and pressure driven flow would probably open space even for scientifically more interesting applications as for example gradient elution ITP described previously in Chapter 1, section 1.1.6 [3]

Some specific future directions proposed in this research area are listed below:

6.2.1 **Chip-CE of oligosaccharides**

As was shown in Chapter 2, section 3 the CE of small molecules on the Bioanalyzer and DNA chips is possible. To compare with classical CE, the faster analysis on the chip did not offer the same resolution. Nevertheless, the obtained results showed possible applications for fingerprint analysis. The further investigation in this field may separate into two independent projects:

1. Building a database of electropherograms obtained from separation of APTS labelled oligosaccharides released from serum of patients. This project would require a proper utilisation of statistics and a close cooperation between University and Hospital.
2. A new chip design development. With respect to the sensitivity of the detector built in Bioanalyzer, to increase the resolution power of the CE on the chip for small molecules analysis would require to extend the length of the separation channel. In this case I agree that would be interesting to develop chip with better resolution and compatible with Bioanalyzer, but at the same time it is necessary to remind that longer separation channel will extend separation time. This mean that this research would be always a compromise between resolution and the separation time as one of the µTAS aims is to decrease analysis time.

6.2.2 On-a-chip isotachophoresis

As was shown in Chapters 3, 4 and 5 the chip ITP on the Bioanalyzer has a great potential especially when the special purpose chip design was used (Chapter 5). Nevertheless, this research still showed space for future improvements, such as multiple sample ITP analysis in sense of using all sixteen electrodes presented in the Bioanalyzer. This idea has already been suggested during this research but the time available within this PhD project did not allow pursuing this project from the drawing board into reality. The design of suggested multiple sample ITP chip compatible with Bioanalyzer is shown in Figure 6.1. Another direction for developing new chips might be focused on using different materials that allow fast fabrication of disposable or reusable microfluidic chips; such materials might be PDMS, PMMA, polycarbonate, polyolefins, etc.
Fig. 6.1) A new generation chip design with a capacity for ITP analysis of up to four samples. The chip structure and dimensions were derived from the design shown previously in Figure 5.3C.
6.3 References


Appendix

General Experimental Section

A.1 Introduction

This chapter contains general information about conditions used for experiments described in this thesis. The first part is focused to instruments used in this research. The most importantly, the detailed description of an Agilent Bioanalyzer 2100 is here. The description of Bioanalyzer includes description of the analytical platform, commercial DNA chips, detection system and Assay developer mode. Another instrument described in section A.2 “General instrumentation” is fluorescent microscope. This was used for capturing images and videos of fluorescently visualised isotachophoretic zones.

Second part of this chapter is focused to general chemicals used in this research.

A.2 General instrumentation

A.2.1 Agilent Bioanalyzer 2100

In accordance with quality health care, appropriate medication for patients is essential. To maintain the correct health care, patients’ health conditions should be continuously monitored. At present, the analytical instruments used in biochemical laboratories are expensive, with large dimension platforms, usually requiring their own air-conditioned room. Miniaturisation enables us to construct portable analytical platforms capable of achieving and maintaining excellent accuracy and reproducibility. The main advantage of these portable instruments is the possibility of field deployability, which shortens the time necessary to obtain results as no sample transportation is required. One of the
instruments offering small dimensions but high performance is the analytical platform Agilent Bioanalyzer 2100. The main aim of the research presented here was to broaden the spectrum of applications for this portable analytical platform. A short description of this instrument is provided below.

A.2.1.1 Design and dimensions

The Agilent Bioanalyzer 2100 is designed to be easily utilized by any user. A slightly uneventful grey box with wide range of applications is just 410 mm long, 165 mm wide and 290 mm high (Figure A.1A). The only movable part of the bioanalyzer is a top lid. The Bioanalyzer contains either electrophoretic or pressure cartridge, which depends on an application used.

The electrophoretic cartridge contains 16 PIN electrodes enabling electrophoretic analysis of DNA, RNA and protein samples. The Bioanalyzer is equipped with a power supply, which is capable of maintaining a constant voltage in the range of 50 to 1500V and a constant current in the range of -20 to +20 µA with 5 nA resolution. Voltage and current are driven and monitored by the Agilent software 2100 Expert. The voltage and current data acquisition is 0.625 Hz for each of the 16 electrodes. The broadening of electrophoretically driven applications was the main aim of this research.

Electrophoretic cartridge is shown in Figure A.1B.

The pressure cartridge is determined for analysing cells by flow cytometry. The pressure is ensured by in Bioanalyzer integrated pressure pump. The pressure used to drive the flow in the chip is 140 mbar. The aim of this work was not focused to utilising pressure cartridge, and therefore more details to this analytical extension are not available here.
Fig. A.1) A. Agilent Bioanalyzer 2100 and B. Electrophoretic cartridge with 16 PIN electrode
A.2.1.2 Commercially available microfluidic chips

So far, four chips compatible with Agilent Bioanalyzer 2100 are commercially available. DNA, RNA, proteins and flow cytometry analyses require specific chips, which are shown in Figure A.2.

