On-line Electrochemistry Electrospray Ionisation Mass Spectrometry

Method Development and Applications

CAMILLA ZETTERSTEN
Dissertation presented at Uppsala University to be publicly examined in BMC, B42, Husargatan 3, Uppsala, Friday, April 24, 2009 at 10:15 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract

This thesis deals with studies of on-line electrochemistry electrospray ionisation mass spectrometry (EC/ESI-MS). It is shown that the use of EC/ESI-MS demands optimal coupling characteristics.

Pre-concentration and desalting, due to matrix exchange, were demonstrated for the model substance 1-hexanethiol in an EC/ESI-MS setup. The setup was also used for investigations of the oxidation states of the manganese complex [Mn(bpmp)(μ-OAc)3][ClO4], where bpmp is a 2,6-bis[N,N-di(2-pyridylmethyl)amino(methyl)-4-methylphenol compound. The manganese complex, which is relevant to artificial photosynthesis, was found to be a good model compound for the EC/ESI-MS studies, thanks to its many oxidation states. For the first time, the presence of the Mn(III,IV) state of the manganese complex was demonstrated in the studies.

During the experimental work, the importance of the electrode positioning within the electrochemical cell was investigated. Different EC cell configurations were studied using the manganese complex as a model substance. It was clearly shown that the EC cell design influences the distribution between the peaks in the mass spectra - not only for manganese complexes and Olsalazine but also for 4-chloroaniline.

A previously unknown comproportionation reaction was found for 4-chloroaniline involving the oxidised dimer, 4-[(4-chlorophenyl)imino]-2,5-cyclohexadien-1-imine. This reaction explained the unexpected presence of the signal due to the reduced dimer, 4-amino-4'-chlorodiphenylamine, in the mass spectra.

Furthermore, it was shown that EC/ESI-MS was successful in conjunction with miniaturised gold wire electrodes in a PDMS chip within which dopamine was oxidised with a conversion efficiency of 30%. The oxidation products of dopamine were detected after 0.6-1.2 seconds for 1.0 and 0.5 μl/min, respectively. The combination of electrochemically controlled solid-phase extraction (EC-SPE) with ESI-MS was found to be less straightforward than detecting anions pre-concentrated on a polypyrrole coated electrode with EC-SPE/ICP-MS.

The on-line combination of liquid chromatography with EC/ESI-MS/MS for studying antioxidants in yellow onion extracts was shown to be fast and a relatively easy complement to classical antioxidant activity determinations.

Keywords: thin-layer electrochemical flow cell, electrospray ionisation, mass spectrometry, high voltage decoupling, cell design, electrochemistry, liquid chromatography, miniaturisation, chip, PDMS, polypyrrole, 4-chloroaniline, antioxidants, isolation transformer, manganese complex, 1-hexanethiol, Olsalazine

Camilla Zettersten, Department of Physical and Analytical Chemistry, Analytical Chemistry, Box 599, Uppsala University, SE-751 24 Uppsala, Sweden.

© Camilla Zettersten 2009

ISSN 1651-6214
ISBN 978-91-554-7459-1
urn:nbn:se:uu:diva-99329 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-99329)
"Tid har förlöpt
dagar har gått
månen har vandrat sin väg
genom vitt och svart och grått"1

Den här doktorsavhandlingen
är tillägnad min familj
This thesis is based on the following publications, which are referred to in the text by their Roman numerals.

I  
A setup for the coupling of a thin-layer electrochemical flow cell to electrospray mass spectrometry  
C. Fredrik Bökman, Camilla Zettersten, Per J. R. Sjöberg, and Leif Nyholm  
Reproduced with permission from American Chemical Society. Copyright (2004).

II  
Ligand exchange upon oxidation of a dinuclear Mn complex – detection of structural changes by FT-IR spectroscopy and ESI-MS  
Gerriet Eilers, Camilla Zettersten, Leif Nyholm, Leif Hammarström, and Reiner Lomoth  
Reproduced by permission of The Royal Society of Chemistry  
http://www.rsc.org/Publishing/Journals/DT/article.asp?doi=b415148h

III  
The influence of the thin-layer flow cell design on the mass spectra when coupling electrochemistry to electrospray ionisation mass spectrometry  
Camilla Zettersten, Reiner Lomoth, Leif Hammarström, Per J. R. Sjöberg, and Leif Nyholm  
Reprinted with permission from Elsevier. Copyright (2006).
IV On-line coupling of a microelectrode array equipped poly(dimethylsiloxane) microchip with an integrated graphite electrospray emitter for electrospray ionisation mass spectrometry
Gustav Liljegren, Andreas Dahlin, Camilla Zettersten, Jonas Bergquist, and Leif Nyholm
Reproduced by permission of The Royal Society of Chemistry
http://www.rsc.org/Publishing/Journals/LC/article.asp?doi=b506289f

V On-line electrochemically controlled solid-phase extraction interfaced to electrospray and inductively coupled plasma mass spectrometry
Gustav Liljegren, Niklas Forsgard, Camilla Zettersten, Jean Pettersson, Malin Svedberg, Merja Herranen, and Leif Nyholm
The Analyst, 2005, 130, 1358-1368.
Reproduced by permission of The Royal Society of Chemistry
http://www.rsc.org/Publishing/Journals/AN/article.asp?doi=b508388e

VI The oxidation of 4-chloroaniline studied by on-line electrochemistry electrospray ionization mass spectrometry
Camilla Zettersten, Per J. R. Sjöberg and Leif Nyholm
Analytical Chemistry, submitted

VII Identification of antioxidants using on-line liquid chromatography, electrochemistry, and electrospray ionization tandem mass spectrometry (LC/EC/ESI-MS/MS)
Camilla Zettersten, Sandra Wende, Michelle Co, Charlotta Turner, Leif Nyholm, and Per J. R. Sjöberg
In manuscript
Author contribution

**Paper I**  
I carried out the EC/ESI-MS experiments in cooperation with Fredrik Bökman.

**Paper II**  
I planned and performed the EC/ESI-MS experiments and the interpretation of the mass spectra.

**Paper III**  
I took active part in the experimental planning, performed all experiments and contributed significantly to the interpretation of the results. I also wrote a major part of the manuscript.

**Paper IV**  
I performed the on-line EC/ESI-MS experiments of dopamine in cooperation with Gustav Liljegren.

**Paper V**  
I carried out the solid-phase extraction in the on-line EC/ESI-MS experiments together with Gustav Liljegren.

**Paper VI**  
I took active part in the experimental planning, performed all experiments and contributed significantly to the interpretation of the results. I prepared all the figures and I wrote the major part of the manuscript.

**Paper VII**  
I took active part in the experimental planning, and performed the single quadrupole MS experiments in cooperation with Sandra Wende and Michelle Co. I performed the LC/EC/ESI-ion trap MS experiments and the major part of the results interpretation. I prepared the figures and wrote the major part of the manuscript.
Contents

1 Introduction......................................................................................................................1

2 Electrochemistry (EC) ..................................................................................................3
   2.1 Electrochemical methods .......................................................................................5
      2.1.1 Potentiometry ..................................................................................................6
      2.1.2 Amperometry ..................................................................................................6
      2.1.3 Coulometry .....................................................................................................7
      2.1.4 Voltammetry ...................................................................................................7
   2.2 Flow-electrolysis .................................................................................................8
      2.2.1 Amperometric cells ......................................................................................10
      2.2.2 Coulometric cells ........................................................................................10

3 Mass spectrometry (MS).............................................................................................12
   3.1 Electrospray ionisation (ESI) .............................................................................13
   3.2 Electrochemical processes in ESI .......................................................................14

4 On-line electrochemistry  mass spectrometry (EC/MS) ..............................................17
   4.1 Historic overview ...............................................................................................17
   4.2 On-line electrochemistry electrospray ionisation mass spectrometry (EC/ESI-MS) ..........................................................................................................................18
      4.2.1 Identification, ionisation, and derivatisation of electroactive species ............18
      4.2.2 Short transfer times between electrochemical generation and MS detection ...20
      4.2.3 Sample preparation for EC/MS ....................................................................21
      4.2.4 Studies of reaction mechanisms and kinetics ..............................................22
      4.2.5 The use of electrochemical reactions for tagging purposes .......................24
      4.2.6 Miniaturised ESI systems ..........................................................................26
      4.2.7 EC/ESI-MS to mimic metabolic reactions ..................................................27
      4.2.8 Use of ESI backward currents for EC/MS applications .........................29
   4.3 Liquid chromatography on-line with EC/MS and electrochemistry on-line with LC-MS/MS ..............................................................................................................31
      4.3.1 LC/EC/API-MS ............................................................................................32
      4.3.2 LC/EC/MS for determination of antioxidant activity ..................................33
      4.3.3 EC/LC/MS for the simulation of drug metabolism ....................................35
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 Practical aspects and applications</td>
<td>37</td>
</tr>
<tr>
<td>5.1 ESI high voltage and backward currents</td>
<td>37</td>
</tr>
<tr>
<td>5.2 Influence of the solution composition in EC/ESI-MS</td>
<td>41</td>
</tr>
<tr>
<td>5.3 Transfer of solution from the electrochemical cell to ESI-MS</td>
<td>43</td>
</tr>
<tr>
<td>5.4 Aspects of the electrochemical cell design</td>
<td>45</td>
</tr>
<tr>
<td>6 Conclusions and future work</td>
<td>50</td>
</tr>
<tr>
<td>7 Acknowledgements</td>
<td>53</td>
</tr>
<tr>
<td>8 Summary in Swedish</td>
<td>55</td>
</tr>
<tr>
<td>8.1 Användningsområden och begränsningar med EC/ESI-MS</td>
<td>55</td>
</tr>
<tr>
<td>8.2 Metodutveckling av EC/MS-sammankopplingen och design av EC-celler</td>
<td>56</td>
</tr>
<tr>
<td>8.3 Slutsatser</td>
<td>59</td>
</tr>
<tr>
<td>9 References</td>
<td>61</td>
</tr>
</tbody>
</table>
## Lists of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag/AgCl</td>
<td>silver/silver chloride reference electrode</td>
</tr>
<tr>
<td>APAP</td>
<td>acetaminophen</td>
</tr>
<tr>
<td>APCI</td>
<td>atmospheric pressure chemical ionisation</td>
</tr>
<tr>
<td>API</td>
<td>atmospheric pressure ionisation</td>
</tr>
<tr>
<td>APPI</td>
<td>atmospheric pressure photoionisation</td>
</tr>
<tr>
<td>ASV</td>
<td>anodic stripping voltammetry</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>AuxE</td>
<td>auxiliary electrode or counter electrode</td>
</tr>
<tr>
<td>BESI</td>
<td>biphasic electrospray ionisation</td>
</tr>
<tr>
<td>BDD</td>
<td>boron-doped diamond</td>
</tr>
<tr>
<td>bpmp</td>
<td>2,6-bis[[N,N-di(2-pyridylmethyl)amino]methyl]-4-methylphenol</td>
</tr>
<tr>
<td>bpy</td>
<td>2,2’-bipyridine</td>
</tr>
<tr>
<td>4-CA</td>
<td>4-chloroaniline</td>
</tr>
<tr>
<td>CCE cell</td>
<td>controlled-current electrochemical cell</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CE</td>
<td>capillary electrophoresis</td>
</tr>
<tr>
<td>CEM</td>
<td>ceramic electrochemical reactor</td>
</tr>
<tr>
<td>CPE cell</td>
<td>controlled-potential electrochemical cell</td>
</tr>
<tr>
<td>CRM</td>
<td>charge residue model</td>
</tr>
<tr>
<td>CSH</td>
<td>cysteine</td>
</tr>
<tr>
<td>CV</td>
<td>cyclic voltammetry</td>
</tr>
<tr>
<td>DPD</td>
<td>N,N-dimethyl-p-phenylenediamine</td>
</tr>
<tr>
<td>DEEP</td>
<td>differential electrospray emitter potential</td>
</tr>
<tr>
<td>DEMS</td>
<td>differential electrochemical mass spectrometry</td>
</tr>
<tr>
<td>3,4-DHBA</td>
<td>3,4-dihydroxybenzoic acid</td>
</tr>
<tr>
<td>DimOx</td>
<td>oxidised dimer</td>
</tr>
<tr>
<td>DimRed</td>
<td>reduced dimer</td>
</tr>
<tr>
<td>DMM2F</td>
<td>methyl-2,5-dihydro-2,5-dimethoxy-2-furan-carboxylate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl</td>
</tr>
<tr>
<td>EC</td>
<td>electrochemistry, electrochemical</td>
</tr>
<tr>
<td>ECE</td>
<td>electrochemical-chemical-electrochemical</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionisation</td>
</tr>
<tr>
<td>ESR</td>
<td>electron spin resonance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>FAB</td>
<td>fast atom bombardment</td>
</tr>
<tr>
<td>FEM</td>
<td>N-(2-ferroceneethyl)maleimide</td>
</tr>
<tr>
<td>FI</td>
<td>flow injection</td>
</tr>
<tr>
<td>FT</td>
<td>Fourier transform</td>
</tr>
<tr>
<td>GC</td>
<td>glassy carbon</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>ICAT™</td>
<td>isotope-coded-affinity-tag</td>
</tr>
<tr>
<td>ICP</td>
<td>inductively coupled plasma</td>
</tr>
<tr>
<td>ICR</td>
<td>ion cyclotron resonance</td>
</tr>
<tr>
<td>IEM</td>
<td>ion evaporation model</td>
</tr>
<tr>
<td>ITIES</td>
<td>interface formed between two immiscible electrolyte solutions</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>M2F</td>
<td>methyl-2-furoate</td>
</tr>
<tr>
<td>MRM</td>
<td>multiple reaction monitoring</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MS^n</td>
<td>tandem mass spectrometry</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>ocp</td>
<td>open circuit potential</td>
</tr>
<tr>
<td>OX</td>
<td>oxidant; oxidising agent</td>
</tr>
<tr>
<td>PAH</td>
<td>polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PB</td>
<td>particle beam</td>
</tr>
<tr>
<td>PDMS</td>
<td>poly(dimethylsiloxane)</td>
</tr>
<tr>
<td>PEEK</td>
<td>polyaryletheretherketone</td>
</tr>
<tr>
<td>PFT</td>
<td>porous flow-through</td>
</tr>
<tr>
<td>PGC</td>
<td>porous graphitic carbon</td>
</tr>
<tr>
<td>PMMA</td>
<td>polymethylmethacrylate</td>
</tr>
<tr>
<td>PPy</td>
<td>polypyrrole</td>
</tr>
<tr>
<td>Q3G</td>
<td>quercetin-3-glucoside</td>
</tr>
<tr>
<td>Q4´G</td>
<td>quercetin-4´-glucoside</td>
</tr>
<tr>
<td>Q3,4´G</td>
<td>quercetin-3,4´-diglucoside</td>
</tr>
<tr>
<td>QRE</td>
<td>quasi reference electrode</td>
</tr>
<tr>
<td>RE</td>
<td>reference electrode</td>
</tr>
<tr>
<td>RED</td>
<td>reductant; reducing agent</td>
</tr>
<tr>
<td>RP</td>
<td>reversed-phase</td>
</tr>
<tr>
<td>SHE</td>
<td>standard hydrogen electrode</td>
</tr>
<tr>
<td>SIM</td>
<td>selected ion monitoring</td>
</tr>
<tr>
<td>S/N</td>
<td>signal-to-noise ratio</td>
</tr>
<tr>
<td>SPE</td>
<td>solid-phase extractions</td>
</tr>
<tr>
<td>TIC</td>
<td>total ion chromatogram</td>
</tr>
<tr>
<td>TOF</td>
<td>time-of-flight</td>
</tr>
<tr>
<td>TSP</td>
<td>thermospray ionisation</td>
</tr>
<tr>
<td>WE</td>
<td>working electrode</td>
</tr>
<tr>
<td>XIC</td>
<td>extracted ion chromatogram</td>
</tr>
</tbody>
</table>
List of symbols

<table>
<thead>
<tr>
<th>symbol</th>
<th>meaning</th>
<th>usual unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C$</td>
<td>concentration</td>
<td>(mol / dm$^3$)</td>
</tr>
<tr>
<td>$E$</td>
<td>electrode potential</td>
<td>V</td>
</tr>
<tr>
<td>$F$</td>
<td>Faraday constant (96485)</td>
<td>(C / mol)</td>
</tr>
<tr>
<td>$\Delta G^0$</td>
<td>free energy change</td>
<td>(kJ / mol)</td>
</tr>
<tr>
<td>$i$</td>
<td>current</td>
<td>A</td>
</tr>
<tr>
<td>$i_{\text{back}}$</td>
<td>backward current</td>
<td>A</td>
</tr>
<tr>
<td>$i_{\text{ESI}}$</td>
<td>electrospray ionisation current</td>
<td>A</td>
</tr>
<tr>
<td>$i_{\text{ext}}$</td>
<td>backward current in external loop</td>
<td>A</td>
</tr>
<tr>
<td>$iR$</td>
<td>ohmic potential drop</td>
<td>V</td>
</tr>
<tr>
<td>$K$</td>
<td>equilibrium constant</td>
<td></td>
</tr>
<tr>
<td>$m/z$</td>
<td>mass-to-charge ratio</td>
<td></td>
</tr>
<tr>
<td>$n$</td>
<td>number of electrons</td>
<td></td>
</tr>
<tr>
<td>$N$</td>
<td>number of moles</td>
<td>mol</td>
</tr>
<tr>
<td>$q$</td>
<td>amount of charge</td>
<td>C</td>
</tr>
<tr>
<td>$R$</td>
<td>gas constant (8.31451)</td>
<td>J / (K $\times$ mol)</td>
</tr>
<tr>
<td>$T$</td>
<td>temperature</td>
<td>K</td>
</tr>
<tr>
<td>$t$</td>
<td>time</td>
<td>s</td>
</tr>
<tr>
<td>$v$</td>
<td>volumetric flow rate</td>
<td>(cm$^3$ / s)</td>
</tr>
<tr>
<td>$Y$</td>
<td>conversion efficiency</td>
<td>%</td>
</tr>
</tbody>
</table>
1 Introduction

The interest in using electrochemical cells coupled on-line with mass spectrometry (MS) has increased during the last decade (see Figure 1) as the amount of published reviews shows.\(^2\)\(^-\)\(^9\) The on-line coupling of electrochemistry with mass spectrometry (EC/MS) is applicable for the study of redox reactions in biological systems,\(^10\) for example the drug metabolism in the human body.\(^7\) Though many oxidation reactions are enzymatic and occur with various mechanisms, one can mimic the reactions and increase the metabolic understanding.\(^11\) The identification of proteins and peptides can also be simplified by EC/MS through specific analyte tagging.\(^12\) Moreover, EC/MS offers the capability to facilitate the analysis by producing or increasing signal intensities, and to pre-concentrate and separate analytes. EC/MS also has the aptitude to directly monitor reactants, short-lived intermediates, and the products of electrochemical reactions as a function of electrode potential \(E\) or current \(i\) with \(m/z\) specificity, and to provide structural information for reactants, intermediates and products.

