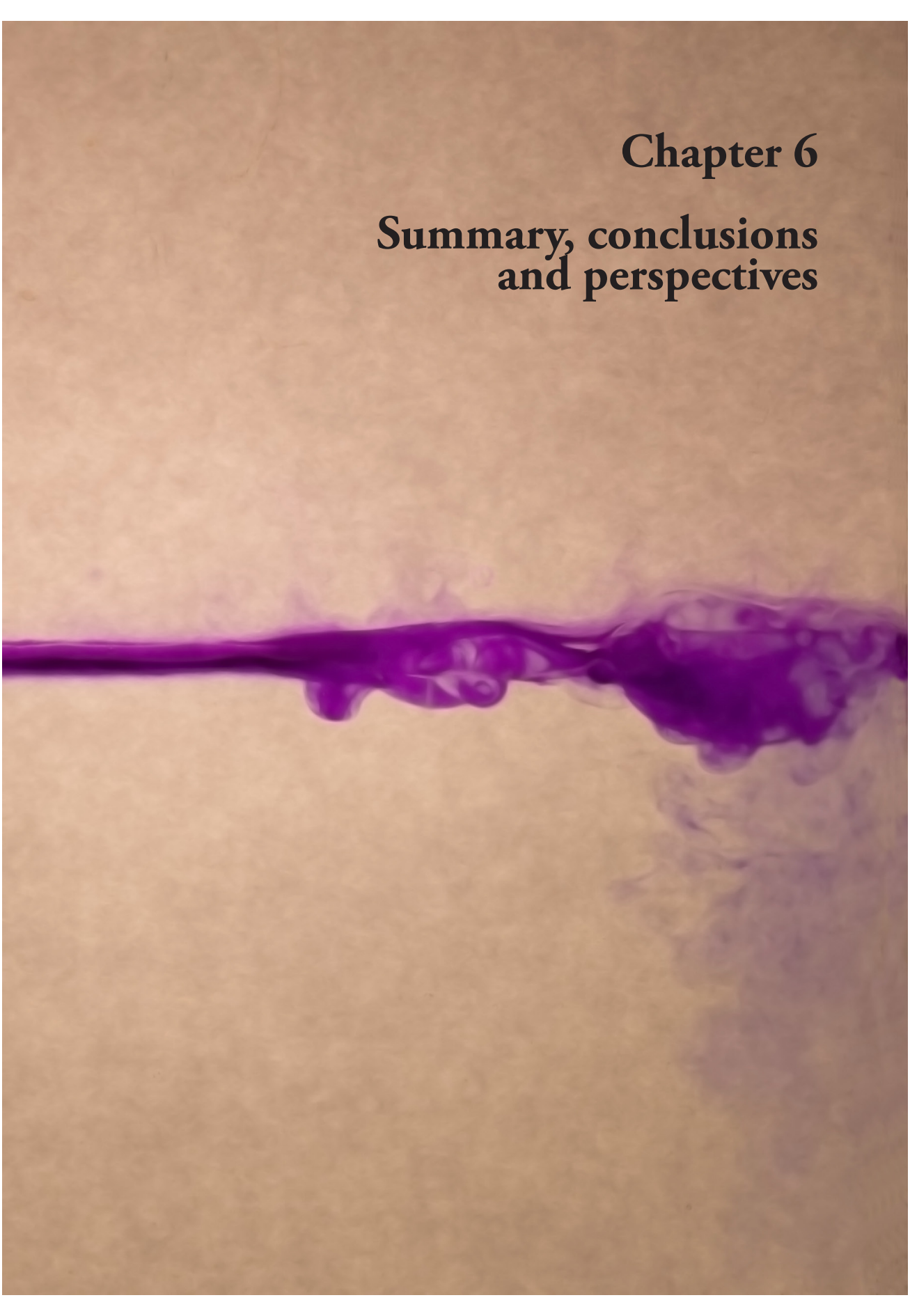
Chapter 6

Summary, conclusions and perspectives



The paradigm shift from reductionism towards systems thinking has stimulated the field of analytical chemistry to develop bio-analytical techniques that are faster, more comprehensive, and have lower detection limits.