The commercial chip used for this research was DNA chip shown in Figure A.2A. In literature the specifications of the chips can be found elsewhere [1-2]. The DNA chip is made from two soda-lime glass wafers. The microfluidic channels shown in Figure A.3A are made in the bottom wafer by using photolithography and wet etching. The wet etched channels are 10 µm wide at the bottom, 36 µm wide at the top and 13 µm deep. The length of the channels is described in Figure A.4. Hot embossing is used to enclose the microchannels. The cover glass wafer contains 16 holes, which are used as electrolytes reservoirs and enable to entry the channels in the chip. The glass chip shown in Figure A.3 is glued in a black PMMA plastic caddy, which ensures the right position of the chip in bioanalyzer and extend volume of chips reservoirs for loading of samples and electrolytes.
Fig. A.2) A. DNA, B. RNA, C. proteins and D. flow cytometry chips
A. the glass DNA chip contains microstructures and 16 reservoirs in the area of ~3 cm². Reservoirs in the column 4 are reserved for background electrolyte (B4, C4 and D4 are waste reservoirs). Vials in columns 1, 2 and 3 are reserved for samples (maximum 12 samples on a chip). Samples from row A and B are electrokinetically injected by applying voltage between injected sample reservoir and waste reservoir D4. Samples from row C and D are electrokinetically injected by applying voltage between injected sample reservoir and waste reservoir B4. Electrophoretic separation is initiated by applying separation voltage between reservoirs A4 and C4. The fluorescence detection is placed 3.2 mm in front of the reservoir C4. B. Enlarged injection cross.
Fig.A.4) DNA chip dimensions. The total and effective length of separation channel is 39.9 and 14 mm, respectively. Channels are 10 µm wide at the base, 36 µm wide at the top and 13 µm deep.
A.2.1.3 Detection system

The instrument is equipped with red laser and blue LED to detect fluorescently labelled samples by fluorescence detection. The 10 mW red laser (\(\lambda_{\text{exc}} = 635\text{ nm}\), \(\lambda_{\text{em}} = 685/22\text{ nm}\)) is commercially used in both electrophoresis and cell cytometry mode. The blue 2 mW blue LED (\(\lambda_{\text{exc}} = 470/25\text{ nm}\), \(\lambda_{\text{em}} = 525/30\text{ nm}\)) is commercially used just for the cell cytometry mode. Every chip used by the Bioanalyzer can be slightly different. Therefore, the Bioanalyzer focuses detection optic prior every analysis to ensure that the detection system is always focused in the middle of the separation channel. The schema of the detection system is depicted in Figure A.5. The data acquisition is 20 Hz.

A.2.1.4 Assay developer mode

The Bioanalyzer 2100 is driven by a software “2100 Expert”. This software offers different protocols for commercial applications. The full control over the Bioanalyzer is provided by using 2100 Expert software in so called “Assay developer mode” (ADM). ADM can be accessed just with special permission of Agilent Technologies and requires software key capable to open this mode. ADM in open software platform can be accessed through the Help menu as it is shown in Figure A.6. ADM offers additional functions in “Assay” and “Data” menu in the left hand side bar shown in Figure A.6.
The detection system uses A. red laser and B. blue LED (DM – dichroic mirror).

Fig.A.5)
Fig. A.6) Access of assay developer mode through the help menu in 2100 expert software.
In “Assay menu” the ADM allows to access a script editor. The script editor contains commands for driving the analysis and allows to:

- set the constant current on any of the 16 electrodes independently,
- set the constant voltage on any of the 16 electrodes independently,
- set the time of each step of the separation program,
- choose the required light source for induced fluorescence detection,
- set the focusing step for LIF detectors optics
- set the temperature of the chip.

In “Data menu” ADM allows to watch progress of analysis and:

- current at each of the 16 electrodes,
- voltage at each of the 16 electrodes,
- red laser induced fluorescence signal,
- blue LED induced fluorescence signal,
- position of detection optics during the focusing step.

Moreover, in “Data mode” ADM allows to:

- access the raw data
- and export data as text file.

Except mentioned benefits, ADM allows for example:

- extend and shorten the time of each step in analysis protocol,
- 10 fold increase intensity of the red laser,
- stop the parking of detection optics at the end of analysis protocol,
- move the detection optics in any direction.
A.2.2 Other instruments

A.2.2.1 Experiments visualisation

An inverted confocal fluorescence microscope (Nikon Eclipse Ti-U, Tokyo, Japan) with Semrock 390/482/563/640 nm BrightLine quad-band bandpass excitation filter, Semrock DI01 – R405/488/561/635 nm dichroic filter and Semrock 446/523/600/677 nm multiband emission filter (Rochester, NY, U.S.A.) was used for experiments visualisation. The connection of Agilent Bioanalyzer 2100 with fluorescent microscope shown in Figure A.7A, was requiring extension cable shown in Figure A.7B.