Figure 1. The number of publications on EC/ESI-MS within the period 1991 to 2008 [“electrochem* AND electrospray AND mass spectrometry” search on ISI Web of Knowledge http://pcs.isiknowledge.com 2009-02-24].
Mass spectrometry, which offers a wide range of possible applications, is one of the most important techniques in analytical chemistry today. Development of different atmospheric pressure ionisation (API) techniques has lead to an enormous increase in the use of mass spectrometry. One of the most commonly used API techniques, electrospray ionisation (ESI), will be discussed in this thesis. Electrospray ionisation transfers ions from solutions into the gas-phase where they are introduced into the mass spectrometer. The ESI technique is sensitive and has the ability to analyse molecules of a wide range of size. Small molecules such as drugs, pesticides and carbohydrates, as well as large molecules like proteins and nucleic acids are applicable to electrospray ionisation. This soft ionisation technique minimises the risk of fragmentation and has the advantage of being possible to combine with separation techniques, such as liquid chromatography (LC) and capillary electrophoresis (CE), as well as electrochemistry (EC).

Though the coupling of EC to MS is rather new, its use has recently increased due to several reasons. EC/MS offers the possibilities to generate oxidation and reduction products and detect them, before they have time to undergo further reactions, and to study reaction mechanisms in general. Furthermore, the opportunity to use limited sample volumes is an advantage compared to off-line EC-MS analysis. The use of EC/ESI-MS emerged in 1995 and has grown since then (see Figure 1), mainly due to the possibility to use sample pre-treatment (e.g. desalting and pre-concentration), separation, and miniaturisation as well as chip-based electrochemical devices for the detection of biologically interesting species without loss of performance. Improvement of the electrospray ionisation technique has also resulted thanks to EC/ESI-MS, due to studies of the inherent EC reactions in the ESI process and the use of EC reactions to facilitate the detection of different compounds by ESI-MS.

The coupling of EC to ESI-MS has, however, been complicated by the fact that common EC cells and equipments have been used for the coupling with ESI mass spectrometry without adequate optimisation. The ESI high voltage can be hazardous to the EC equipment and some possible ways to avoid such problems are reported and discussed in this thesis. One should also be aware of that ESI demands volatile sample solutions with low concentrations of analyte and electrolyte and high content of organic modifier. This particular situation is not common in off-line EC analysis. When coupling EC to ESI-MS, there are hence some factors to pay attention to, for example the ESI high voltage and interfering redox reactions, the flow rate and transfer time between the EC and ESI-MS systems. Solution composition – including conductivity and ionic strength, type of supporting electrolyte, and concentration of analyte and electrolyte – are other important aspects to bear in mind. When interfacing the two techniques, EC and ESI-MS, one can improve the coupling by using different EC flow cell designs. Some of these aspects are discussed in this thesis.
Electrochemistry (EC) is the branch of chemistry that studies electron transfer reactions, which take place at electrode-solution interfaces. There are two main types of electrochemical cells. If the cells are used to produce electrical energy, for example in batteries, they are called galvanic cells. The galvanic cell is named after Luigi Galvani and often consists of two different metals (copper and zinc for example) connected by a salt bridge between the individual half-cells. Alessandro Volta invented the first modern electric battery, also called a voltaic cell. When electrochemical cells consume electricity from an external source they can be classified as electrolytic cells. Electrolytic cells (as well as galvanic cells) are composed of an electrolyte, a cathode and an anode. When a species loses electrons it is said to be oxidised, and when it gains electrons it is reduced. The whole process, the redox reaction, involves transfer of electrons (e\(^{-}\)) from the reductant (\(\text{RED}\); the reducing agent that is oxidised) to the oxidant (\(\text{OX}\); the oxidising agent that is reduced):

\[
\text{OX} + n\text{e}^- \leftrightarrow \text{RED}
\] (1)

Electrolysis is a process in which an electrochemical reaction is forced – in its non-spontaneous direction – to occur at an electrode by an imposed potential (or electric current). Any chemical species that is electroactive (i.e., that can be reduced or oxidised) can therefore be studied in electrolytic cells.

Electrochemical reactions take place at the interface between an electrode (which can be a metal, carbon or a semiconductor) and an ionic conductor (the electrolyte). The working electrode (WE) is where the analytical reaction occurs. A reference electrode (RE) is used for measuring the potential of the working electrode against a reference potential. The constant reference potential is achieved by maintaining a constant composition of the species involved in the redox reaction e.g. \(\text{Ag}^+\), \(\text{AgCl}\), and \(\text{Cl}^-\) in the reference electrode. A third electrode, the auxiliary (also called counter) electrode (AuxE), is generally used as the current-supporting partner of the working electrode.

Quasi (also called pseudo) reference electrodes (QRE) are sometimes used when it is difficult to find an appropriate reference electrode and when one expects that neither the bulk solution nor the QRE will undergo changes during the measurements. This QRE can for example be a metal wire or a
stainless steel rod. It is important to calibrate the actual potential of the QRE vs. a true reference electrode under open circuit potential (ocp) conditions, i.e. when no electrochemical potential is applied.19

At working electrode potentials \( E \) that are more negative than the standard potential, \( E^0 \), a net reduction will occur. A net oxidation, on the other hand, takes place when the working electrode potential is made more positive than the standard potential. The anode is the positive electrode, while the cathode is the negative electrode in an electrolytic cell. In other words, oxidation (loss of electrons) occurs at the anode and electrochemical reduction (gain of electrons) occurs at the cathode.17 Electrochemical reactions are composed of an oxidation and a reduction; each of which is called a half-cell reaction. The relationship between the potential and the concentrations of the oxidised and reduced species is expressed by the Nernst equation:

\[
E_{eq} = E^{0}_{OX/RED} + \frac{RT}{nF} \ln \left( \frac{[OX]}{[RED]} \right)
\]  

(2)

where \( E^{0}_{OX/RED} \) is the standard potential. The latter is a thermodynamic parameter given versus the potential of a standard hydrogen electrode (SHE), usually at 25 ºC and \([OX]\) and \([RED]\) represent the concentrations of each specific species. The standard potential is related to the change in the standard reaction free energy:

\[
\Delta G^0 = -nFE^0
\]  

(3)

where \( \Delta G^0 \) is the free energy change. The standard potential is also related to the equilibrium constant, \( K \), via equation (4) or (5):

\[
\Delta G^0 = -RT \ln K
\]  

(4)

\[
K = e^{(nFE^0)/(RT)}
\]  

(5)

Nernst reaction is, however, only valid for reversible reactions which proceed in both directions. For irreversible reactions one of the steps is impossible on the time scale of the experiment.

The potential between the working and the reference electrode is kept constant by an electronic device, a potentiostat.17 One can view the potentiostat as an active element whose job is to force through the working electrode whatever current is required to achieve the desired potential at any time.19 Chemically, the current is the flow of electrons needed to support the active electrochemical processes at rates consistent with the potential. Thus, the response from the potentiostat – the current – is the experimental observable.20 A potentiostat was used in Paper I-VII.
A galvanostat (synonymous to amperostat) can be used to maintain a constant current in e.g. coulometric titrations (amperostatic coulometry). The galvanostat reacts to changes in the resistance of the cell by altering its output potential. In comparison, a potentiostat changes the current to maintain a constant potential in potentiostatic coulometry.

The reactions in the electrochemical cell will generally not occur at the potentials described by the Nernst equation due to the following three phenomena: ohmic potential \((iR)\) drop, concentration polarization, and kinetic overpotential. The ohmic potential drop is the product of the current \(i\) and the resistance \(R\) of the electrolyte between the reference electrode and the working electrode. In a potentiostatic experiment, a potential is imposed and the resulting current is recorded. The potential of the working electrode is expected to be equal to the applied potential. In the presence of an ohmic drop, the potential of the working electrode is different from the expected potential. The potentiostatic control of the working electrode compensates for some of the cell resistance.

Concentration polarization is the difference between the concentration at the electrode surface and the bulk concentration due to the presence of faradaic reactions. This difference in concentration is equivalent to a potential that is called the concentration polarization potential. This phenomenon occurs at both the working and auxiliary electrodes – but not at reference electrodes since no current is drawn through the latter – and tends to limit the flowing current.

The third phenomenon is called kinetic overpotential and is described as a difference between the expected potential (for example 1.23 V to oxidise water) and the actual potential where the reaction actually starts (e.g. 1.7 V). The kinetic overpotential is significantly dependent on the electrode material and the state of the specific electrode surface.

2.1 Electrochemical methods

The electrochemical methods can be classified based on the controlled parameter i.e., either the potential \(E\) or current \(i\), by the measured quantities or the occurring processes. Electrochemical methods can also be classified according to their purpose. See Figure 2 for a classification schematic of the different methods. The following techniques: potentiometry, amperometry, coulometry, voltammetry as well as flow-electrolysis will be described in more detail in this chapter. If one desires to alter the composition of the bulk solution then bulk electrolysis is the method of choice. These methods are characterised by effective mass-transfer conditions.

Electroanalytical chemistry is the branch of analytical chemistry that uses electrical measurements for analytical purposes. There are two major types of electroanalytical measurement techniques. The potentiometric technique
(constant or zero current methods) is also called controlled-current techniques,\textsuperscript{19} where the current passing through the electrochemical cell is held constant.\textsuperscript{18} In the potentiostatic (or amperometric) technique (controlled-potential or dynamic and non zero current methods) – also known as the controlled-potential technique – the potential of the working electrode is controlled with respect to that of the reference electrode.\textsuperscript{18,19} The current is monitored as a function of time or potential. The potential controls which reaction that can occur. Controlled-potential techniques are therefore often preferred for bulk electrolysis.\textsuperscript{19}

Figure 2. Classification of electrochemical methods.

2.1.1 Potentiometry
The measurement of potentials between two electrodes (i.e., the indicator and reference electrodes) provides chemical information and is called potentiometry.\textsuperscript{19} Chronopotentiometry is a controlled-current technique with usually a three-electrode arrangement in which the interesting reaction occurs at the working electrode.

2.1.2 Amperometry
Amperometry involves the determination of the quantity of a material (analyte) in a sample by measuring the electric current passing through a cell containing the solution, at a constant applied potential.\textsuperscript{19} In chronoamperometry, a current change is induced by a potential step. The current is recorded as a function of time while the potential between the working and the reference electrode is maintained at a preset value.\textsuperscript{19} This was performed in Paper VII in which amperograms were acquired.
2.1.3 Coulometry

In coulometry, the total quantity of electricity required to carry out a complete electrolysis is determined. The quantity of material (or number of electrons) involved in the electrode reaction can be determined by Faraday’s law – if the reaction occurs with 100 % current efficiency. The charge (number of coulombs) $q$ is related to number of moles $N$ that is converted to the product and the number of electrons $n$ involved in the reaction:22

$$ q = nFN $$

(6)

Coulometry is based either on the use of a controlled current (amperostatic coulometry):

$$ q = it $$

(7)

or a controlled potential (potentiostatic coulometry). The latter is more selective since the potential controls the reactions. The charge is measured by integrating the current with respect to time $t$:

$$ q = \int i \, dt $$

(8)

Chronocoulometry is an example of a coulometric approach19 that is analogous to chronoamperometry.

2.1.4 Voltammetry

Voltammetry is a collection of techniques in which the relationship between current and potential is studied for electrochemical processes.20 Information about an analyte is obtained by measuring the current as the potential is varied.19 In amperometry, as a comparison, current alone was used to provide information of the analyte quantity.

There are different types of voltammetry, such as cyclic voltammetry (CV), polarography (normal pulse, differential pulse and square wave), rotating disk voltammetry and differential pulse voltammetry.17 In cyclic voltammetry, the potential is swept at a given scan rate (e.g. 100 mV/s) between anodic and cathodic potential limits while the current is measured. By using cyclic voltammetry and changing the scan rate or the potential limits, it is possible to study the kinetics of the electrochemical reactions involved.

Cyclic voltammetry was used in Paper II and IV-VII.
2.2 Flow-electrolysis

Methods of flow electrolytic nature can yield high efficiencies and rapid electrochemical conversions. These methods are very convenient for large solution volumes. Flow methods can be used in industries to remove metals from waste streams. Flow electrolysis has also found applications in electro-synthesis, separations, and analysis.19 The flow electrolysis cell usually contains a working electrode of large surface area and carefully positioned auxiliary and reference electrodes to minimise the ohmic potential \((iR)\) drop.19

The conversion efficiency, \(Y\), is defined by equation (9). The inlet concentration of \(OX\) is \(C_{OX}(in)\), while the concentration of \(RED\), \(C_{RED}(in)\), is assumed to be zero. At the outlet of the cell, the concentrations are \(C_{OX}(out)\) and \(C_{RED}(out)\). The overall conversion from \(OX\) to \(RED\) is \(i/nF\) (mol/s) or when the volumetric flow rate is \(v\) (cm\(^3\)/s) \(i/nFv\). \(Y\) thus denotes the fraction of \(OX\) converted, and when \(Y = 0\), no conversion has occurred and when \(Y = 1\), 100 % conversion has been reached:

\[
Y = \frac{i}{nFvC_{OX}(in)} = 1 - \frac{C_{OX}(out)}{C_{OX}(in)}
\]  (9)

The conversion efficiency increases with decreasing flow rate and increasing specific area and length of the working electrode. The time for the solution to transfer along the working electrode is called the residence time, and the conversion efficiency is higher for longer residence times. For flow cells, the current is directly proportional to the concentration of the electrolysed substance. These cells are convenient for continuous analyses of liquid streams, e.g. in coulometric analysis. In the latter cases, the analytical method is absolute and no calibration and knowledge of the electrode area is required.19,21

In the early 1960s, the first thin-layer electrochemical cell was utilised. During the years the theory and applications of the cells have been reviewed.23-28 Examples of application, where thin-layer cells have been used in electrochemical studies, include investigations of adsorption, electrodeposition, complex reaction mechanisms, and spectroelectrochemical studies.19

Important applications of electrochemical flow cells also include the use of liquid chromatography (LC), capillary electrophoresis (CE) and flow injection (FI) methods. Electrochemical detectors cells may be coulometric (in which all the material is electrolysed) or amperometric. The LC detectors may be designed in various ways with different cell geometries and flow arrangements. Some general requirements are: well-defined hydrodynamics, low dead volume, high mass-transfer rate, high signal-to-noise ratio (S/N), robust design, and reproducible responses.29
The detection limit is related to the produced current compared to the background current at the electrode. For oxidisable species, detection limits of about nM concentration can often be reached. The sensitivity is poorer for reducible substances, mainly because of oxygen reduction yielding higher background currents. When the redox couple is reversible, it is possible to increase the sensitivity by redox cycling through passage of more electrons per molecule between the working and auxiliary electrodes.19

The working electrodes of the thin-layer electrochemical flow cell can be of dual electrode type. The dual working electrodes can be placed either in parallel or in series, see Figure 3. In the case of parallel electrodes, one electrode can be used to measure the background current while the other electrode detects the desired species. When the electrodes are arranged along the solution flow (in series) then the downstream electrode can monitor (collect) products from the upstream electrode. By detecting products at another potential, selectivity can be improved. There can also be discrimination between compounds that produce and do not produce electroactive products.19

Figure 3. Placement of working electrodes in a dual working electrode cell with (a) series and (b) parallel crossflow.30

By decreasing the cell volume, bulk electrolysis can be obtained. In this way, a very small solution volume (μl) can be confined into a thin layer (2-100 μm) at the electrode surface where the cell thickness (defined by a thin gasket) is thinner than the diffusion layer so that mass transfer effects can be neglected. Otherwise, diffusion in the cell must be considered. Problems with high uncompensated $iR$ drops are especially prominent in thin-layer cells when non-aqueous solutions and/or very low concentrations of supporting electrolyte are used.19

Even if UV absorption detection still dominates detection in LC, the advantages of electrochemical detection are significant. UV absorption detects μM concentrations and has poorer detection limit than EC detection, which works well also for nM concentrations. Decreased detection limits can be obtained with fluorescence detection, however, it generally demands derivatisation procedures. Electrochemical detectors are relatively inexpensive, but if one wishes to study structural information, then the more costly mass spectrometry (LC/MS) is the technique of choice. Species as dopamine, neurotransmitters, and endogenous aromatic substances are well detected by
LC/EC. There are of course significant differences regarding dead volumes of different electrochemical detectors, and miniaturised separation systems are generally not compatible with conventional detection in thin-layer electrochemical cells. For instance, in capillary column LC, the thin-layer cell is normally replaced by microelectrodes. The ionic strength, pH, electrochemical reactivity of the solvent and electrolyte, and the presence of electroactive impurities (dissolved oxygen etc) are important considerations for LC/EC.22

Weber and Purdy32 have reviewed the detector design for LC/EC, and thoroughly discussed both amperometric and coulometric detectors. Schieffer33 reported on increased selectivity of LC/EC by the use of a dual coulometric-amperometric cell provided the oxidation potentials of the components are sufficiently different. Boyer and Goddard34 showed efficient detection of toxins in seafood with LC and post-column EC oxidation.

2.2.1 Amperometric cells
For amperometric detectors, the conversion efficiency is relatively low (1-10 %), but the detection limit is almost the same as for coulometric detectors. The signal-to-noise ratios are thus in analogy with those of coulometric cells. Most amperometric detectors consist either of a tubular electrode or a thin-layer cell. In practice, the latter cell is more popular, since it is easier to obtain a small cell volume (μl) and to use various electrode materials.22

The uncompensated $iR$ drop can be reduced – even for low ionic strength (< 10 mM) solutions by positioning the auxiliary electrode opposite to the working electrode.22 On the other hand, the position of auxiliary electrode close to the working electrode may result in undesired interfering reactions, which is discussed in Paper I, III-VI.