In this thesis, it is demonstrated that electrophoretic methods can be exploited to achieve these developments. For this, carefully chosen electrolyte systems were used, leading to conditions that were suitable for isotachophoresis (ITP) or electroextraction (EE). In such a suitable electrolyte system, an analyte flux can be generated by an electric field. This analyte flux can be utilised to carry out on-line preconcentration of the analytes. In addition, in ITP the analytes are simultaneously separated. All peptides and a large part of the known metabolites are charged or can become charged by varying pH; consequently, migration of these compounds can be manipulated by application of an electric field.

In **Chapter 2**, the potential of capillary isotachophoresis (cITP) coupled to mass spectrometry (MS) of peptides using spacer molecules was explored as a strategy to detect peptides one by one. cITP-MS combines the concentrating power of cITP with the selective and sensitive detection power of MS. In principle, cITP-MS has great potential for the analysis of low-abundant peptides and metabolites, due to its ability to concentrate and separate analytes simultaneously. However, a major limitation has to be overcome, namely the mixing of ITP zones during transfer to MS: as soon as ITP conditions are abandoned, the concentrating effect, which is the key characteristic of ITP, is diminished and the isotachophoretic zones start to collapse and mix. When the isotachophoretic zones are very narrow, as is the case with low-abundant analytes, the collapsing of these zones results in zones that contain multiple peptides. Proof-of-principle experiments are described in which spacer compounds are added to a peptide sample prior to cITP separation. As expected, the peptides are physically separated by the spacer molecules and enter the MS one by one instead of all at once, as happened when no spacer molecules were added.

During the study, it was noticed that the spacer compound mixture that was used (carrier ampholytes (CA)) needed improving. CA mixtures were originally developed to generate stable pH gradients in isoelectric focusing (IEF). However, the currently available CA mixtures have not been well defined and contain high levels of neutral contaminations which are present in all ITP zones, frustrating MS detection and resulting in less favourable detection limits or even no analyte detection at all. A solution for this could be to clean up the CA mixture prior to use, for example by performing weak cation exchange solid phase extraction. A more elegant solution would be to develop a well-defined spacer compound mixture dedicated to ITP use and of which the exact composition is known, including the

electrophoretic mobilities of all the individual spacers. It is interesting to note that during the period IEF was invented, the theoretic concept of IEF was introduced first, and practical IEF was only possible after a chemist took the challenge of synthesizing appropriate CA [1]. In the case of ITP, suitable spacer compounds should possess electrophoretic mobilities in the range of the analytes, they should cause no ion suppression and they should be volatile in order not to clog the spray needle. In proteomics, transient ITP-CZE has been successfully used to concentrate and fractionate complex peptide samples prior to LC-MS analysis. Direct coupling of cITP to MS, using spacer molecules, will result in an analytical method which has improved detection limits, which covers a high dynamic range, is easy to set up, uncomplicated, not labour-intensive and cheap.

The second electrophoretic approach that was studied in this thesis is electroextraction (EE) **(Chapters 3-5)**. It was demonstrated that EE is a fast sample preconcentration technique that is quantitative, applicable to peptidomics and metabolomics, easy to automate, easy to hyphenate with LC-MS, and able to deal with large sample volumes (10-100 µL, with the possibility to further enlarge the volume).

With EE, sample molecules can be extracted rapidly into a small volume. Therefore, the amount of sample injected into an analytical separation can be increased without increasing the injected sample volume. Increasing the sample volume to achieve lower detection limits is often undesirable, since large amounts of contaminations are injected along with the compounds of interest. EE also offers selectivity; in one extraction either cations or anions are extracted and all other compounds, including neutrals, remain largely behind, since they only slowly passively migrate by diffusion into the acceptor phase.

To carry out EE, the charged sample molecules should be dissolved in a low-conductive (organic) donor phase that is immiscible with the (aqueous) acceptor phase, which has a high conductivity. When a high voltage is applied over such a liquid-liquid system, a very high electric field strength will be present in the low-conductive phase, causing the charged compounds that are present there to migrate very fast towards the acceptor phase. In the highly conductive acceptor phase, a very low electric field strength exists and the charged compounds are slowed down as they enter; therefore, they are concentrated just after they have passed the liquid-liquid interface.