A.2.2.2 Applying pressure

To fill the microchannels of the microfluidic chips described in this thesis, an in-house system made from a 1 mL plastic syringe, a syringe needle and a rubber seal was used. The syringe shown in figure A.8 was used to apply both positive and negative pressure.
Fig.A.7)  A. The Agilent Bioanalyzer connected with fluorescent microscope Nikon Eclipse Ti-U by using B. extension cable. The in-house plastic holder, made from a Petri dish, positioned the electrodes in the chip placed at the microscope stage.
Fig.A.8) In-house equipment used for applying positive or negative pressure on the chip was fabricated from 1 mL plastic syringe, syringe needle, needle cover and rubber seal.
A.3 General chemicals

Hydrochloric acid (32%) and propionic acid (99%) were purchased from Merck (San Diego, CA, U.S.A.). Oxalic acid (99.8%) was purchased from BDH Chemicals (Victoria, Australia). Sodium acetate (99%) was purchased from Ajax Chemicals (N.S.W., Australia). Sodium benzoate (99%) was purchased from Chem-supply (S.A., Australia). Sodium pyruvate (99%), sodium lactate (syrup, 60% (w/w)), sodium 3-hydroxybutyrate (99%), β-alanine (99%), polyvinylpyroldione (PVP, 1.3 MDa), hydroxypropylmethyl cellulose (HPMC, 3500-5600 cP), R6G chloride salt (99%), tris(hydroxymethyl)aminomethane (TRIS), 2-hydroxyisobutyric acid, salicylic acid (99%), fumaric acid (99%), malic acid (99%), mandelic acid (99%) and succinic acid (99%) were all purchased from Sigma Aldrich (St. Louis, MO, U.S.A.). Milli-Q water was used for all experiments.

A.3.1 Electrolytes evaporation

Microfluidic chips use microlitre volumes of electrolytes, and the evaporation of electrolytes can cause significant problems during analysis. In pursuit of achieving good repeatability when using microfluidic chips, the evaporation effect must be minimised. Specifically in ITP, the length of the ITP steps and the position of the steps can dramatically change if the evaporation effect is not diminished.

In all the experiments presented in the previous chapters the effect of evaporation was minimised by using electrolytes containing different polymers. The main reason for adding polymers in electrolytes in this work was to reduce EOF. This effect is usually obtained simply with the increased viscosity of the electrolytes after a polymer is added. Another positive effect of viscose electrolytes is their low evaporation.
The repeatability results presented in previous sections proved that the polymers used in this research (PVP, HPMC) were able to prevent evaporation of electrolytes in commercial microfluidic chips as well as in in-house designed and fabricated microfluidic chips.

A.3.2 **Analyte adsorption**

Analyte adsorption on the wall surface of a separation channel is another effect with negative impact on the repeatability of analyses. In this research, while using different chips made from different materials good repeatability was obtained even without chemical pretreatment of the separation channels’ wall surfaces.

A.3.3 **Leading and terminating electrolyte selection**

In ITP the LE and TE must be chosen for analyte or analytes with specific electrophoretic mobilities. The electrophoretic mobility of the LE must be always higher than the mobility of targeted analytes. The electrophoretic mobility always must be lower than the mobility of targeted analytes.

In this work, the targeted analytes included different carboxylic acids with a broad range of mobilities. The chloride as LE was used for all ITP experiments. Electrophoretic mobility of this anion is $79.08 \times 10^{-9} \text{ m}^2\text{s}^{-1}\text{V}^{-1}$, which is greatly higher than electrophoretic mobility of any existing carboxylic acid. The concentration of LE anion was always set in the millimolar range according to the expected concentration of target anions in different samples. The pH of LE was set to 3.3 by using counterion β-alanine. The value of pH and the counterion used were found in relevant literature [3]. The electroosmotic flow and evaporation effect were minimised by adding polymer in LE. The TE used in this work was propionic and nicotinic acid. Those carboxylic acids
have similar electrophoretic mobilities, specifically propionic acid $8.46 \times 10^{-9} \text{m}^2\text{s}^{-1}\text{V}^{-1}$ (pH 3.3) and nicotinic acid $\sim 8.37 \times 10^{-9} \text{m}^2\text{s}^{-1}\text{V}^{-1}$ (pH 3.3). These electrophoretic mobilities were lower than the mobility of the target carboxylic acids. This ensured that the ITP would be running correctly. First the experiments were done with propionic acid. Later experiments were done with nicotinic acid as TE anion. Nicotinic acid is a solid crystalline powder and it is easier to prepare solutions with a precise concentration. Concentrations of both used anions were kept in the millimolar range. In chapter three and four the TE contained just TE anion. In chapter 5 TE and sample contained the same concentration of HPMC as LE. The addition of HPMC in TE and sample was required to maintain similar viscosity with LE. This was required for ensuring precise distribution of all electrolytes in DFR chips after applying a vacuum.
A.4 References