For a thin-layer cell with two working electrodes (dual electrode detector, see Figure 3), one can use the electrodes independently or the down-stream electrode can detect the product of the upstream electrode.35 A thin layer of solution flows parallel to the planar electrode surface imbedded in a channel.18 A PEEK block with a dual working electrode and a stainless steel block often form the flow cell, see Figure 4. These blocks are separated by a thin Teflon™ spacer gasket, which defines the flow channel. The dead volume of the cell is therefore rather small (~ 1 μl). A thin-layer EC flow cell was used in Paper I-III, and V-VII.

2.2.2 Coulometric cells
Coulometric detector cells are defined as cells for which the conversion efficiency is 100 % due to rapid mass transfer. In order to reach such conversion efficiency, a working electrode with large area, for example a porous working electrode, is often used. Another advantage of coulometric cells is that
the charge $q$ is independent of the flow rate. As already mentioned, the de-
tection limits of coulometric detection are still similar to those for am-
perometric detection.\textsuperscript{32} Large electrode areas, result in large background
current, and lower signal-to-noise ratios.\textsuperscript{22}

By control of the electrode potential, one can achieve great selectivity
with a coulometric detector cell.\textsuperscript{22} If an easily oxidisable compound disturbs
the electroanalysis, one can eliminate it at the first working electrode (WE1),
and then detect the analyte at the second electrode (WE2). This approach is
especially convenient for cells with two working electrodes or more in se-
ries,\textsuperscript{36} see a schematic picture in Figure 5.

**Figure 4.** Schematics of a thin-layer amperometric flow cell\textsuperscript{30} with reference elec-
 trode (RE), working electrode (WE) and auxiliary electrode (AuxE) blocks, viewed
from top and front, respectively.

**Figure 5.** Schematics of a coulometric flow cell with porous working electrodes
(WE1 and WE2), as well as auxiliary and reference electrodes (AuxE and RE).
3 Mass spectrometry (MS)

A mass spectrometer is an instrument that effectively separates moving ions on the basis of their mass-to-charge ratios ($m/z$) and records the intensities of the ions.\textsuperscript{37} See Figure 6 for the principal components of an atmospheric pressure ionisation (API) mass spectrometer.\textsuperscript{38,39}

\textbf{Figure 6.} Schematics of an API mass spectrometer.

After introducing the sample via the inlet system, the sample is converted into gaseous ions in the ion source. Ionisation can be performed by the bombardment with electrons, photons, ions, or molecules. Thermal or electrical energy can also be used for the ionisation. Through the mass analyser, the gaseous ions are accelerated and dispersed on the basis of their mass-to-charge ratios.\textsuperscript{37} To convert the beam of ions into an electrical signal, a detector is used. There are several types of commercially available detectors, e.g. the electron multiplier and the Faraday cup collector.\textsuperscript{37} Within the range of API\textsuperscript{38,39} mass spectrometers, the main ionisation techniques are electrospray ionisation (ESI),\textsuperscript{40-44} atmospheric pressure chemical ionisation (APCI)\textsuperscript{45} and atmospheric pressure photoionisation (APPI).\textsuperscript{46-49}
Several types of mass analysers are commercially available, including for example quadrupole\textsuperscript{50} and time-of-flight (TOF)\textsuperscript{51-55} mass spectrometers. There are also ion trap analysers,\textsuperscript{56-60} ion cyclotron resonance (ICR)\textsuperscript{61} based, and Fourier transform (FT)\textsuperscript{62-65} based instruments.

ESI-MS was used in Paper I-VII. A triple quadrupole mass spectrometer was used in Paper I-V whereas a single quadrupole was the mass analyser in Paper VI and VII. Moreover, a linear ion trap quadrupole MS was also used in Paper VII.

3.1 Electrospray ionisation (ESI)

Electrospray ionisation is a soft technique, which means that gas-phase ions can be produced with very little (or without) fragmentation of thermally labile molecules, such as peptides or proteins and other organic molecules. Polar, fragile, or non-covalent complexes can also be ionised by this popular and versatile technique. Dole\textsuperscript{66} and Fenn\textsuperscript{43} were pioneers in the field of electrospray with their ionisation of large polymers and biomolecules. The principle of electrospray is based on a solution flowing through a small opening at atmospheric pressure. When high voltage (± 2-5 kV) is supplied between the (conducting) spray capillary with solution and the entrance of the mass spectrometer,\textsuperscript{41-43,66-72} dispersion of solution into fine droplets occurs. At last, singly or multiply charged ions of analytes, so called gas-phase ions, are produced.\textsuperscript{73-76}

In positive electrospray, positive ions accumulate at the liquid surface and a Taylor cone is established.\textsuperscript{44} When the surface tension of the liquid is exceeded, positively charged droplets are formed. The diameter of the droplets decreases due to solvent evaporation. Exactly how the gas-phase ions are produced is not yet fully understood. Two theories have been presented during the years by Iribarne and Thomson,\textsuperscript{77} the ion evaporation model (IEM), and by Dole and co-workers,\textsuperscript{66} the charge residue model (CRM). The electric field on the droplet surface is so strong that an ion from the droplet surface is directly moved by emission into the gas-phase. This very shortly described model (IEM) is valid for droplets having a radius smaller than 10 nm. The other model (CRM) assumes that solvent is evaporated successively and that Rayleigh fission occurs due to coulombic repulsions of charges at the droplet surface. Each produced droplet only contains one analyte molecule. As its last solvent molecule evaporates, some charge is retained and the molecule is becoming a free gas-phase ion. Irrespective of which model that dominates during the whole process, the result in the end is qualitatively similar.\textsuperscript{40} So, the distinction between Rayleigh fission (CRM) and ion evaporation (IEM), for droplets with radius smaller than 1 nm, is difficult to make.
3.2 Electrochemical processes in ESI

Since electrochemical reactions have been found to take place at the interface between the liquid and spray capillary in electrospray ionisation, there are many publications in which ESI is described as an electrolytic cell.\textsuperscript{71,78-91} The electrochemical reactions can be oxidative, Figure 7(a), or reductive, Figure 7(b). In the positive electrospray mode, oxidation of metals (present in the ESI emitter electrode), oxidation of solvents, analytes, and other solutes occur. In the negative electrospray mode, reduction of solvents, analytes and other solutes take place. As a result of the oxidative reaction at the interface between the solution and metal capillary (usually made of stainless steel), a reduction must occur at the large sampling aperture plate of the mass spectrometer. It is therefore possible to make use of the inherent redox reactions in ESI for electrochemical applications and for further developments of the ESI technique. The oxidation of metal (zinc or stainless steel) ESI capillaries has been demonstrated by Blades et al.\textsuperscript{78} Since stainless steel is not electrochemically inert, an oxidation of Fe to Fe\textsuperscript{2+} is possible. It was hence concluded that the ESI device could be seen as an electrolytic cell.\textsuperscript{78,85} Van Berkel et al.\textsuperscript{79} have also shown that solution species can be involved in the charge-balancing redox reactions.

\begin{figure}[h]
\centering
\begin{subfigure}{0.4\textwidth}
\centering
\includegraphics[width=\textwidth]{positive_ESI}
\caption{(a) Positive ESI}
\end{subfigure}
\begin{subfigure}{0.4\textwidth}
\centering
\includegraphics[width=\textwidth]{negative_ESI}
\caption{(b) Negative ESI}
\end{subfigure}
\caption{Schematics of (a) positive and (b) negative ESI, depicted as electrolytic cells.}
\end{figure}

Some advantages with the use of the inherent electrolysis in an ESI metal capillary emitter, compared to traditional EC analysis, are that less sample is required, and that it is possible to obtain molecular weight and structural information. In comparison with a separate EC flow cell on-line with ESI-MS, the instrumental setup is simpler – but less flexible. One cannot control the potential without influencing the high voltage on the ESI emitter. The ESI current, the material of the capillary, and the amount of the electroactive
species present in the sample solution, will all influence the electrochemical potential of the spray capillary.\textsuperscript{92}

With an appropriate solvent system, Van Berkel et al.\textsuperscript{79} generated radical cations and expanded the utility of ESI to compounds not normally amenable to the technique.\textsuperscript{93} Alkyl-substituted metalloporphyrins and polycyclic aromatic hydrocarbons (PAH) were oxidised electrochemically in the electrospray ionisation emitter.\textsuperscript{93} Cole and co-workers\textsuperscript{80} used ESI-MS for the study of metallocenes and their derivatives through electrochemical oxidation and nucleophilic addition reactions. It was concluded that their ESI source functioned as an electrolytic cell, as already proposed by Blades et al.\textsuperscript{78} While Van Berkel et al.\textsuperscript{79} preferred methylene chloride as solvent for radical cation formation in ESI-MS, Cole et al.\textsuperscript{80} found that protic solvents and nucleophilic solvents (water, methanol) are less suitable for this purpose and concluded that the choice of solvent composition determines whether protonation or oxidation of the analytes occurred.

The idea of the ESI source as an electrolytic cell\textsuperscript{78} was further refined by Van Berkel and Zhou,\textsuperscript{81} who experimentally verified that the ESI source operates as a controlled-current electrolytic (CCE) cell. The metal (stainless steel) ESI capillary emitter and the atmospheric sampling aperture plate of the mass spectrometer (usually stainless steel) serve as the electrodes of this CCE cell. Van Berkel et al.\textsuperscript{92} also proved the electrolytic nature of ESI; by showing that solution species can be involved in the redox reactions, and by ionising neutral analytes.

According to Van Berkel et al.,\textsuperscript{92} efficient ionisation of neutral analytes with ESI as a CCE flow cell require: a sufficient ESI current $i_{\text{ESI}}$, analyte reaction at the metal/solution interface in the ESI capillary, sufficiently low flow rates to yield efficient electrolysis, and gas-phase ion formation from electrolytically generated ions in solution. If these requirements are fulfilled, ESI-MS can be used in routine work for efficient ionisation of neutral analytes such as metallocenes, metalloporphyrins, and polycyclic hydrocarbons. The transfer time is also important to consider for short-lived species. For instance, Cole and co-workers\textsuperscript{84} presented a probe of low volume and with minimal distance to decrease the time between EC generation of ions and mass spectrometric detection of polycyclic aromatic hydrocarbons.

In 1991, Blades et al.\textsuperscript{78} concluded that electrolytic reactions affect the composition of the solution sprayed from a metal ESI capillary. For example, the pH of the solution can decrease significantly due to oxidation of water in the positive electrospray mode.\textsuperscript{86} Alteration of the bulk solution pH will increase as the solution flow rate decreases and/or the ESI current, $i_{\text{ESI}}$, increases. In negative ESI, the pH increases due to electrochemical reduction reactions.

Alteration of bulk solution pH is one example of a change in the solution composition induced by electrolytic reactions.\textsuperscript{86} Another example was discussed by Van Berkel,\textsuperscript{87} in which it was shown that the ions in the gas-phase
could be influenced by the nature and extent of the electrochemical reactions in the emitter. Ions such as Ag⁺, Cu²⁺, and Hg²⁺ can be electrolytically reduced and deposited on the electrospray ionisation emitter. The deposited metals were then electrochemically oxidised in the positive ESI mode, and detected in the mass spectrometer. The results showed more efficient deposition of Ag⁺ than of Cu²⁺ and Hg²⁺. When analysing metals by ESI-MS, the electrochemical processes in the emitter influence both the concentration and oxidation state of the metals.⁸⁷

To investigate the electrochemical aspects of ESI, Cole et al.⁹⁴,⁹⁵ recently carried out some interesting differential electrospray emitter potential (DEEP) experiments that display variations in the electrochemical potential in the ESI capillary and the Taylor cone. Cole and co-workers examined these DEEP maps in positive,⁹⁵ as well as in negative electrospray⁹⁴ ionisation mass spectrometry. They found that the measured potential was the highest at the points furthest into the Taylor cone, and that the values descended to zero at distances beyond 15 mm within the ESI capillary. Cole and co-workers⁹⁴,⁹⁵ concluded that the results are consistent with the characterisation of the ESI device as a controlled-current electrolytic flow cell.

Recently, Van Berkel et al.⁸⁹,⁹⁶,⁹⁷ described a new approach for emitter design. A porous flow-through electrode emitter enhanced the mass transport to the electrode surface and oxidised the analytes very efficiently in the electrospray ion source at flow rates up to 800 μl/min.⁸⁹ The efficient oxidation was possible due to an upstream current loop in the electrospray ionisation source circuit.⁸⁹,⁹⁶-⁹⁸
4 On-line electrochemistry mass spectrometry (EC/MS)

4.1 Historic overview

Electrochemistry and mass spectrometry were combined for the first time by Bruckenstein and Gadde\textsuperscript{14} in 1971, and the technique was then limited to the analysis of volatile species. A porous electrode was used and the products of the electrochemical reactions were studied by mass spectrometry. Later on, several ionisation types and interfaces were developed which have facilitated the analysis of non-volatile species present in liquid solutions. In 1986, Hambitzer and Heitbaum\textsuperscript{99} coupled EC on-line with thermospray ionisation mass spectrometry (TSP-MS) and studied non-volatile solution species. The EC/TSP-MS technique was further improved by Brajter-Toth and co-workers.\textsuperscript{100,101} The method was also combined with liquid chromatography in order to be able to separate and study the products of biological redox reactions.\textsuperscript{102} Electrochemistry have also been combined with other mass spectrometric ionisation techniques, for example fast atom bombardment (FAB) by Bartmess and Phillips\textsuperscript{103} in 1987, particle beam (PB) by Regino and Brajter-Toth,\textsuperscript{104,105} and atmospheric pressure chemical ionisation (APCI) by Henion and co-workers\textsuperscript{38} in 1990.

In 1995, electrochemistry was combined with electrospray ionisation (ESI) by Bond and co-workers,\textsuperscript{15} who studied metal-diethylthiocarbamate complexes with use of a two-electrode system, and by Van Berkel and co-workers,\textsuperscript{16} who combined three types of electrochemical cells with ESI-MS. The latter authors studied ionisation of the neutral analyte perylene, and products of electrode reactions for nickel(II) octaethylporphyrin, and determined silver (Ag\textsuperscript{+}) by anodic stripping voltammetry (ASV) on-line with ESI-MS.

ESI is a soft ionisation technique with a lot of possibilities\textsuperscript{40} so it is not surprising that several groups have reported on EC/ESI-MS systems, in which the ESI acts as an EC cell\textsuperscript{78-81,92} and where EC cells are coupled to ESI-MS, as was comprehensively described by Van Berkel and co-workers.\textsuperscript{81,106-109} Except studies of the electrochemical nature of ESI, Van Berkel and co-workers\textsuperscript{92} have also used electrochemical reactions to ionise neutral species. It has also been shown that an EC cell can be used for sample pre-concentration and cleanup under potential control for both organic\textsuperscript{106,107} and inorganic\textsuperscript{108,109} compounds. An important reason for the in-
Increasing interest in combining EC with MS is the development of the ESI technique. The EC/ESI-MS combination is clearly very powerful for the study of electrochemically active species and electrochemical reactions.

4.2 On-line electrochemistry electrospray ionisation mass spectrometry (EC/ESI-MS)

Although there are some inherent restrictions regarding the combination of EC and ESI-MS, the interest in this combination has increased during the last decades. Some examples of applications are discussed below. EC/ESI-MS was used in Paper I-VII.

4.2.1 Identification, ionisation, and derivatisation of electroactive species

Important reasons for the growing attention in the field of EC/ESI-MS are the possibilities for identification of generated species by electrochemical reactions also demonstrated in Paper I, II, and III and the use of EC reactions for ionisation and derivatisation of analytes. Hayen and Karst have reviewed the strategies for LC/MS analysis of non-polar compounds, and one way is to use EC for on-line derivatisation.

Van Berkel and co-workers have reported on radical cations, and chemical derivatisation of aromatic and highly conjugated molecules, as well as alkenes and alkynes. Ionisations of neutral analytes clearly facilitate the analysis with ESI-MS. Zhou and Van Berkel ionised perylene, anthracene, ferrocene, tetrabutylammonium tetrafluoroborate, decamethylferrocene and nickel(II) octaethylporphyrin.

A neutral thiol (1-hexanethiol) was detected with ESI-MS in negative ion mode, after spontaneous adsorption on a gold working electrode in Paper I. Ionisation by production of sulfinates or sulfonates followed upon the oxidative desorption, see Figure 8.

Williams and Young have analysed neutral isomeric carbohydrates of low molecular weight using ferrocenyl boronate derivatisation in the EC cell on-line to the ion trap mass spectrometer. It was demonstrated that a distinction between the diastereomers of mono- and disaccharides is possible to make. Quirke and Van Berkel have studied ferrocene carbamate ester derivatives of saturated primary, secondary and tertiary alcohols by EC oxidations inherent in the ESI capillary of the mass spectrometer.
Guo et al.\textsuperscript{123} have demonstrated an electrochemical cell design and a systematic EC/ESI-MS study of electrochemical oxidation of iodide and thiocyanate at platinum and gold electrodes in the negative ESI mode.

Duckworth and co-workers\textsuperscript{124} have reported on studies of electrochemically induced reactions of ionic liquids. Negative ESI-MS was applied to indirectly measure the water content in undiluted ionic liquids, thereby providing a rapid assessment of their purity.

Differential electrochemical mass spectrometry (DEMS)\textsuperscript{125} can be used for both on-line detection of EC reaction products and to study adsorbates on polycrystalline and single crystal electrode surfaces by means of their desorption. The advantage or disadvantage is that only volatile products can be detected.

Johnson et al.\textsuperscript{126} have observed the EC reduced forms of several metal-containing proteins by ESI-FTICR-MS and stated that the EC method have advantages compared to the technique of chemically reduced metalloproteins. The laboratory-fabricated two-electrode EC cell (comprised of Pt-wires inside two plastic syringes) was connected to the ESI emitter tip. The reoxidation of reduced protein was greatly diminished, provided exclusion of oxygen in sample solution prior to analysis. Johnson et al.\textsuperscript{126} concluded that EC/ESI-MS was useful for characterisation of metalloproteins and their oxidation states.