In this thesis, two on-line set-ups for EE-LC-MS were successfully demonstrated: capillary EE (cEE) (**Chapter 3**) and large volume cEE (**Chapter 4 and 5**). The main differences between cEE and large volume cEE were the positioning of the liquid phases and the volume of organic donor phase that could be extracted.

In cEE (**Chapter 3**), the aqueous acceptor phase was positioned in a capillary and the organic donor phase in a sample vial, in which the grounded capillary was immersed as well as an electrode. When an electric field strength was applied, approximately 10 µL organic phase could be depleted from cationic analytes, which migrated into the aqueous phase in the capillary. The collected sample plug was transferred to an LC-MS system via a switching valve. A peptide enrichment factor of around 100 times in comparison to a conventional reversed phase LC injection of the organic donor phase was achieved, demonstrating the capacity of EE to increase the loadability of LC-MS. With this set-up, it was shown for the first time that EE is suitable for enrichment of biomolecules (peptides) from a biological matrix (urine).

In **Chapter 4 and 5** cEE-LC-MS was further improved, so that it could extract larger sample volumes (100 µl instead of 10 µL, e.g. large volume cEE-LC-MS) in order to achieve higher enrichment factors (and therefore improve detection limits). Moreover, the repeatability of EE was improved. These improvements were achieved by performing EE in a large bore capillary, in which the whole extraction volume of organic phase is injected. Then, extraction from this large, well-defined and controlled organic sample volume took place. Peptide enrichment factors were improved to approximately three orders of magnitude and it was demonstrated that the method is also suitable for also acylcarnitines. With on-line large volume cEE-LC-MS, peptides in plasma as well as low abundant metabolites in urine were determined.

In this thesis, large volume cEE was used to hyphenate SPE off-line with reversed phase LC-MS. Normally, SPE extracts have to be evaporated to dryness and reconstituted prior to reversed phase LC-MS in order to be able to inject a sample amount large enough. Both evaporation and reconstitution are labour intensive, result in sample loss and introduce errors in the measurement. In this thesis, it is demonstrated that due to the implementation of cEE, these steps can be eliminated. The construction of a fully automated, closed analytical system in which SPE is coupled on-line to large volume cEE-LC-MS is foreseeable. Such a system minimises experimental errors, sample loss and labour, since no human interference is required after filling the autosampler with the appropriate samples. For full hyphenation of SPE to LC-MS via large volume cEE, the organic solvent should be able to elute the compounds of interest from the SPE material as well as to serve as a donor solvent in EE.

In **Chapter 5**, the combination of large volume cEE with CZE-MS for the analysis of low abundant urine metabolites, using acylcarnitines as example, is demonstrated and shown to be promising. The on-line coupling with CZE is attractive, since large volume

cEE can overcome a major limitation of CZE, namely its low volume loadability. To obtain a reliable on-line coupling of large volume cEE to CZE, a dedicated valve could for example be developed.

In all the EE work described in this thesis, only ethyl acetate (EtOAc) was used as organic donor solvent. EtOAc is a suitable solvent, since it can form a two-phase system with water and it can contain some (up to 3.5%) water to enable the presence of ions. The range of compounds that can be extracted (in terms of polarity, for example) can be further expanded by using other organic solvents or solvent mixtures.

The EE process lends itself very well to microfluidic applications. In an EE-chip, very small volumes can be extracted and the use of high voltage equipment is unnecessary since in these small dimensions high field strengths can already be achieved with low voltages. When on-chip EE is successfully developed, the consecutive development of small, easy-to-operate EE equipment coupled on-line to analytical separations is foreseeable.

To summarise, we have demonstrated in this thesis that electromigration-based sample pretreatment techniques for bio-analysis have great potential, showing cITP and EE as examples. It can be expected that such techniques will be implemented in analytical instrumentation in the near future. Both cITP and cEE can be expected to contribute to an increased coverage of the peptidome and the metabolome, which will result in the discovery of new biomarkers. Therefore, cITP as well as cEE have great potential to become new additions to the repertoire of the modern analytical chemist.

References

1 Vesterberg, O., Svensson, H., *Acta Chem Scand,* **1966**, *20*, 820-834