Towards fast multidimensional separations in microfluidic devices
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Chapter 1

General Introduction
1.1 Introduction

Liquid chromatography is the workhorse of most analytical laboratories including those involved in proteomics and metabolomics. The instrumentation involved is continuously being improved, amongst others by miniaturization, column technology and hyphenation. These improvements are mainly driven by the increasing demands from the application areas. Liquid chromatography and other techniques used in this thesis are briefly described in this chapter.

1.2 Liquid Chromatography

Liquid chromatography (LC) is one of the most powerful separation techniques currently available. The separation is achieved by partitioning of the analytes between the stationary phase and the mobile phase which is directed through the stationary phase. It is a very versatile technique because many different mobile and stationary phases are available so that the selectivity of the separation can easily be changed. Separations can be based on differences in size, polarity, charge state or affinity to a certain (ad)sorbent. Reversed phase LC was used in the experiments described in this thesis and this mode of operation implies that the stationary phase is non-polar and the mobile phase is a mixture of an aqueous buffer and an organic solvent, mostly methanol or acetonitril. Polar compounds will elute early in the chromatogram before the less polar analytes.

1.3 Miniaturization

Miniaturization of analytical instrumentation is an important research topic that has drawn much attention in recent years. Micro- and nanotechnology research has resulted in techniques to produce analytical equipment in a chip-based format in which different aspects of the analytical workflow can be integrated such as sample preparation, injection, separation and detection. Its expected increase in sensitivity, selectivity and sample throughput indicates that these devices are required to face the challenges of complex samples originating from the “life sciences”. With respect to the
separation channel, a number of options are available. The most sophisticated use of
micromachining technologies is the production of a perfectly ordered array of pillars
which serve as stationary phase. It has been demonstrated that these stationary phases
can be used for separations with plate heights lower than with conventional liquid
chromatography phases. Alternatively, channels can be etched in a chip and these can
be filled with for instance a monolithic stationary phase or an empty channel can be
used for electrophoretic separations.

1.4 Multidimensional Liquid Chromatography

The separation power of a separation system can be increased by the addition of a
second separation dimension in which the analytes present in the sample are separated
based on a different mechanism. When the different mechanisms are not correlated,
the separation is called orthogonal. It is very important to make sure that the separation
obtained in the first dimension is still intact when the second dimension is executed.
For this purpose, a switching valve is used which injects the first dimension eluate into
the second dimension 1 to 4 times per peak.

1.5 Monoliths

The word “monolith” means “one stone” and stationary phases of this type are
produced in one piece. Various different types of monolithic stationary phases are
available and these can be categorized in two classes: silica and polymeric monoliths.
Both types have the same advantages over traditional packed stationary phases, which
is low resistance to flow and the ease of production from liquid precursors. Both these
advantages are very favorable for its use in microfluidic devices because these cannot
withstand high pressures and are difficult to fill with particles. A special advantage of
the methacrylate monoliths prepared in this work is that the polymerization reaction is
initiated with UV light. This allows patterning for the stationary phase and direction to
a specific part of the microfluidic device. In monolithic stationary phases an enormous
number of different functionalities can be created. Different types of functional groups
can be added during the polymerization process or these can be added afterwards by
grafting. Also, specific molecules can be attached to create a stationary phase for affinity chromatography.

1.6 Proteomics

Since the early nineties the number of publications on the subject of proteomics has increased almost exponentially. This indicates how this field of research has become an important scientific area in a short time. The focus in this area is on the identification and characterization of proteins expressed in a certain species at a certain time. This last addition is important because the proteome changes continually under the influence of food, stress, hormones and others. One of the ways of studying a proteome is by simply separating and identifying the proteins present in a sample. This approach is called top-down proteomics and can be performed using two-dimensional gel electrophoresis, possibly in combination with MALDI-ToF-MS. Alternatively, a bottom-up approach can also be chosen, in which the proteins are first enzymatically cleaved into smaller fragments that are identified, after which the original proteins can be virtually reconstructed. This is mostly performed using LC-MS/MS of the trypsin digestion products of the proteins of interest. The last technique requires significant computational power in order to link the peptides analyzed to the respective proteins.

1.7 Peak capacity

In the optimization of separation systems, it is of high importance to have a metric that can objectively quantify the separation power of the system at hand. For this purpose, the separation efficiency can be expressed as the number of theoretical plates (for isocratic separations) and the peak capacity (for separations in the gradient mode). As all separation systems discussed in this thesis are operated in the gradient elution mode, only the peak capacity will be discussed here in more detail. The peak capacity can be defined as the maximum number of peaks that can be separated with a resolution of 1. This can approximately be calculated by dividing the gradient time by the average peak width at the base of the peak (4σ). The resulting peak capacity is a theoretical value which can, in practice, hardly ever be achieved because most sample constituents do not fill the entire gradient time. For practical method development, the
conditional or sample peak capacity (difference in retention time of the first and the last eluting compound divided by the average peak width at the base of the peak) may be of more use as the peak capacity for a certain sample is calculated. This can be done by calculating the difference in retention time between the last eluting compound and the first eluting compound and dividing this retention window by the average peak width at the base.

1.8 Scope of the thesis

The main objective of the research work presented in this thesis was to explore the feasibility of multidimensional separation systems in microfluidic devices. To this end, different stationary phases were prepared and tested in capillary columns and in chips for liquid chromatography. Standard gradient elution theory was used to describe the chromatographic behavior and efficiency in order to define critical steps in the instrumental configuration.

Special interest was in the separation of biologically relevant samples such as occur in proteomics, metabolomics and pharmaceutical samples, which should be the type of samples that would theoretically benefit the most from the expected increase in sensitivity, separation power and sample throughput a multidimensional separation system could offer. The accurate identification can be provided of the compounds present in the complex samples can be provided by MS, so interfacing with mass spectrometry was very important.

The introduction of the analytical techniques described in this chapter is followed by an overview of the literature published on the use of pressure driven liquid chromatography separations of biologically relevant samples in microfluidic chips. Chapter 3 describes the optimization of a comprehensive two-dimensional separation system for the characterization of the impurities of alprazolam formed by degradation in the tablet formulation in the presence of certain excipients. The work described in chapter 4 deals with the evaluation of various packed and monolithic columns for the separation of peptides. Gradient theory was used to characterize the performance of the stationary phases. Chapter 5 describes the production of different methacrylate monolithic stationary phases in microfluidic devices. These separation devices were tested for the separation of peptides. The effect of the stationary phase density and
chemistry was investigated. The separation of proteins on chip-based monoliths is described in chapter 6. The stationary phase density as well as instrumental settings were optimized.