Gun et al.\textsuperscript{114,115} have studied electrochemical reductions of organometallic molybdenum complexes both in methanol/water/acetate solutions, and in methanol/water/trifluoroacetate solutions, while Arakawa et al.\textsuperscript{127} have investigated oxidative electrolysis reactions of metal complexes using EC/ESI-MS. The ligand oxidation reaction of [Ru(bpy)$_2$(en)](ClO$_4$)$_2$, where bpy = 2,2'-bipyridine, and en = ethylenediamine, formed two dehydrogenation products; [Ru(bpy)$_2$(en-2H)]$^{2+}$ and [Ru(bpy)$_2$(en-4H)]$^{2+}$, that were successfully detected by the method of Arakawa et al.\textsuperscript{127}

In \textbf{Paper II}, electrochemical oxidation of dinuclear manganese complexes by on-line EC/ESI-MS was studied. It was possible to monitor the

---

**Figure 8.** Molecular structure of a neutral 1-hexanethiol that was ionised by EC reactions on-line to ESI-MS in \textbf{Paper I} as well as the resulting oxidation products.
loss of acetate ligands from $[\text{Mn}_2(\text{bpmp})(\mu-\text{OAc}_2)]^{n+}$ (bpmp = 2,6-bis[bis(2-pyridyl)methyl]amino)methyl-4-methylphenol anion) in different oxidation states and to identify products of the ligand-exchange reactions. Furthermore, the Mn(II,II) and Mn(II,III) states were found to be less prone to acetate loss in solutions of low water content while the Mn(III,III) state releases acetate ligands even in solutions with low water content [Paper II]. In Paper II, the Mn(III,IV) oxidation state was generated and identified for the first time.

In Paper IV, dopamine was analysed by a novel EC device, while Paper V discusses the oxidation of anions for the extraction and desorption on a polymer coated working electrode. The oxidation products of 4-chloroaniline (4-CA) was identified and studied in Paper VI, while Paper VII discussed the oxidation of antioxidants such as kaempferol, catechin, resveratrol as well as quercetin and its glucosides.

4.2.2 Short transfer times between electrochemical generation and MS detection

Van Berkel and co-workers\textsuperscript{106-108} have identified EC generated species present in small cell volumes. They studied biological redox reactions in a commercial three-electrode thin-layer flow-by cell with a volume of 1.1 $\mu$l.\textsuperscript{106} Dopamine was oxidised at pH 4.0 and tandem mass spectrometry showed that 5,6-dihydroxyindoline and 5,6-hydroxyindole are two of the major dopamine oxidation products. The transfer time between the EC cell and the ESI-MS was 5.1 s at a flow rate of 30 $\mu$l/min and 2.4 s at 62 $\mu$l/min.\textsuperscript{106}

Another important reason for the interest in electrochemistry on-line with mass spectrometry is the possibility to detect unstable reaction products with transfer times of a few seconds between the EC cell and the entrance of the mass spectrometer.\textsuperscript{10,128,129} Methylene blue has been electro-polymerised\textsuperscript{128} and polycyclic aromatic hydrocarbons were investigated\textsuperscript{129} with a low-volume probe. In the review by Brajter-Toth and co-workers,\textsuperscript{10} the time resolution was discussed among other subjects. It is important to attain sufficient time resolution to detect unstable and short-lived reaction intermediates. The transfer time and the cell dead volume are therefore crucial parameters\textsuperscript{10}

To obtain a short transfer time, one can position the EC electrodes on chip with an on-chip ESI emitter prior to the mass spectrometer as was performed in Paper IV. The transfer times between the Au working electrode within the PDMS chip and its ESI emitter (5 mm distance) were very short; 0.6 and 1.2 s for flow rates of 1.0 and 0.5 $\mu$l/min, respectively. Such transfer times facilitate the detection of unstable reaction products.
4.2.3 Sample preparation for EC/MS

Thin-layer flow-by cells with small volumes were used in publications where tamoxifen and 4-hydroxitamoxifen were pre-concentrated by adsorptive stripping,\textsuperscript{107} and where pre-concentration of copper(II) was examined.\textsuperscript{108} Van Berkel and co-workers\textsuperscript{106-108} showed that electrochemical pre-concentration and sample cleanup (i.e., matrix elimination) may be coupled on-line with ESI-MS thereby facilitating the analysis of metals and organic analytes. Desalting, sample clean-up/matrix elimination, and pre-concentration are advantageous on-line with the ESI-MS.\textsuperscript{107,108}

In Paper I, examples of sample preparations were presented. It was demonstrated that uncharged (neutral) thiols can be detected in ESI-MS after spontaneous adsorption on a gold working electrode, followed by oxidative desorption to yield sulfinates or sulfonates. Adsorption and potential-controlled desorption have been used for the pre-concentration of micromolar concentrations of 1-hexanethiol. Furthermore, the possibility of on-line matrix exchange was performed by desalting of solutions containing micromolar concentrations of 1-hexanethiols.

Paper V contains a description of electrochemically controlled solid-phase extractions (EC-SPE) interfaced on-line to ESI-MS and inductively coupled plasma mass spectrometry (ICP-MS), using polypyrrole (PPY) coated working electrodes and a thin-layer EC flow cell. The molecular structure of PPY is shown in Figure 9. It was beneficial to use a polymer-coated electrode due to its large active surface area yielding significant pre-concentration. It was found that EC-SPE can be used for sample preparation of anions, such as Fe(CN)\textsubscript{6}\textsuperscript{3-} and Br\textsuperscript{-}, by electrochemical potential control. Furthermore, it was found that the properties of the PPY coatings could be modified by altering the electrodeposition conditions. Another benefit was that the use of polymer coated electrodes enabled the extraction of charged electroinactive species while classical anodic/adsorptive stripping voltammetry requires electroactive species.

![Figure 9. The structure of polypyrrole.](image)

The selected ion monitoring (SIM) signal for the singly charged K\textsubscript{2}Fe(CN)\textsubscript{6}\textsuperscript{-}, \textit{m}/\textit{z} 290, clearly decreased during the extraction step (+0.8 V) and increased during the desorption step (-0.8 V) when the PPY polymer was reduced to its neutral state. Furthermore, a linear relationship (\textit{R}\textsuperscript{2} value of 0.99) was found between the analyte concentration (50-500 μM) and the signal (\textit{m}/\textit{z} 290 in
SIM) intensity [Paper V]. It was also found that the $m/z$ 290 peak area $(K_2\text{Fe(CN)}_6^-)$ increased with increasing EC-SPE extraction time.

A drawback with the EC-SPE/ESI-MS setup was that changes in the ionic strength, as a result of the desorption of the extracted ions, influenced the spray stability. A more straightforward way was the use of EC-SPE/ICP-MS by which it was found that tap water samples spiked with different bromide concentrations were possible to analyse [Paper V]. The conducting polymer PPY has also found other areas of application, for example in EC stimulated adenosine-5’-triphosphate (ATP) release from PPY/ATP films coupled online to ESI-MS.130

Duckworth and co-workers131 have also discussed some already presented applications for the EC cell.132-134 In analogy with conventional stripping methods, plutonium can be retained on and released from anodised glassy carbon (GC) electrodes in the EC flow cell on-line prior to ICP-MS.131 The phenomenon is useful for separation, concentration, and detection. The detection limit was improved due to analyte pre-concentration, and elimination of matrix and isobaric ions.

4.2.4 Studies of reaction mechanisms and kinetics

Li and co-workers135 showed that ESI tandem mass spectrometry (ESI-MS$^n$) is an excellent method to identify the structures of DNA-recognising polyamide products after electrochemical oxidation (by bulk electrolysis). Mechanistically, it was found that the EC reactions take place on the imidazole ring of the polyamide. The double bound of the imidazole ring was shown to be oxidised into a carboxyl group.135

Arakawa et al.136 used a micro flow electrolytic cell on-line with ESI-MS to study the oxidation of the highly antioxidative agent, caffeic acid. Dimer and trimer products were found, and the dimers where distinguished from hydrogen-bonded complexes by MS/MS. To determine the number of hydroxyl and carboxyl groups in the dimers, experiments with hydrogen/deuterium exchange were performed. Recently, Arakawa and co-workers116 investigated an anti-psychotic drug, zotepine, and its EC oxidation products and fragmentation using on-line EC/ESI-MS.

Lev and co-workers137 have studied the oxidation mechanism of $N,N$-dimethyl-$p$-phenylenediamine (DPD) in several aqueous electrolyte solutions (at pH 1.4 to 9.7) with on-line EC/ESI-MS. The coupling reactions of DPD with quinonediimine and quinonemonoimine were found to be strongly pH dependent. At pH 3.6, Lev and co-workers, studied the kinetics of the coupling reaction between DPD and quinonemonoimine. EC/ESI-MS systems have also found applications in the field of ligand-exchange reactions of dinuclear manganese complex in acetonitrile environment [Paper II]. The design of a thin-layer flow cell has also been evaluated by mechanistic analysis of manganese complexes [Paper III].
Kertesz and Van Berkel\cite{138} have monitored ionic adducts to elucidate reaction mechanisms. On-line EC/ESI-MS was used to reduce tetracyanoquinodimethane and oxidise triphenylamine prior to detection. Gu et al.\cite{117} recently tested several nitriles, i.e., acetonitrile, isobutyronitrile, and benzonitrile, in their laboratory. It was found that these nitriles (R-CN) were electrochemically reduced to their respective amines (R-CH$_2$NH$_2$) during ESI-MS in the positive mode. Both the phenomenon and the mechanism of nitrile reduction were discussed, and Gu et al.\cite{90} pointed out the ability to identify nitrile-containing unknown compounds.

Brajter-Toth and co-workers\cite{139} have used EC/ESI-MS to enhance the sensitivity of uric acid detection, thanks to a higher ES ionisation efficiency. The results indicated that negatively charged urate ions were oxidised during positive ion mode ESI yielding neutral uric acid radicals, which gave rise to the uric acid dimer. Furthermore, Brajter-Toth and co-workers\cite{139} showed that EC/ESI-MS, with an on-line two-electrode EC cell voltage (9 V from a battery) floating on the ES high voltage, allowed the detection of uric acid in 1000-fold diluted human urine, due to the high sensitivity of a novel conical capillary inlet configured for ESI-MS.

A similar EC setup was used in an earlier work by Brajter-Toth and co-workers,\cite{140} where analysis of dopamine was used for the evaluation of the cone-shaped capillary inlet compared to the cylindrical capillary inlet for ESI-MS. It was found that dopamine, $m/z$ 154, underwent an one-electron oxidation to radicals, which rapidly dimerised to singly charged, $m/z$ 307, or doubly charged, $m/z$ 153.5, ions. The dopamine quinone, $m/z$ 152, was also detected in positive ESI-MS after inherent EC oxidation.\cite{140}

In Paper IV, the oxidation of dopamine (Figure 10) was used for evaluation of the PDMS-chip coupled on-line to ESI-MS/MS. In agreement with Van Berkel et al.\cite{106} and Brajter-Toth et al.,\cite{140} the signal at $m/z$ 154 was assigned to unreacted protonated dopamine, while the peak at $m/z$ 152 was assigned to the dopamine oxidation product(s) dopamine o-quinone and/or 5,6-dihydroxyindoline. The peak at $m/z$ 123 originated from a loss of CH$_2$NH from the product at $m/z$ 152. A very small signal was also found for $m/z$ 150 in Paper IV due to the oxidation product(s) dopamino-chrome and/or 5,6-dihydroxyindole.\cite{106}

![Figure 10. Molecular structure of dopamine.](image-url)
By using a micromixer chip prior to ESI-MS, chemical kinetic studies were easily performed.\textsuperscript{141} The reaction intermediates and products can then be investigated after adjustment of the reaction time by controlling the microchannel length and the flow rate.\textsuperscript{141}

In \textit{Paper VI}, the oxidation of 4-chloroaniline was studied by EC/ESI-MS and the results verified previously suggested pathways for the EC processes.\textsuperscript{142-146} In addition, an unrecognised comproportionation reaction was found in \textit{Paper VI}. The oxidation of 4-CA gave rise to both an oxidised dimer 4-[(4-chlorophenyl)imino]-2,5-cyclohexadien-1-imine (\textit{m/z} 217) and a reduced dimer 4-amino-4’-chlorodiphenylamine (\textit{m/z} 219) in addition to a dimer intermediate (\textit{m/z} 253). The unexpected formation of the reduced dimer was found to stem from a comproportionation reaction involving 4-CA and the oxidised dimer [\textit{Paper VI}].

### 4.2.5 The use of electrochemical reactions for tagging purposes

Recently, some reviews on electrochemical analyte tagging have been published.\textsuperscript{12,147} Girault and co-workers\textsuperscript{148} have modified free cysteine residues by means of quinone addition. The electrochemical tagging is performed prior to the MS analyses and different mechanisms have been investigated. The tagging strategy takes advantage of the electrochemical nature of the electrospray. EC mass tags are infused employing microfabricated polymer microspray.\textsuperscript{149} The mass tags undergo redox reactions on the microspray microelectrode and become reactive toward a specific amino acid. Discussions about the microspray design and how it influences the tagging processes,\textsuperscript{150} and optimisations of electrochemical probes\textsuperscript{151} for nanospray mass spectrometry were recently published by Girault and co-workers. One of the great advantages, with this nanospray based approach used, is that the tagging is protein specific. In the proteome research protein identification, expression, regulation, and function are studied. Of importance is the information of cysteine content. In the usual tools for protein identification and quantitative analysis – 2D-gel electrophoresis, and peptide mass fingerprinting or isotope-coded-affinity-tag (ICAT™) coupled with LC/MS/MS – cysteine play important roles.

Roussel et al.\textsuperscript{152} elucidated the electrochemical tagging reaction mechanism using cyclic voltammetry. They found that the oxidation product of hydroquinone is benzoquinone, and when the latter is protonated, it acts as an electrophile for the free cysteine residues. When cysteine is substituted a reduction of adduct occurs and during further oxidation, cysteine is even higher substituted. This ECE (electrochemical-chemical-electrochemical) mechanism was confirmed by simulations of the cyclic voltammograms.\textsuperscript{152} It was also proved that a protein without cysteine residues does not react with benzoquinone present in the solution. For example, β-lactoglobulin A (from
bovine milk) – with one free cysteine residue – was tagged using hydroquinone, while myoglobin (from horse heart) – without cysteine – was not.

Some applications demonstrate the use of the tagging strategies for protein identification, i.e., counting of cysteine units in peptides and identification of four proteins in a model mixture. The tagging efficiency of different hydroquinone compounds were tested and it was found that 2-carboxymethylhydroquinone is the most reactive probe suitable for cysteine quantification of peptides containing three cysteine residues or two consecutive cysteines. Optimal conditions for determination of up to five cysteine groups were numerically simulated, and yielded the relative distribution and concentration of tags, untagged and tagged species in the microchannel. The number of cysteine groups corresponds to the number of characteristic mass shifts observed with respect to the unmodified peptide. It is an advantage that the native protein and the modified one have the same retention time since the cysteine-specific adduct formation occurs just before MS analysis. The occurrence and the location of the tagging reaction can therefore be chosen.

Girault and co-workers developed an analytical kinetic model to predict the tagging extent. It was shown that the extent of the EC tagging reaction depends mainly on the reactivity of the cysteine residues in the proteins and not on the ionisation properties of the adducts.

Another type of tagging applicable for peptide on-line complexation, is based on the generation of transition metal ions, Cu(II), Zn(II), Ni(II), Fe(II), and Ag(I), from sacrificial electrodes. An advantage with this strategy is that additions of metallic salts are avoided, and problems with signal suppression in ESI-MS can be circumvented.

Van Berkel et al. applied hydroquinone tagging of an initially disulfide-linked peptide, in order to demonstrate use of two high surface area flow-through electrodes (up-stream grounding point and emitter electrode) prior to the mass spectrometer. It was concluded that their tagging approach was similar to that of Girault and co-workers – except for the additional possibility to reduce disulfide linkages to free cysteine residues prior to the tagging.

Dayon and Girault have further developed the methods for the derivatisation of cysteine-containing peptides by benzoquinone compounds using diagonal reversed-phase liquid chromatography (RP-LC). This diagonal LC method consist of one primary and several secondary chromatographic separations before and after the tagging process. Furthermore, the cysteinyl peptides of a bovine serum albumin (BSA) tryptic digest was modified with 1,4-benzoquinone and analysed by the diagonal chromatographic method.

Very recently, some inorganic applications have been published. The polymer micro-sprayers with built-in electrodes have been used for studying bioinorganic systems, such as dinuclear zinc(II), copper(II), and lead complexes. Another interesting application of on-line EC/MS
was presented by Girault and coworkers,\textsuperscript{161} in which biphasic electrospray ionisation mass spectrometry (BESI-MS) and a dual-channel microchip were used for identification of peptide-lipid complexes. It was found that the EC studied peptides, angiotensin III and Leu-enkephalin, formed interfacial complexes with the phospholipid, dipalmitoylphosphatidylcholine, at the interface formed between two immiscible electrolyte solutions (ITIES).\textsuperscript{161} McClintock et al.\textsuperscript{162} have reported on the novel use of EC for generation of covalent oxidative labels on intact proteins. Two different working electrode types were evaluated, the porous flow-through (PFT) graphitic carbon and the boron-doped diamond (BDD) electrode, for off-line protein oxidation and LC/ESI-FTICR-MS/MS. It was demonstrated that the BDD electrode could be useful in protein structure studies, with less protein adsorption problems than the PFT graphitic carbon electrode.\textsuperscript{162}

4.2.6 Miniaturised ESI systems

Miniaturisation by use of microfabricated devices,\textsuperscript{163} lab-on-a-chips,\textsuperscript{164} microfluidic devices,\textsuperscript{165} and miniaturised total chemical analysis systems\textsuperscript{166} have emerged due to the need for short transfer times, small sample volumes, reduced reagent consumption, and high-throughput possibilities. Several microchip applications have been published in which on-chip ESI emitters have been used.\textsuperscript{167,168} These chips have, however, not been used to study electrochemical reactions. Instead, they were the devices that introduced the liquid stream to the MS. During the years, many publications have discussed interfaces for mass spectrometry, mainly with liquid streams from capillary electrophoresis\textsuperscript{169-174}

Principally, there are three kinds of interfaces for ESI-MS i.e., sheath-flow,\textsuperscript{170} liquid junction,\textsuperscript{171} and sheathless\textsuperscript{169} interfaces. Nilsson et al.\textsuperscript{175} have evaluated the stability of sheathless electrospray emitters, and improved the technique by overcoming the instabilities of the conductive coatings.\textsuperscript{172} The improvements included the development of polymer imbedded gold (fairy dust) and graphite (black dust) emitters.\textsuperscript{176,177}

Advantages with miniaturised systems,\textsuperscript{178} for example low dead volume and fast transfer time, make it easier to perform electrochemical reactions prior to the MS. Mengeaud et al.\textsuperscript{179} have developed a ceramic electrochemical reactor (CEM) for electrosynthesis and connected it on-line to ESI-MS. The CEM was allowed to perform on-line sampling and analysis of the reaction mixture, methyl-2-furoate (M2F) and methyl-2,5-dihydro-2,5-dimethoxy-2-furan-carboxylate (DMM2F), in the positive ion mode. It was found that the inherent electrodes of the CEM successfully performed the methoxylation of M2F to DMM2F.\textsuperscript{179}

Girault and coworkers\textsuperscript{141} have recently described an electrospray micromixer chip for on-line derivatisation and kinetic studies. It was found that the protein identification was significantly enhanced by counting the cys-
teines after on-line derivatisation of albumin tryptic peptides in the micromixer unit. Furthermore, the micromixer chip, that composed two microchannels and a liquid junction, was almost as efficient as an ideally mixed reactor.\textsuperscript{141}

In Paper IV, a novel method for the successful manufacturing of microchips, for on-chip combinations of EC and graphite-based sheathless ESI emitter prior to the MS/MS, was thus described. An array of gold (Au) microcoil electrodes was incorporated into a poly(dimethylsiloxane) PDMS microflow channel (of 50 μm inner diameter), where the manufacturing was based on a polymethylmethacrylate (PMMA) mould. It was found that the manufacturing process was reproducible and that the interelectrode distances in the EC chip could be adequately controlled. Furthermore, the novel EC chip technique was straightforward, inexpensive and did not require clean room facilities.

Regarding the positioning of the Au electrodes, it was found \textsuperscript{[Paper IV]} that an interelectrode distance of 100 μm was needed to avoid significant $iR$ drop effects in conjunction with the 50 μm id microchannel.

The EC/ESI-MS device was evaluated by multiple reaction monitoring (MRM) of dopamine and its oxidation products. It was found that the on-chip device enabled full potentiostatic control of the EC cell with a conversion efficiency of 30% at a flow rate of 0.5 μl/min \textsuperscript{[Paper IV]}.

4.2.7 EC/ESI-MS to mimic metabolic reactions

Permentier et al.\textsuperscript{180} have recently reviewed the use of EC/MS for applications in important research areas such as drug metabolism and enzymes, as well as protein analysis and biomarker discovery. Bruins and co-workers\textsuperscript{11} used electrochemistry coupled on-line with mass spectrometry to simulate metabolic oxidation reactions. The aim of the study was to mimic phase I oxidative reactions in drug metabolism, in which cytochrome P450 isoenzymes are the most important enzymes. To characterise these enzymes, some standard substrates, lidocaine and 7-ethoxycoumarin, were tested in the EC/MS system.\textsuperscript{11} Drug metabolism is normally divided into three metabolic phases (I, II, and III), where phase I metabolism involves oxidation, reduction, hydrolysis, and isomerisation.\textsuperscript{11} Enzyme-catalysed oxidations are the most important pathway in phase I metabolism, and cytochrome P450 is the far most important enzyme system.

Drug development is expensive, and to limit the cost it is important to eliminate many compounds at an early stage of the discovery procedures. According to Jurva et al.,\textsuperscript{11} EC/MS is a complementary screening method to \textit{in vitro} studies with purified enzymes. It was concluded that EC/MS could be a useful tool to characterise the metabolic pathways of new chemical species. Information about possible metabolites to look for \textit{in vivo} may be provided from EC tests. Bruins and co-workers\textsuperscript{11} aimed at a systematic com-
parison of electrochemical oxidation reactions and oxidation reactions catalyzed by cytochrome P450 isoenzymes.

To increase the understanding of drug metabolism, Jurva et al. \(^{181}\) introduced an electrochemical flow-through system on-line with liquid chromatography/tandem mass spectrometry (LC/MS/MS), suitable for on-line generation and characterisation of potential drug metabolites resulting from hydroxylation of double bonds and aromatic systems. The device allowed the generation of hydroxyl radicals for EC-assisted Fenton reaction with xenobiotics and detection of the oxidation products. This enabled investigations of new radical scavengers and antioxidants. One drawback with the EC-Fenton system\(^ {181}\) is the requirement of significantly lower flow rates compared to in other electrochemical oxidation.\(^ {11}\) On the other hand, EC-assisted Fenton system provides a different type of oxidation, and may be useful when electrochemistry fails to mimic the enzymatic oxidation.\(^ {181}\)

Jurva et al.\(^ {182}\) made a thorough comparison between enzyme-catalysed oxidations and electrochemical oxidations. It was found that EC mimics one-electron oxidation reactions with success. N-dealkylation, S-oxidation, P-oxidation, alcohol oxidation and dehydrogenation are all mechanisms initiated by a one-electron oxidation and this provides the possibility to mimic P450 catalysed reactions in these cases. The EC system is not able to mimic all oxidations performed by cytochrome P450. One reason for this, is that O-dealkylation and hydroxylation of unsubstituted aromatic rings (i.e., P450 catalysed reactions initiated with direct hydrogen atom abstraction) take place at a potential above the oxidation potential limit of water.\(^ {182}\) Cytochrome P450-catalysed oxidations of cyclic tertiary allylamines were studied by Jurva et al.\(^ {183}\) It was found that aminyl radical cations are not obligatory intermediates in the cytochrome P450 catalysed α-carbon oxidations. Johansson et al.\(^ {184}\) have investigated how EC oxidation, EC assisted Fenton reactions and synthetic metalloporphines can be used to mimic the most important cytochrome P450 catalysed oxidations. It was found that these three mimic systems are complementary and can be used for drug discovery by elucidating the metabolite structure without interferences from complex biological matrices.

Getek et al.\(^ {185}\) have reported on on-line formation and detection of glutathione (GSH) and cysteine (CSH) conjugates of acetaminophen (APAP) at different pH values by coupling a coulometric EC cell on-line to TSP-MS. It was found that the APAP-SG conjugate was easier formed at pH 8.5 than at lower pH values, which indicated that the rate of reaction of GSH and N-acetyl-\(p\)-benzoquinoneimine (the EC oxidation product of APAP) was enhanced at higher pH values.\(^ {185}\) Madsen et al.\(^ {186}\) have developed an EC method for generating reactive phase 1 metabolites that were trapped with GSH and then analysed by LC-MS/MS and nuclear magnetic resonance (NMR). High similarities between conjugates produced \textit{in vivo} and \textit{in vitro}
were observed and indicated that the developed method was useful for metabolic mimicking and scaling up synthesis of GSH conjugates.

Gamache and co-workers\textsuperscript{187} have combined an EC-array with mass spectrometry for multi-component analysis of metabolites. Measurements of redox active compounds make the EC-array/MS device able to elucidate biomarkers. Furthermore, Gamache et al.\textsuperscript{188} have used a coulometric flow cell on-line with ESI-MS for ADME/Tox profiling, which comprises absorption, distribution, metabolism, excretion and toxicity, to diminish the late-stage compound failure in pharmaceutical development.

The group of Santana da Silva\textsuperscript{189} have studied the off-line EC oxidation of albendazole (ABZ) and it was found that the same products were obtained by \textit{in vivo} and \textit{in vitro} metabolism. The bulk-electrolysis of ABZ yielded the oxidation products albendazole sulfoxide and albendazole sulfone and structural confirmation was elucidated by LC-MS/MS.

Thevis et al.\textsuperscript{190} have demonstrated the value of EC/ESI-TOF-MS in negative ion mode for doping control purposes with a class of anabolic agents (selective androgen receptor modulators) and the electrochemically generated metabolites were further identified by LC/NMR. It was found that the EC/MS method gave rapid insight into the oxidation behaviour of a drug and made useful simulations of the therapeutic compound metabolisms. Thevis et al.\textsuperscript{190} stated that EC/MS performed simple and clean synthesis of metabolites in a coulometric EC cell.

On-line EC/ESI/MS systems have also found to be useful in the field of electrochemical oxidation and cleavage of peptides\textsuperscript{191} or cleavage of proteins.\textsuperscript{192} The results confirmed already known mechanisms and reaction products of chemical and electrochemical peptide oxidation. The tyrosine-specific cleavage reaction enables the EC/MS system to be operated as an on-line protein digestion and peptide mapping system.\textsuperscript{191} Oxidation of tyrosine and tryptophan can cleave the peptide bond of a protein, and make EC/MS an alternative protein digestion method.\textsuperscript{192} The speed, the distinct amino acid specificity, and no need to remove excess reagents, enzyme or buffers, make the electrochemical approach more advantageous than the enzymatic and chemical methods. On the other hand, EC/MS also suffers from some drawbacks, for example side reactions of impurities and protein oxidation reactions that do not produce protein backbone cleavage, which complicate the mixture of oxidation products.\textsuperscript{192}

### 4.2.8 Use of ESI backward currents for EC/MS applications

In EC/ESI-MS, one has to consider the ESI high voltage and backward current, $i_{\text{back}}$ (also known as $i_{\text{ext}}$), among other aspects (as is further discussed in Chapter 5). In LC/MS generally, a ground point is inserted to avoid backward currents to reach the LC instrument. The current can be harmful to the instrumentation and the personal working with the instrument. If a grounded
stainless steel union is used electrochemical reactions will, however, take place. Sometimes the result of such an inserted ground point, upstream in the system, is neglected and undesired electrochemical reactions occur without control. In the latter case, result interpretation and correct comparisons with results from EC/ESI-MS without ground point are naturally less straightforward.

The insertion of one or more ground points – under strict control – has on the other hand recently found important applications by Konermann and co-workers, and by Van Berkel and co-workers. The latter authors used a PFT electrode emitter to obtain high conversion efficiencies. Konermann et al. added an external loop to the ESI circuit by connecting the metal needle of the sample injection syringe to ground. Konermann and co-workers denoted the backward current due to the external loop, $i_{\text{ext}}$. The total current in the system, $i_{\text{tot}}$, is the sum of the current in the ESI circuit, $i_{\text{ESI}}$, and the current in the external circuit, $i_{\text{ext}}$, (where $i_{\text{ext}} \neq 0$) i.e., $i_{\text{tot}} = i_{\text{ESI}} + i_{\text{ext}}$. When the syringe needle is not connected to ground, no external circuit appears in the system, and $i_{\text{tot}} = i_{\text{ESI}}$, since $i_{\text{ext}} = 0$. Konermann and co-workers found that the external loop and EC reactions of water induced pH changes and the extent of protein unfolding of for example cytochrome $c$ was increased. The effects were clearly shown in the mass spectra.

Later on, Konermann and co-workers also showed that the external loop and the external current influences the signal intensity in ESI-MS. According to the study, the magnitude of $i_{\text{ext}}$ could be controlled by the electrolyte concentration in the analyte solution, and by the dimensions of the silica capillary delivering analyte solution from the syringe needle to the ESI source. Significantly enhanced signal intensities were found for analytes such as ferrocene and reserpine. It was concluded that careful control of the EC parameters is of great importance in ESI-MS and/or EC/ESI-MS.

Van Berkel and co-workers used an upstream ground point and consequently an upstream current loop – in analogy to Konermann et al. – to increase the oxidation efficiency of reserpine, and ferroceneboronate derivatives of pinacol, and to study reduction of methylene blue (in the positive mode) and oxidation of 3,4-dihydroxybenzoic acid (3,4-DHBA) (in the negative mode). The ability to selectively ionise (oxidise) analytes with different standard electrochemical potentials was illustrated using a mixture of nickel and cobalt octaethylporphyrin, thereby overcoming overlapping isotopic clusters due to different charge states. The upstream current loop approach found application to a multistep reaction including consecutive electrochemical reduction and oxidation reactions, followed by a chemical reaction, as for the hydroquinonone tagging of an initially disulfide-linked peptide (discussed in Chapter 4.2.6). It was also found that reserpine was irreversibly oxidised and 3,4-DHBA was reversibly oxidised in the negative mode, and that thionine was reversibly reduced in the positive mode.
A modification of the PFT electrode emitter cell, used in CPE (controlled-potential electrochemistry) ESI studies, was made in a recent publication.97 Electrochemical reactions of analytes were controlled by a battery-powered, controlled-current (CC), two-electrode EC cell. The high surface area working electrode of porous graphitic carbon (PGC) and four palladium auxiliary electrodes were incorporated into the CCE (controlled-current electrochemistry) emitter. Reserpine and methylene blue illustrated the easy use of this emitter cell of small size and low cost.97 Electrochemical reduction in the positive mode and oxidation in the negative mode (see Figure 11) was possible due to an upstream ground point yielding an external current loop.89,96-98

Figure 11. Schematics of analyte oxidation in the negative ESI mode.

4.3 Liquid chromatography on-line with EC/MS and electrochemistry on-line with LC-MS/MS

Some applications of hyphenated techniques, such as liquid chromatography on-line with electrochemistry (LC/EC) and mass spectrometry as well as chromatographic separation of electrochemically generated products (EC/LC) prior to MS, have recently been published.198

By combining electrochemistry (EC) on-line with LC/MS, it is possible to separate electrochemical oxidation products (EC/LC/MS) or to form more polar compounds and thereby facilitate ionisation in the electrospray source (LC/EC/MS). The first attempt to employ this latter hyphenation was made by Volk et al.,102 as EC/LC/TSP-MS was used for the separation and study of the products of biological redox reactions. Dewald et al.,199 were the first to use LC/EC/TSP-MS for the separation and identification of isoflavones. Iwahashi and Toshihiro200 and Iwahashi201 used EC/LC/MS in combination with UV detection and electron spin resonance (ESR), to chromatographi-
cally separate the oxidation products of 3-hydroxy-\textit{dl}-kynurenine\textsuperscript{200} and 3-hydroxyanthranilic acid compounds,\textsuperscript{201} respectively. The group of Karst have made significant progresses in the field of LC/EC/MS and/or EC/LC/MS.\textsuperscript{6,198,202-209} Tahara et al.\textsuperscript{210} have used EC/LC/MS for analysis of (±)-\textit{\alpha}-tocopherol. Two of the oxidation products were suspected to be diastereomers and confirmation was obtained by use of a circular dichroism (CD) detector. It was concluded that EC/LC/MS in combination with EC/LC/CD was useful for studies of chiral drug metabolism.\textsuperscript{210}

4.3.1 LC/EC/API-MS

In 2001, Karst and co-workers\textsuperscript{202} described the first hyphenation of LC/EC on-line to atmospheric pressure ionisation MS using either ESI or APCI interfaces. They found that the ionisation was influenced by the EC pre-treatment. Karst et al.\textsuperscript{202} described the synthesis, separation and coulometric oxidation (ionisation) of ferrocenecarboxylic acid esters (of various alcohols and phenols) with excellent detection limit employing an LC/EC/MS system. Diehl et al.\textsuperscript{203} have demonstrated a rapid LC/EC/MS method for RP chromatographic separation of ferrocenecarboxylic acid esters of various alcohols and phenols, which were oxidised electrochemically to form charged ferrocinium species and thereby could be easily detected by APCI-MS. The APCI interface was used as a heated nebuliser – without discharge (because the radical cations provided by oxidation in solution were efficiently transferred into the gas-phase at low probe voltages).\textsuperscript{203} Meyer et al.\textsuperscript{204} have carried out LC separation of some phenols on-line with EC derivatisation and the detection of the reaction products was made fluorometrically. These fluorescent dimers and higher oligomers were then identified by on-line EC/ESI-MS. Hayen and Karst\textsuperscript{205} have further developed the LC/EC/MS method for rapid investigations on the electrochemical oxidation pathways of phenathiazine to strongly fluorescent sulfoxides in a coulometric flow cell. By this on-line approach to APCI- and ESI-MS, enhanced sensitivity was achieved and disproportionation reactions were detected. Diehl et al.\textsuperscript{206} have determined several alcohols and alkylphenols qualitatively in gasoline and diesel fuels with LC/EC/ESI-MS and LC/EC/APCI-MS as important screening techniques. Diehl et al.\textsuperscript{206} also demonstrated that LC/EC/MS/MS could be used for quantitative analysis of derivatives.

Karst and co-workers\textsuperscript{211} have likewise made important progress in the field of LC/EC/MS where they used ferrocenoyl piperazide as a derivatising agent for the analysis of isocyanates and related compounds in air samples after thermal degradation of polyurethane foam. Excellent separation was achieved and EC oxidation was used to obtain charged products that were analysed by APCI-MS.
4.3.2 LC/EC/MS for determination of antioxidant activity

There are many classical assays to determine the total antioxidant capacity (TAC). These assays can be classified in two categories; the hydrogen atom transfer and the single electron transfer reaction based assays. Niederländer et al. have recently reviewed antioxidant activity assays (electro-chemical and chemistry-based) on-line with liquid chromatography for antioxidant screening of complex mixtures.

A common assay for the determination of antioxidant capacity is the DPPH (2,2-diphenyl-1-picrylhydrazyl radical) assay. This assay is based on the reduction of the purple chromogen radical, DPPH, to the corresponding pale yellow hydrazine by antioxidants. The DPPH is stable and commercially available, but the assay time is very long; about 4h. Instead, it would be convenient to be able to study the antioxidant activity (ease of oxidation) with flow methods, for example a thin-layer EC flow cell. Low oxidation potentials $E_p$ of specific compound(s) indicate that the analyte could be oxidised easily and thereby could provide a high antioxidant activity.

In food samples, the most readily oxidisable compounds are those containing (poly)phenolic groups. These compounds are thus the most abundant antioxidants in our diet. This explains why the total phenolic content has been used as a measurement of the antioxidant capacity.

An electrochemical assay cannot give more information than the potential and the total current, but when combined with LC and MS, specific information concerning half-wave potentials $E_{1/2}$ and peak currents $i_{peak}$ can be related to the structural information given by MS/MS of the chromatographically separated compounds. Paper VII is the first report of the successful coupling of LC/EC/MS resulting in fast and easy antioxidant activity determinations. An advantage with the LC/EC/MS/MS setup is that the retention times in the amperograms can be related to the peaks in the mass spectra, see Figure 12. The amperogram (recorded with applied EC potential of 1.4 V vs. Ag/AgCl) in Figure 12(a) shows four major peaks, labelled 1-4, and are ascribed to quercetin-3,4´-diglucoside (Q3,4G, 1, 3.3 min), quercetin-3-glucoside (Q3G, 2, 5.2 min), quercetin-4´-glucoside (Q4´G, 3, 6.5 min), and quercetin (Q, 4, 8.8 min), with the retention times given in the brackets. From the total ion chromatogram (TIC), not shown, the most interesting ions can be extracted, i.e. in positive ESI, the protonated analytes [$Q + H]^+$, [Q3G + H]$^+$, [Q4´G + H]$^+$, and [Q3,4´G + H]$^+$. The extracted ion chromatogram (XIC) in Figure 12(b) show four peaks with similar retention times as was found for the analytes in the amperogram, see Table 1. The quercetin glucosides (Q3,4´G, Q3G, and Q4´G) were found to be fragile. Due to up-front fragmentation, neutral losses of sugar (162 u) were seen [Paper VII].

---

* amount
† ease of oxidation
Table 1. Retention times, protonated ions and identities using LC/EC/MS in positive ion mode for 10 μM of the analytes in 10% methanol, gradient elution at 200 μl/min with ammonium formiate buffer, pH 3.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Time / min</th>
<th>Analyte</th>
<th>Protonated ion</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.3</td>
<td>Q3,4´G</td>
<td>m/z 627</td>
<td>[Q3,4´G + H]⁺</td>
</tr>
<tr>
<td>2</td>
<td>5.2</td>
<td>Q3G</td>
<td>m/z 465</td>
<td>[Q3G + H]⁺</td>
</tr>
<tr>
<td>3</td>
<td>6.5</td>
<td>Q4´G</td>
<td>m/z 465</td>
<td>[Q4´G + H]⁺</td>
</tr>
<tr>
<td>4</td>
<td>8.8</td>
<td>Q</td>
<td>m/z 303</td>
<td>[Q + H]⁺</td>
</tr>
</tbody>
</table>

(a) amperogram

(b) XIC

Figure 12. LC/EC/MS, with positive ESI, for 10 μM Q3,4´G (1), Q3G (2), Q4´G (3), and Q (4) shown as (a) amperogram and (b) XIC with extracted ions (see inset). The injection volume was 10 μl, using gradient elution at a flow rate of 200 μl/min (ammonium formiate buffer, pH 3). The applied potential was 1.4 V vs. Ag/AgCl.
4.3.3 EC/LC/MS for the simulation of drug metabolism

Jurva et al.\textsuperscript{181} introduced an electrochemical flow-through system that allowed the generation of hydroxyl radicals (for reaction with xenobiotics) and subsequent detection of the oxidation products on-line with LC/MS/MS. It was found that the system was useful for investigations of new radical scavengers and antioxidants. It was concluded that the EC/LC/MS/MS technique could be useful also for on-line generation and characterisation of potential drug metabolites.

Some applications of hyphenated techniques, such as electrochemistry on-line with liquid chromatography (EC/LC) and ESI-MS,\textsuperscript{224,225} have been published and the approach seems to be a good tool for drug metabolism research. Karst and co-workers\textsuperscript{224} have studied the clozapine metabolism, while Chen et al.\textsuperscript{225} quantified ($p$-chlorophenyl) aniline in biological samples. Lohmann and Karst\textsuperscript{207} used EC/LC/MS to simulate the detoxification mechanism of paracetamol in the body. After electrochemical oxidation, a quinoneimine intermediate was formed which then underwent further reactions with glutathione and/or $N$-acetylcysteine. Thereby, isomeric adducts were produced via the thiol function and these were subsequently detected with mass spectrometry.

Seiwert and Karst\textsuperscript{208} have used $N$-(2-ferroceneethyl)maleimide (FEM) as an electroactive derivatisation agent for thiol functionalities in proteins. No unspecific labelling of free amino functions was observed and LC/EC/ESI-MS was used for detection of the derivatised reaction products. According to Seiwert and Karst,\textsuperscript{208} FEM was used for the first time as a reagent in LC/MS analysis of thiol-containing proteins. It was found that FEM was a useful tool for determination of the number of free thiol groups or the total number of free and disulfide-bound thiol groups in proteins.\textsuperscript{208}

Lohmann and Karst\textsuperscript{209} have showed that EC/LC/MS can be a complement to the HRP/LC/MS method, in which horseradish peroxidase (HRP) was immobilised on magnetic microparticles. It was found that both on-line techniques could be valuable complementary methods and promising tools for the identification of metabolites in drug development.\textsuperscript{209} Lohmann and Karst\textsuperscript{198} have also reviewed the biomimetic modelling of oxidative drug metabolism on-line in LC/MS for systematic investigations and simulation of phase II reactions.

Tahara et al.\textsuperscript{226} have used a coulometric EC cell on-line with LC/APCI-MS in the negative ion mode for successful preparation of oxidation products of the drug Troglitazone and additional confirmation was obtained with nuclear magnetic resonance ($^1$H- and $^{13}$C-NMR). As demonstrated by Gamache et al.,\textsuperscript{187} mass spectrometric and electrochemical array (EC-array) detection can be useful for multi-component analysis of metabolites. It was found that LC post-column flow splitting between the MS and EC-array gives complementary quantitative and qualitative information, and that bio-
markers can be elucidated through measurements of redox active compounds. Once again it was showed that EC/LC/MS is a good tool for drug metabolism research.

Recently, Lohmann et al.\textsuperscript{227} presented covalent protein modification by reactive drug metabolites (paracetamol, amodiaquine, and clozapine) using on-line EC/LC/ESI-TOF-MS and nano-ESI-FTICR-MS. It was found that this rapid method is an interesting tool for high-yield and high purity synthesis of covalent drug–protein adducts. The previously reported EC/LC/MS system\textsuperscript{227} constituted a new tool for risk assessment of drug candidates, and very recently Lohmann et al.\textsuperscript{228} came up with the complementary use of ESI-MS and ICP-MS for the qualitative and quantitative analysis of drug metabolites after coulometric oxidation in an EC flow-through cell.
5 Practical aspects and applications

The coupling of electrochemistry on-line with electrospray ionisation mass spectrometry is a rather new technique and several practical aspects have not yet been fully explored. In EC/ESI-MS one has to consider the ESI high voltage and backward currents, solution composition, flow rate, transfer times, dead volumes, and the EC cell design.

5.1 ESI high voltage and backward currents

There are several types of ESI mass spectrometers available on the commercial market. The ESI emitter is grounded in some of these instruments. The discussion, which follows below, is focused on instruments in which the ESI emitter is not grounded.

When coupling an electrochemical cell on-line with ESI-MS, the EC equipment must in some way be decoupled from the ESI high voltage source or be floated at the potential induced by the ESI high voltage, otherwise backward currents\(^{195}\) will float between the ESI emitter and grounded EC instruments damaging the latter. A decoupling is possible to obtain if one uses long transfer lines (~ 30 cm)\(^{11,16,81,85,110,114-116,123,136,137,181-183,191}\) between the electrochemical cell and the mass spectrometer or by inserting a ground point\(^{82,83,89,96-98,106-108,128,138,187,193,195,196,202,225,229-231}\) between the EC cell and the ESI emitter. The decoupling via long transfer line as well as insertion of a ground point were evaluated and discussed in Paper I. The disadvantage with a long transfer line as the decoupling device is obvious, since the electrochemically-generated products may not be detected fast enough.

For inherent EC processes in the ESI emitter, the use of an upstream external loop\(^{89,96-98,195,196}\) (also known as a ground loop or current loop as discussed in Chapter 4.2.9) due to an upstream ground point has some advantages. Some benefits are the enhancement of the oxidation rate of charge-balancing reactions (e.g. oxidation of water) in the ES ion source, and the enhancement of the signal intensity of analytes, such as ferrocene – that undergo electrochemical ionisation, and reserpine – that form protonated molecules.\(^{195,196}\) The advantage is that the ion source has to supply electrons for both of the circuits (ESI circuit and external loop circuit). In this way, it is possible to overcome the current limits due to the enhancement of the magnitude of the total current and therefore to increase the oxidation efficiency.\(^{89,195}\)
An insertion of a ground point [Paper I] causes the ESI emitter and the ground point to act as a cathode and anode, respectively, depending on the ESI mode, due to the backward current. In positive electrospray ionisation (oxidation at the emitter), an electrochemical reduction will occur at the upstream ground point. If the desired reaction in the electrochemical cell is oxidation, there can be a serious problem for the study of electrochemically generated species. A ground point in the system is therefore undesirable.

A more preferable way to decouple the electrochemical device from the mass spectrometer high voltage is to float the EC device on the ESI high voltage, either using a battery-operated potentiostat as in Paper IV, VI and VII and/or an isolation transformer as in Paper I, II, III, IV, V, VI, and VII. During the experimental work included in this thesis, an isolation transformer (type PVM 440 0019, Tufvassons, Sweden) was used to decouple the EC cell and the ESI high voltage, see Figure 13.

![Figure 13. Schematics of the experimental setup. [Figure reproduced by courtesy of Andreas Dahlin.]](image)

To decouple the EC cell from the ESI-MS high voltage and to increase the userfriendliness, the on-line EC/ESI-MS/MS experiments in Paper IV were performed using a Bluetooth battery-powered instrument with the PDMS chip floated at the potential induced by the ESI high voltage. This wireless Bluetooth setup was also used in Paper VI and Paper VII.

Since a battery-powered potentiostat was used in the experiments of Paper IV, VI, and VII, the use of an isolation transformer would not have been necessary. On the other hand, the recharging of the battery and its capacity were not satisfactory and the PalmSens potentiostat was therefore connected via the isolation transformer. Thanks to the Bluetooth wireless connected HP iPAQ Pocket PC, the EC instrument could be operated without risk for hazardous shocks when the EC flow cell and the PalmSens instrument were kept floating at the potential induced by the ESI high voltage [Paper IV, VI, and VII].
When EC was combined with LC and MS in Paper VII, see the experimental setup in Figure 14(a), two different software products were used for the PalmSens potentiostat (Figure 14(b)); PalmScan software for acquiring current as a function of potential, and PalmTime for acquiring amperograms, i.e. current as a function of time.

![Experimental setup](image)

(a)

![PalmSens potentiostat](image)

(b)

Figure 14. Experimental setup (a) LC/EC/ESI-MS (b) PalmSens potentiostat.

In Paper VII, the influence of an inserted ground point on the results was investigated. It was found that some oxidation products (corresponding to m/z 331, 361, 364, and 385) were seen in mass spectra although no oxidation was expected in the EC cell (when the applied potential was –0.2 V vs. Ag/AgCl). The results verify that oxidation did occur at the ground point between the EC cell and the negative ESI emitter, see Figure 15(a). The ground point can also interfere with EC/ESI-MS measurements in the positive ion mode, where the oxidation reactions in the emitter induce electrochemical reduction reactions at the ground point, see Figure 15(b). This fact may result in changes of the intensities for the oxidation products that were generated in the EC cell, for example as a result of a regeneration of the
original compound. Since the experimentally used thin-layer cell had a con-
version efficiency of 50%, it was difficult to see the intensity changes of the
original species the in mass spectra (positive ESI) and much easier to find
new peaks appearing in mass spectra as in the case with a ground point in
negative ESI [Paper VII].

Figure 15. Schematics of an EC cell coupled on-line to (a) negative ESI and (b)
positive ESI, with inserted ground points in between where EC reactions take place.
5.2 Influence of the solution composition in EC/ESI-MS

Addition of supporting electrolyte to the sample solution significantly increases the conductivity of the solution and, consequently, decreases the ohmic potential drop causing the electrode potentials to depart from the value imposed by the electrochemical instrument (as discussed in Chapter 2). In order to obtain a good electrochemical conversion in the EC cell, some kind of supporting electrolyte is added to increase the conductivity. Most electrochemical studies are carried out in the presence of a supporting electrolyte selected based on the solvent and electrode process of interest. For aqueous media, many acids, bases, and salts are available. For organic solvents, like acetonitrile, tetra-alkylammonium salts are often used. As is widely known, non-volatile salts are not desirable in ESI-MS to avoid chamber contamination or plugging of the sampling orifice. Phosphate, sulfate, or borate additives are therefore not suitable for ESI-MS. The solvent additives should not form ion pairs that are so strong that charge neutralisation reactions occur in the ESI process. Appropriate volatile solvent additives include ammonium acetate, ammonium formiate, acetic acid, formic acid, and ammonia. Some suitable solvents for ESI, that permit formation of ions in solution, are methanol, ethanol, propanol, isopropanol, acetonitrile, and water. The choice of solvent suitability also affects the nebulisation and desolvation processes. Water supports ion formation in solution, but the high surface tension makes the ion desorption more difficult than when organic modifiers are added to the sample solution.

The ESI process may be limited by the amount of excess charge, by the space on droplet surfaces, or by a combination of these two factors. These limitations will yield competition and ion suppression effects at high concentrations. Surface-active (hydrophobic, non-polar) analytes suppress the response of more polar (hydrophilic) analytes. At high concentrations, the signal is saturated due to limitations in the number of ions that can be produced in the ESI process or due to instrumental factors such as the transmission of ions from atmospheric pressure into the mass spectrometer. Regardless of the mechanism of saturation, it is important to consider the competition effects when working with high analyte or electrolyte concentrations. For sufficiently high concentrations, the response of the mass spectrometer will be independent of the analyte concentration. The ESI process and the ion transmission through (the mass analyser of) the mass spectrometer limit the sensitivity of ESI. The ion transmission is dependent of the type of mass analyser and varies non-linearly with the mass. For increasing \( m/z \) values, the ion transmission efficiency will decrease.

It is sometimes difficult to obtain enough conductivity in the thin-layer EC cell if one has to consider the demands from the ESI process. High concentration of salt in EC cell will yield good conductivity, but can introduce
problems with the ESI-MS, such as ion suppression and signal saturation. Extremely low conductivities will give very high ohmic drops as well as unstable Taylor cones. Therefore a compromise has to be made when determining the content of the sample solution, since good conductivity is desirable and ion suppression (in ESI process) and signal saturation (in the detector) is undesirable. Sometimes an addition of supporting electrolyte is disadvantageous with respect to the reactions in the EC cell [Paper II]. For example, acetate loss was studied during oxidation of a dinuclear manganese complex in the EC/ESI-MS setup. During off-line experiments 0.1 M tetrabutylammonium perchlorate was used, but this electrolyte does not fulfill the demands of the ESI processes. Adding ammonium acetate as a supporting electrolyte to increase the solution conductivity was obviously not suitable in this particular study [Paper II]. In the lack of appropriate supporting electrolytes, the conductivity was increased by enhancing the analyte concentration, to 50-100 μM and 1 mM of [{Mn}_2(bpmp)(μ-OAc)_2][ClO_4], in Paper II and Paper III, respectively. This can unfortunately introduce other problems such as signal saturation of the detector and detection in the non-linear range. With an increased sample concentration in the solution, the abundance of ESI created sample ions will increase until a plateau is reached. Generally, 10^{-5} M is the limit of concentration if one requests work in the dynamic range of the mass spectrometer. The actual linear and dynamic ranges are however dependent on the mass spectrometer, the specific analytes and presence of other charged species as electrolytes and buffers.

In Paper V, where Fe(CN)_6^{3-} was preconcentrated on a PPY coated electrode, the connection between EC-SPE and ESI-MS was not straightforward, since the electrospray was not stable and totally collapsed after the desorption step. This effect was especially prominent for longer extraction times and was ascribed to the sudden increase in the conductivity as a change in the salt content of the solution may affect the stability of the electrospray [Paper V]. Furthermore, a conductive sheath flow was introduced to stabilise the spray but analyte ion suppression was unfortunately observed instead as discussed in Paper V.

The salt content influenced the half-wave potentials of the antioxidant oxidation reactions in Paper VII. The E_p values found for the CV oxidation peaks and the E_{1/2} values, obtained from EC/ESI-MS experiments, differed since the conductivity of the solutions varied. Extra supporting electrolyte was used in the off-line CV experiments and it was found that the half-wave potentials were less positive compared to when lower amount of salt was used and the resistance was higher [Paper VII].

It is important to consider the pH dependence of the desired electrochemical reaction and the buffer capacity of the solution when choosing a suitable electrolyte. The result of the reaction can be modified if the pH of the solution is changed. For ESI-MS, solvent additives are also important in the controlling the pH, since protonation or deprotonation of the analyte in
solution affect the ion formation. Generally, basic species (e.g., amines) should be analysed at a low solution pH (pH < pKₐ) and detected in the positive mode, while acidic species are best analysed at high pH (pH > pKₐ) in the negative mode.²³²

The pH of the electrospray droplets may be considerably different from that of the bulk solution²³² and the pH of the solution will be more stable if a buffer with good buffer capacity is chosen. Sometimes this is not possible; as for example for Olsalazine in acetate buffer of pH 6.8 [Paper I] and a change in the local pH in the EC cell can lead to misleading mass spectra. Oxidation of water:

\[ 2 \text{H}_2\text{O} \leftrightarrow \text{O}_2 + 4 \text{H}^+ + 4 \text{e}^- \] (10)

in the ESI positive mode generates protons and can decrease the droplet pH.⁸⁶ Acidifying processes decrease the solution pH and have been found to cause the protein cytochrome \( c \) to unfold in the ion source resulting in the fact that mass spectra were shifted to higher charge states.¹⁹⁵

Further considerations of local pH have been done. When the 4-CA oxidation was studied with two different cell designs, it was found that the counter electrode reactions influenced the reactions at the working electrode and thereby the local pH [Paper VI]. The generation of the reduced dimer was favoured in the modified EC cell, i.e. where the counter and working electrodes were separated and the onset of water oxidation resulted in a decrease of local pH [Paper VI].

5.3 Transfer of solution from the electrochemical cell to ESI-MS

After the reactions in the EC cell, the solution is pumped through the transfer line to the ESI-MS. One therefore has to consider the transfer time between the EC cell and the ESI emitter. If the aim is to analyse unstable EC reaction products, the transfer time (and transfer line) should be as short as possible. The dead volume in the EC cell and between the EC cell and the ESI emitter should be as small as possible to enable detection of low concentrations of EC reaction products. On the other hand, one may desire a large transfer volume and time (4-480 s) when aiming at completed homogenous reactions.¹³⁷

Electrospray ionisation is usually performed at flow rates of 5-10 μl/min,⁴⁰ while LC/EC often involve flow rates of 1 ml/min or more. The conversion efficiency is defined as the percentage of reactant converted into the product in the EC cell.¹⁹ High conversion efficiencies are often desirable, but the efficiency depends of different factors, for example the flow rate.¹³⁷
Generally, the use of a lower flow rate will increase the conversion efficiency. So a decreased flow rate increases the ionisation degree of ESI. Unfortunately, a lower flow rate increases the transfer time of the species between the EC cell and the ESI emitter. To obtain a short transfer time, one can however use an on-chip device in front of the MS as was done in Paper IV, where it was found that the conversion efficiency of dopamine could be increased from about 10% to 30% by decreasing the flow rate from 1.0 μl/min to 0.5 μl/min.

The electrospray ionisation flow rate is limited due to nebulisation through charging of the sample solution by electrical fields. Using pneumatically assistance, ESI can be carried out at enhanced flow rates, due to better droplet-formation. With SCIEX mass spectrometers such interfaces, with pneumatically assisted ESI, are called IonSpray interfaces. The nebuliser gas was zero grade air (generated in house) or nitrogen. In some of the experimental work [Paper I and II], the IonSpray interface consisted of a fused silica spray capillary positioned inside a stainless steel capillary. The high voltage was applied to the stainless steel capillary and the necessary electrical contact to the liquid was due to a thin liquid film between the stainless steel capillary and ESI capillary. This approach yields a much lower electrochemical conversion efficiency in the ESI than for the electrochemical reactions inside the EC cell or when a metallic spray capillary is used for the high voltage connection. The oxidation of analytes in the electrospray ionisation process can hence be neglected – if the IonSpray interface is used in the experiments [Paper I, II, III, V, VI, and VII].

When the Turbo V source was used in Paper VII, the high flow rate of 200 μl/min minimised the conversion in negative ESI (electrochemical reduction) so that this did not influence the oxidation products generated in the thin-layer EC flow cell. When lower flow rates were used in combination with the split (6 μl/min, and 31 μl/min) and positive ESI, it was expected that the conversion efficiency of the stainless steel Turbo V source was higher [Paper VII]. But these expectations were not so easily verified, since the higher the flow rate was, the more amount of analyte entered the MS and thereby were the signals more intense in the mass spectra.

In Paper V, a split was introduced after the EC cell, to obtain a stable electrospray at a flow rate of 1.5 μl/min. The EC cell volume was about 8 μl, but at a flow rate of 20 μl/min band broadening was not a problem [Paper V].

In Paper VII, the influence of a split between LC and EC/ESI-MS was evaluated for quercetin and its glucosides using isocratic elution. It was found that the flow rate through the LC column (200 μl/min) was suitable for oxidative conversion in the EC cell. When the flow rate was reduced, by using a split after the LC column, flow rates of 31 μl/min or 6 μl/min were obtained and significant band broadening and tailing peaks (Figure 16) were seen [Paper VII]. These results indicated that the 200 μl/min flow rate was the most optimal one for the LC/EC/MS setup and that splitting was not
necessary with the novel method described in Paper VII. In addition, it was found that gradient elution was possible to use, as a complement to isocratic elution profiles, when complex mixtures were separated, oxidised and introduced into the ESI-MS system [Paper VII].

![Figure 16. Amperograms acquired at different flow rates (6, 31, 66, and 200 μl/min, see inset) when a split was introduced between the LC column and the EC cell of the on-line LC/EC/MS system.](image)

**Figure 16.** Amperograms acquired at different flow rates (6, 31, 66, and 200 μl/min, see inset) when a split was introduced between the LC column and the EC cell of the on-line LC/EC/MS system.

### 5.4 Aspects of the electrochemical cell design

A thin-layer electrochemical flow cell contains three electrodes: the working, auxiliary, and reference electrodes. The current is passed between the working and the auxiliary electrode while the potential of the working electrode is monitored relative to the reference electrode. In a thin-layer EC flow cell, the thin spacer gasket (e.g. 16 μm) defines the flow channel and the distance between the working electrode and the auxiliary electrode. When an EC cell is coupled to a mass spectrometer, for detection and identification of reaction products, there will always be a risk that the EC reactions at the auxiliary electrode gives rise to products that appear as undesired peaks in the mass spectra [Paper I]. In analogy with coulometric cells, in which the working and counter electrodes are separated, it is desirable to eliminate the influence of such interfering reactions also in EC/ESI-MS [Paper III and VI]. Another possibility is that species produced at the working electrode react with species generated at the auxiliary electrode. Modifications of the electrode positions can be employed to decrease these interferences. This was partly discussed by Deng and Van Berkel, who used a modified cell
in which the working electrode was positioned between the reference electrode and the outlet of the cell. In this way, species produced at the working electrode were prevented to react with species generated at the auxiliary electrode. However, there was still a risk that species generated at the auxiliary electrode gave rise to peaks in the mass spectra and thus were mistaken as reaction products from the working electrode. Gun et al.\textsuperscript{114} have discussed cell design for their on-line concentric EC cell, in which the auxiliary electrode and its products were separated from the flow reaching the mass spectrometer. This separated the EC reduced compounds of molybdenum complexes (at the working electrode) from the oxidised substances generated at the auxiliary electrode.\textsuperscript{114} A discussion about electrode modifications of thin-layer EC cells was published already by Hambitzer and co-workers\textsuperscript{251,252} who coupled the EC cell to thermospray ionisation mass spectrometry. The working electrode was composed of a small gold sheet and the auxiliary electrode was a gold wire. The compartments of these two electrodes (working and auxiliary) were separated by a porous glass frit preventing mixing of electrolyte solution. One of the benefits with this design was that products formed at the auxiliary electrode did not enter the working electrode compartment.\textsuperscript{251}

A thin-layer electrochemical flow cell from BAS (Bioanalytical Systems, Inc.), with a series dual electrode (glassy carbon or gold) embedded in PEEK block, together with an Ag/AgCl reference electrode and a stainless steel block as auxiliary electrode, constituted the conventional EC cell design [Paper I]. In order to separate the auxiliary electrode from the working electrode, the stainless steel block was used as a quasi reference electrode (pseudo reference electrode).\textsuperscript{19} A stainless steel auxiliary electrode rod was made to fit the reference electrode compartment of the flow cell as described in Paper I. In this way, the distance between the working electrode and the auxiliary electrode was increased and the influence of interfering EC reactions at the auxiliary electrode was minimised. This flow cell will further on be referred to as the modified EC cell design. For the schematics of the cell designs, see Figure 17.

The potential of the quasi reference electrode versus the Ag/AgCl reference electrode must be measured when reporting potentials with reference to the QRE.\textsuperscript{19} Under the present experimental conditions, in Paper I and III, the potential difference between the stainless steel QRE and the Ag/AgCl reference electrode was found to be less than 100 mV in the used solutions.

Differences between the results for the conventional and modified EC cells were seen for the oxidation of Olsalazine [Paper I]. A detailed study and comparison of the two EC cell designs can be found in Paper III, in which oxidation products of [Mn\textsubscript{2}(bpm)(\mu-OAc)\textsubscript{2}]\textsuperscript{+} were investigated with respect to their potential dependence for the two cell designs.
Significant differences were seen with respect to the peak intensities and number of peaks generated for Olsalazine oxidation in Paper I. Electrochemical reduction of water was proposed to occur at the auxiliary electrode resulting in a local pH increase for the conventional EC cell. The differences seen between the mass spectra generated with the two cell designs were probably dominated by this pH change. The buffer capacity of the acetate buffer system used was poor at pH 6.8 and the electrochemically behaviour of Olsalazine is known to be dependent on the pH.\cite{253} To minimise these problems, a modified thin-layer EC cell – in which the working electrode was separated from the auxiliary electrode – should be used. The oxidation reactions of Olsalazine are rather complex and it was therefore desirable to also investigate the changes in the mass spectra, due to EC cell design differences, using a more electrochemically well-defined species such as the $[\text{Mn}_2(\text{bpmp})(\mu-\text{OAc})_2]^+$ complex,\cite{254,257} also studied in Paper II see Figure 18. In this case, the Greek letter $\mu$ indicates that the oxo ligand is a bridging ligand between the two metal centres.\cite{258}

The manganese complex was well suited for the EC/ESI-MS studies, thanks to its several possible oxidation states and its relevance to artificial photosynthesis (i.e. creation of oxygen and hydrogen from solar energy and water).\cite{259,260}
Figure 18. The molecular structure of \([\text{Mn}_2(\text{bpmp})(\mu\text{-OAc})_2][\text{ClO}_4]\).

In the mass spectra, a singly charged Mn(II,II) ion gave rise to a peak at \(m/z\) 757 (with an isotopic peak at \(m/z\) 758, labelled 1a), see Figure 19, and a doubly charged Mn(II,II) ion at \(m/z\) 349 (labelled 2), after acetate loss from 1a, the \([\text{Mn}_2(\text{bpmp})(\mu\text{-OAc})_2]^+\) ion \([\text{Paper II and III}].\) Upon electrochemical oxidation, some additional products appeared in mass spectra at \(m/z\) 379 (labelled 1b), 358 (labelled 3), 328 (labelled 5), and 336 (labelled 6).

Figure 19. Scheme of the studied complexes discussed in Paper II and III.

It was found that the mass spectra obtained with the two EC cell designs differed significantly with respect to species 6, a Mn(III,IV) complex \([\text{Paper III}].\) A higher potential was needed for the generation of 6 with the conventional cell, most likely due to the presence of interfering reactions at the aux-
iliary electrode in the conventional setup. Such reactions can affect the potential dependences and intensities of the peaks. The electrochemical conversion efficiency was approximately 50% at a flow rate of 1.0 μl/min for the conventional EC cell [Paper III]. Homogenous redox reactions involving species 6 and unreacted 1a can occur in the solution for both EC cell designs, since the Mn(III,IV) complex is a strong oxidation agent and unreacted species exist in the sample solution.

To avoid problems with redox cycling (species generated at the working electrode undergoing a reverse reaction at the auxiliary electrode) and interfering reactions (due to auxiliary electrode generated species reacting with species generated at the working electrode) in solution, the auxiliary electrode should be positioned in a separate compartment of the EC cell.

The EC cell design was also considered in Paper V, in which a 120 μm thick Teflon™ spacer gasket was used. The stainless steel tube positioned at the inlet of the cell, was employed as the counter electrode, while the stainless steel block of the thin-layer cell was used as a QRE, the latter in analogy with the cell described in Paper I. This electrode setup was used to minimise unwanted possible interferences as a result of the EC reactions occurring at the counter electrode during desorption [Paper V].

The positioning of the gold coil electrodes in the PDMS chip of Paper IV was thoroughly considered prior to fabrication. The electrode coil used as the working electrode was placed approximately 5 mm from the electrospray emitter tip [Paper IV]. Just next to the WE coil was a four turn coil used as the quasi reference electrode. The counter electrode was positioned as far as possible from the emitter and was composed of nine turns of Au wire, in analogy with the WE coil. This arrangement of electrodes was made to minimise interferences due to band broadening between the WE and the emitter tip as well as due to the uncompensated ohmic drop between WE and QRE [Paper IV].

The EC cell design (Figure 17) was also addressed in Paper VI, where two different setups were further compared, based on the findings in Paper III. The presence of a comproportionation reaction was clearly seen by comparing the results obtained with the two thin-layer flow cells, both with conversion efficiencies of 50%, but of different design with respect to the influence of the counter electrode reaction on the reaction at the working electrode [Paper VI]. It was found that the formation of the reduced dimer was favoured by a decrease in the local pH in the modified flow cell, e.g. caused by the onset of water oxidation, equation (10), as discussed in chapter 5.2. In Paper VI, it was stated that EC/ESI-MS is a powerful tool for the study of reaction pathways and that these studies are best carried out with thin-layer flow cells with conversion efficiencies smaller than 100%.
6 Conclusions and future work

It has been shown that EC/ESI-MS facilitates analyses by producing or increasing the ESI-MS signals, and by enabling pre-concentration of analytes. EC/ESI-MS also has the ability to directly monitor reactants, short-lived intermediates, and the products of electrochemical reactions as a function of the electrode potential or current with $m/z$ specificity, as well as to provide structural information on reactants, intermediates and products.

Important conclusions drawn from the experimental work [Paper I-VII] are that EC cells can be coupled to ESI-MS without interferences from the ESI high voltage – if the EC cell is made to float at the potential induced by the presence of the ESI high voltage, e.g. through decoupling via an isolation transformer. The latter is much more favourable than the insertion of a ground point or decoupling using a long transfer line. To facilitate the detection of unstable products, the transfer time between the electrochemical cell and the ESI-MS should be as short as possible. This normally requires that the electrochemical cell is made to float on the potential induced by the ESI high voltage [Paper III].

EC/ESI-MS can be used for pre-concentration, desalting, and ionisation of neutral species and also enables studies of manganese complexes and their oxidation states. Thiol samples can be pre-concentrated as well as desalted, on the basis of the spontaneous formation of self-assembled monolayer on gold electrodes [Paper I]. The use of potential-controlled accumulation and oxidative desorption giving rise to negatively charged sulfonates or sulfinites holds great promise for the analysis of many biologically interesting molecules containing thiol (or disulfide) functionalities [Paper I].

When coupling EC cells to ESI-MS, the appearance of artifacts in the mass spectra – as a result of reactions between the reaction products formed at the working electrode and products formed at the auxiliary electrode – can be eliminated by proper design of the EC flow cell [Paper I]. It was shown that contradictory mass spectra [Paper I, III, VI] were obtained when coupling thin-layer cells of different design to ESI-MS. When conventional thin-layer cells (originally designed for electrochemical detection in liquid chromatography) are used, care should be taken to position the auxiliary electrode in a separate compartment [Paper I]. If this is not done, interferences due to the auxiliary electrode reaction may be introduced. These interferences can affect both the potential dependence and signal intensities of the peaks [Paper III, VI].
Additional homogenous redox reactions in the solution between the EC cell and the ESI-MS should also be considered in the absence of 100% conversion efficiency in the EC cell. In Paper III, it was found that the peak intensities of the electrochemically generated products were significantly lower with a conventional thin-layer cell compared to using a modified cell in which the auxiliary electrode had been positioned in a separate compartment. The interferences are likely to be most pronounced for products whose formation requires high oxidation (or reduction) potentials [Paper III].

By the combined use of EC, IR spectroscopy and ESI-MS, it was possible to monitor the loss of acetate ligands from [Mn₂(bpmp)(μ-OAc₂)]ⁿ⁺ in different oxidation states and to identify products of the ligand-exchange reactions [Paper II]. The Mn(II,II) and Mn(II,III) states were found to be less prone to acetate loss in solutions of low water content while the Mn(III,III) state releases acetate ligands even in solutions with low water content.

For a given oxidation state, the ligand-exchange reactions do not lower the overall charge compared to the intact complex, but the water-derived ligands that replace the acetate bridges are increasingly deprotonated in the higher oxidation states thereby facilitating further oxidation [Paper II]. The mechanism of the formation of the di-μ-oxo bridged Mn(III,IV) dimer, [Mn₂(bpmp)(μ-OAc₂)]⁺ seems to depend on the water content of the solution. [Mn₂(bpmp)(μ-OAc₂)]⁺ can be formed via ligand-exchange reactions starting from the intact Mn(III,III) complex [Mn₂(bpmp)(μ-OAc₂)]³⁺ if little water is present but also via ligand-exchange products of the lower oxidation states if high water content favours ligand-exchange in these states [Paper II].

EC/ESI-MS is a promising technique with many exciting possibilities for a wide range of compounds. A particularly interesting approach involves the possibility to miniaturise EC cells in the form of chip-based devices that readily can be interfaced to ESI-MS. This was successfully done in Paper IV, in which the electrochemical oxidation of the biologically interesting dopamine was evaluated using a PDMS chip with inherent gold electrodes and sheathless graphite ESI tip for short transfer times and reasonable conversion efficiencies.

In Paper V, a polypyrrole coated working electrode was used for EC-SPE. Anions were extracted (adsorbed) and desorbed and thereby pre-concentrated due to a large active area of the modified working electrode. The interfacing of EC-SPE to ESI-MS was not straightforward due to the large inherent change in the solution conductivity after desorption process. The combination of EC-SPE/ICP-MS was found to be less complicated, and it was possible to pre-concentrate and detect low levels of bromide anions from spiked tap water samples [Paper V].

It was interesting to couple liquid chromatography to the on-line EC/ESI-MS system in Paper VII for separation and detection of electroactive compounds. This LC/EC/ESI-MS/MS setup facilitated the investigation of anti-
oxidants in yellow onion extract, but can in the future be used for far more complex samples.

With appropriate high voltage decoupling, EC cell design and transfer time reduction, electrochemistry coupled to ESI-MS can be a very powerful and versatile tool to study electrochemical reactions involving pharmaceutical and/or biologically interesting compounds.

By either oxidation or reduction of water it is also possible to modify the local pH and thereby change the signal-to-noise ratio for many compounds. A further evaluation of alternative cell designs is likewise an important topic for future work.
I would like to express my sincere gratitude to past and present colleagues at Uppsala University. Especially, I really want to thank my supervisors and co-authors.

This thesis was completed by invaluable support from my excellent supervisors Leif Nyholm and Per Sjöberg – thank you for professional supervision, encouragement, enthusiasm, and for helping me understand electrochemistry and mass spectrometry! Thanks also for helpful and interesting discussions about science and research methodology, for experimental ideas and proofreading the manuscripts; for always believing in me and supporting me whenever needed. Thanks for accepting me as a Ph.D student – even though some potentiostats were crashed in the beginning of our collaboration…

Thanks to Fredrik Bökman for kind introduction to electrospray ionisation and for valuable collaboration. Thanks to Gerriet Eilers, Reiner Lomoth, and Leif Hammarström for rewarding cooperation with manganese complexes and for introducing me to the field of artificial photosynthesis. Thanks to Gustav Liljegren, Andreas Dahlin and Niklas Forsgard for collaborations regarding PDMS chip applications and polypyrrole extractions and thanks to co-authors Jean Pettersson, Malin Svedberg, Merja Herranen and Jonas Bergquist for rewarding publications. A special thank to Sandra Wende for great collaboration with the LC/EC/ESI-MS experiments and thanks to the co-authors Michelle Co and Charlotta Turner.

Thanks to the administrative, Barbro Nelson, and technical, Bo Fredriksson, Yngve Öst, and Erik Forsman, staff for helpful support of various kinds during the years.

A special thank to all the members of the Green Technology Group for scientific discussions and nice company.

A lot of thanks to Barbro Kollander for being such a nice and supporting room mate – you are the best!

Thanks to former PhD students for all kind help with troubleshooting and instrument assistance.
Thanks for the travel grants provided from *Swedish Chemical Society* (Svenska kemistsamfundets resestipendier), the *Foundation of Liljewalch* (Liljewalchs resestipendium, Uppsala universitet) and the *Foundation of Knut and Alice Wallenberg* (Rektors resebidrag från Wallenbergstiftelsen, Uppsala universitet).

I want to thank Sara Lind (Bergström), Heidi De Brabandere, and Roland Pettersson for great ideas and joint effort in the work with the Alumn Society for Analytical Chemists.

Special thanks to Eva Magnusson and Labbret’s kennel.

Tack vare möjligheten till hundägande, och allt skoj på fritiden, har avhandlingsarbetet flutit på!

I would like to thank my friends for really nice company and for sharing unforgettable memories.

Kram på er allihopa (ingen nämnd – ingen glömd...) och tack för att det finns ett innehållsrikt liv även utanför labbet och skrivrummet på BMC!

At last, I want to thank my family for endless support, encouragement, and motivation. The PhD work would have been much harder without your believe in me.

Stort tack till Bosse & Gunilla, Gunnar och Monica samt tack till mina föräldrar Marita & Björn och min syster Cecilia för att ni alltid finns där för mig när det behövs! Tack för stöttningen jag fick, i och med mina funderingar, att söka till forskarutbildningen. Tack allihopa för att ni tar så väl hand om Atlas när hundvakt har krävts.

Ovärderligt tack till min älskade Lars för allt vad du gör för mig!

Camilla Zettersten
Uppsala, 2009-03-12
8 Summary in Swedish

Elektrokemi kopplat till elektrosprayjoniserings-masspektrometri för metodutveckling och tillämpningar

8.1 Användningsområden och begränsningar med EC/ESI-MS

Sedan den första publikationen år 1991 har intresset för sammankopplingen av elektrokemi (EC) och elektrosprayjoniserings-masspektrometri (ESI-MS) ökat markant det senaste decenniet. En av anledningarna är att EC/ESI-MS har många användningsområden. Man kan studera redoxreaktioner i biologiska system, t.ex hur läkemedel bryts ned (metaboliseras) i människokroppen. Trots att många oxidationsreaktioner är enzymatiska så kan man efterlikna dessa reaktioner och därmed öka förståelsen för metabolismen. Genom att haka på specifika kemiska föreningar, vilka binder till en viss känd aminosyra, kan identifieringen av dessa proteiner och peptider förenklas.

EC/ESI-MS kan även göra så att oladdade föreningar laddas (joniseras) eller derivatiseras; för det första för att ens kunna analyseras med MS och för det andra för att signalintensiteterna i masspektra därmed ökas, vilket kan förbättra signal/brus-förhållandet. Med EC/ESI-MS har man även möjlighet att uppkoncentrera och separera analyter med låga halter. Genom att använda EC/ESI-MS kan man direkt detektera reaktanter, korrivåde intermediat och reaktionsprodukter från elektrokemiska processer samt även erhålla information om reduktions- och/eller oxidationspotentialer, genererad ström som funktion av tiden (amperogram), och förhållandet mellan massa och laddning (m/z). Dessutom kan information om molekylär struktur hos reaktanter och produkter erhållas.

En annan anledning till att EC/MS ökat i popularitet är att tekniker för joniserings vid atmosfärstryck (API) utvecklats de senaste decennierna. Elektrosprayjoniserings, en av flera API-tekniker, överför joner från lösningen till gasfasen tack vare den höga pålagda spänningen (3-5 kV). Dessa gasfajskjoner överför sedan till vakuum i masspektrometern. Både små organiska molekyler, mellanstora metallkomplex och stora proteiner är möjliga att elektrosprayjonisera utan att de fragmenteras i någon större utsträckning. Dessutom är olika separationstekniker, såsom vätskechromatografi (LC) och kapillärelektrofores (CE), möjliga att koppla samman med ESI-MS. Med EC/ESI-MS kan man alltså generera oxidations- och reduktionsprodukter för att sedan detektera dem, och på det viset studera reaktionsmekanism. Vidare kan även LC kopplas samman till EC/ESI-MS. Om man använder
LC/EC/ESI-MS så är det möjligt att separera komplexa sammansättningar, ta reda på när elektroaktiva komponenter passerar EC-cellens för att sedan introduceras i masspektrometern, där retentionstiden avgör vad som är intressant att studera för att ta reda på strukturerna hos de olika komponenterna i blandningen. Om man istället vill studera vilka oxidationsprodukter som skapas, så är EC/LC/ESI-MS att föredra eftersom EC-cellens genererar oxidationsprodukter vilka separeras i LC-kolonnen och sedan dyker upp i massspektra vid olika retentionstider. De m/z, som kommer vid samma retentionstid, härstämmer troligen från en och samma oxidationsprodukt (förutsatt att kromatografin är bra).

Jämfört med off-line EC är on-line EC/ESI-MS fördelaktigt genom att endast begränsad provvolym krävs vid den senare mätningen. En annan orsak till att intresset för EC/ESI-MS ökat är möjligheterna till provupprätting (avsaltning och uppkoncentrering), samt analytseparation och förminsning (miniatyrisering) ledande till chip-baserade elektrokemiska plattformar för biologiskt intressanta föreningar utan förlust av prestanda.

8.2 Metodutveckling av EC/MS-sammankopplingen och design av EC-celler

Sammankopplingen av EC och MS är å andra sidan inte helt enkel, eftersom vanliga, kommersiellt tillgängliga, EC-celler (med tre elektroder) används och kopplas till ESI-MS ibland utan adekvat optimering. Högspänningen hos ESI kan vara skadlig för EC-instrumenteringen. Syftet med den här avhandlingen var därför att vidareutveckla sammankopplingen mellan EC och MS samt att diskutera vilka problem som kan uppstå, hur man skall undvika dessa, vilka faktorer som bör beaktas och vilka kompromisser som kan krävas.

För ESI krävs vanligtvis lättflyktiga provlösningar och låg koncentration för analyt(er) och elektrolyt(er) önskas. Vidare kan en hög halt av organisk modifierare (exempelvis metanol och acetonitril) behövas, eftersom det är svårt att elektrospraya rena vattenlösningar på grund av ytspänningen hos vattendropparna. Dessa önskemål angående lösningens sammansättning är inte vanliga för off-line EC (t ex cyklisk voltammetri), där god konduktivitet och därmed hög halt av ledelektrolyt önskas. Dessutom är dessa ledelektrolyter sällan lättflyktiga.


Vidare användes den modifierade EC-cellen för att skapa laddning på (jonisera) den oladdade 1-hexantiolmolekylen, för att kunna introduceras med elektroprayjonisering i masspektrometern. Genom att självmant adsorbera tiol på en arbetselektrod av guld och sedan använda en kontrollerad potential så kommer EC-oxidationen leda till att tiolen desorberas från elektrodytan och därmed vara laddad (som 1-hexylsulfinat och 1-hexylsulfonat i artikel I). Förutom den elektrokemiska jonisingen, kan även metoden användas för uppkoncentrering av prover med låg halt eller för avsaltning och matrisutbyte till lösningar som passar ESI-MS bättre.

I artikel II användes den utvecklade metoden för att studera hur mangankomplex beter sig rent strukturmässigt. Bland annat undersöktes utbytet av ligander hos ett dinukleärt mangankomplex under elektrokemisk oxidation vid närvaro och frånvaro av vatten. Genom att använda EC/ESI-MS kunde olika produkter med flera olika oxidationstillstånd, Mn(II,II), Mn(II,III), Mn(III,III), Mn(III,IV), genereras och studeras. För första gången kunde man identifiera och förklara uppkomsten av Mn(III,IV), vilken förbryllat vid tidigare studier. Dessutom studerades hur de olika oxidationstillstånden påverkades av mer eller mindre vatten i lösningen. Eftersom flera av dessa mangankomplex är reaktiva är det en fördel med ett system där transportti-
den mellan generering i EC-cellen och detektering i ESI-masspektrometern är så kort som möjligt så att ytterligare följdreaktioner minimeras.

Eftersom kunskapen om hur Olsalazine oxiderades (artikel I) är bristfällig och informationen om hur mangankomplexet oxiderades har utökats (artikel II), ville man i artikel III använda den senare molekylen (Mn-komplexet) för att ytterligare studera hur elektodpositioneringen i EC-cellen inverkar på MS-resultatet. Det visade sig att både fördelningen och intensiteterna hos topparna i masspektrat beror på vilken EC-cell (den konventionella eller den modifierade) som används. Denna skillnad förklaras av att minimera risk för redox-cykling och interfererande reaktorer med motelektrodens produkter för den modifierade EC-cellen. Därför är det viktigt att beakta cell-designen när EC sammankopplas med ESI-MS.


Även i artikel V användes en elektrokemisk cell med modifierade elektrodepositioner tillsammans med masspektrometern. Den här gången ville vi titta närmare på provupparbetning och uppkoncentrering. Som modellsubstans användes den i elektrokemi vanligt förekommande kaliumhexacyanoferrat med oxidationstillstådet Fe(III). För att bättre klara av uppkoncentreringen användes en arbetselectrodel belagd med en ledande polymer, polypyrrol. Denna polymer gör att den aktiva ytan förstoras och att anioner, som K₂Fe(CN)₆⁻ och Br⁻, kan anrikas genom elektrokemiskt kontrollerad fastfaseextraktion (EC-SPE). Denna anrikning av anioner styrdes av EC-potentialen, så att extraktion (vid +0.8 V) och desorption (vid –0.8 V) åstadkoms genom att polypyrrol oxiderades eller reducerades. Däremot visade det sig att sammankopplingen av EC-SPE och ESI-MS försvårades av att konduktiviteten förändrades drastiskt och elektrosprayen därför kollapsade efter en uppkoncentrering (extrahering och desorbering). Av den orsaken användes istället inductoriskt kopplat plasma (ICP) som introduceringsteknik till masspektrometern. För att utvärdera användningsområdet hos EC-SPE/ICP-MS analysera-
des vanligt kranvatten, som spikats med olika koncentrationer av bromid. Det visade sig att 3μM bromid kunde detekteras i närvaron av högre koncentrationer av snarlika specier.

I artikel VI gjordes ytterligare ett försök att studera inverkan av EC-cellar design och om elektrodreaktionerna kan påverka lösningens pH. För detta användes, den av andra forskare väl studerade, molekylens 4-kloroanilin. Genom att använda två EC-celler och jämföra de erhållna masspektra, var det tydligt att nya reaktionsintermediat kunde detekteras. Helt oväntat visade det sig att oxidationen av 4-kloroanilin gav upphov till både en oxiderad och en reducerad dimer. Uppkomsten av denna reducerade dimer var förbryllande, men kunde förklaras av en hittills okänd komproportioneringsreaktion mellan ursprungssubstansen 4-kloroanilin och den oxiderade dimeren. Liknande komproportioneringsreaktioner kan tänkas äga rum även under bildningen av ledande polymerer som polyanilin och polypyrrol.

Vidare visade det sig att den reducerade dimeren i större utsträckning skapades med den modifierade EC-cell. Det förklaras av att vattenoxida-
tion på arbetselectroden ledde till ett lägre lokalt pH. Effekten av pH-
sänkningen var större i den modifierade EC-cell. Det beror på att den bal-
anserande vattenreduktionen på motelektroden då har förflyttats från arbeits-
elektrodens direkta närhet.

I artikel VII kopplades en kromatografisk kolom samman med EC/ESI-
MS. Genom att använda LC/EC/ESI-MS/MS kan blandningar, såsom extrakt av gul lök, separeras i kolonnen och sedan kan varje provplugg (förhopp-
ningsvis innehållande bara en komponent) utsättas för oxidation i den modi-
fierade EC-cell och sedan fragmenteras och detekteras i masspektrome-
tern. Då kan informationen vid vilken retentionstid något elektrokemiskt passerar EC-cellen (amperogram) relateras till vilken tid detta intressanta dyker upp i masspektra. Med hjälp av fragmentering (MS/MS) kan sedan de olika elektroaktiva komponenterna strukturutredas. Resultaten visade att den utvecklade LC/EC/ESI-MS/MS-metoden kan användas som en teknik för att ta reda på om och vilka antioxidanter som finns i verkliga pröver. Detta är en stor fördel jämfört med de tidigare använda kemiska metoderna för att ta reda på den totala kapaciteten hos antioxidanterna. LC/EC/ESI-MS/MS är snabbare och kan dessutom ge information på molekylär nivå.

8.3 Slutsatser
I den här avhandlingen har möjligheter och begränsningar med samman-
kopplingen av EC/ESI-MS påvisats och diskuterats. Att använda en isola-
tionstransformator för att flyta EC-instrumenteringen på ESI-högspänningen
är mycket mer fördelaktigt än att använda långa transportlinor eller införa jordpunkter i systemet. Vidare är det behövligt att handha en batteriupps-
backad potentiosatt och en handdator, vilka kommunicerar via trådlös Blå-
tandsuppkoppling.
9 References


33. Schieffer, G. W. *Dual coulometric-amperometric cells for increasing the selectivity of electrochemical detection in high-performance liquid*


50. Miller, P. E.; Denton, M. B. *The quadrupole mass filter: basic operating
69. Bruins, A. P. Mechanistic aspects of electrospray ionization,


87. Van Berkel, G. J. Electrolytic deposition of metals on to the high-voltage contact in an electrospray emitter: implications for gas-phase ion formation,


96. Van Berkel, G. J.; Kertesz, V. Expanded electrochemical capabilities of the electrospray ion source using porous flow-through electrodes as the upstream ground and emitter high-voltage contact, Anal. Chem., 2005, 77, 8041-8049.


102. Volk, K. J.; Lee, M. S.; Yost, R. A.; Brajter-Toth, A. Characterization of solution-phase and gas-phase reactions in on-line electrochemistry-


140. Mautjana, N. A.; Estes, J.; Eyler, J. R.; Brajter-Toth, A. Antioxidant pathways and one-electron oxidation of dopamine and cysteine in electrospray and on-line electrochemistry electrospray ionization mass spectrometry,


simulation of consecutive reactions in a microchannel,

155. Dayon, L.; Roussel, C.; Girault, H. H. Probing cysteine reactivity in proteins
by mass spectrometric EC-tagging, J. Proteome Research., 2006, 5, 793-800.

156. Rohner, T. C.; Girault, H. H. Study of peptide on-line complexation with
transition-metal ions generated from sacrificial electrodes in thin-chip

157. Dayon, L.; Girault, H. H. Diagonal chromatographic selection of cysteinyl
peptides modified with benzoquinones, Anal. Bioanal. Chem., 2007, 389, 841-
849.

158. Prudent, M.; Rossier, J. S.; Lion, N.; Girault, H. H. Microfabricated dual
sprayer for on-line mass tagging of phosphopeptides, Anal. Chem., 2008, 80,
2531-2538.

159. Prudent, M.; Girault, H. H. On-line electrogeneration of copper-peptide
complexes in microspray mass spectrometry, J. Am. Soc. Mass Spectrom.,
2008, 19, 560-568.

160. Prudent, M.; Méndez, M. A.; Girault, H. H. Biphasic electrospray ionization
for the study of interfacial complexes, Analytical Sciences, 2008, 24, 1399-
1404.

161. Méndez, M. A.; Prudent, M.; Su, B.; Girault, H. H. Peptide-phospholipid
complex formation at liquid-liquid interfaces, Anal. Chem., 2008, 80, 9499-
9507.

162. McClintock, C.; Kertesz, V.; Hettich, R. L. Development of an
electrochemical oxidation method for probing higher order protein structure

163. Lazar, I. M.; Grym, J.; Foret, F. Microfabricated devices: a new sample
introduction approach to mass spectrometry, Mass Spectrom. Rev., 2006, 25,
573-594.

164. Nyholm, L. Electrochemical techniques for lab-on-a-chip applications,
Analyst, 2005, 130, 599-605.

165. Lion, N.; Reymond, F.; Girault, H. H.; Rossier, J. S. Why the move to

166. Manz, A.; Graber, N.; Widmer, H. M. Miniaturized total chemical analysis
systems: a novel concept for chemical sensing, Sens. Actuators B, 1990, 1,
244-248.


168. de Mello, A. J. Chip-MS: Coupling the large with the small, Lab Chip, 2001,
1, 7N-12N.

spectrometric detection for capillary zone electrophoresis, Anal. Chem., 1987,
59, 1230-1232.

170. Smith, R. D.; Barniaga, C. J.; Udseth, H. R. Improved electrospray ionization
interface for capillary zone electrophoresis-mass spectrometry, Anal. Chem.,


197. Van Berkel, G. J.; Asano, K. G.; Granger, M. C. *Controlling analyte electrochemistry in an electrospray ion source with a three-electrode emitter*.


217. Apak, R.; Güclü, K.; Demirata, B.; Özyürek, M.; Celik, S. E.; Bektasogu, B.; Berker, K. I.; Özyurt, D. Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay, Molecules, 2007, 12, 1496-1547.


225. Chen, H.; Zhang, Y.; Mutlib, A. E.; Zhong, M. Application of on-line electrochemical derivatization coupled with high-performance liquid chromatography electrospray ionization mass spectrometry for detection and


239. Bonfiglio, R.; King, R. C.; Olah, T. V.; Merkle, K. The effects of sample


254. Huang, P.; Magnuson, A.; Lomoth, R.; Abrahamsson, M.; Tamm, M.; Sun, L.;


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations
from the Faculty of Science and Technology 622

Editor: The Dean of the Faculty of Science and Technology

A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology”.)