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Electrochemistry on-line with mass spectrometry

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Chapter 1

Introduction

1.1 Electrochemistry coupled on–line with mass spectrometry: An historic overview

Studies of on–line coupling of electrochemistry with mass spectrometry date back to the work by Bruckstein et al. in the early 1970s.¹ In this and other early experiments, porous working electrodes or permeable membranes were used for the measurement of volatile products of electrochemical reactions by means of electron ionization (EI) mass spectrometry.

In 1986, Hambitzer et al. studied the redox reactions of *N,N*-dimethyl-aniline in an electrochemical cell on–line with thermospray mass spectrometry (TSP/MS).² In the same year Getek used electrochemistry on–line with TSP/MS to study the oxidation of acetaminophen.³

In 1989, Volk, Yost and Brajter–Toth used the ESA electrochemical cell on–line with TSP/MS/MS to study the electrochemical oxidation of uric acid.⁴ A couple of years later, the same group used a modified version of this setup in the study of 6–thiopurine.⁵ An HPLC–column was introduced between the electrochemical cell and the TSP/MS to enable separation of the electrochemical oxidation products prior to the TSP/MS. The early developments in electrochemistry on–line with mass spectrometry in the field of drug metabolism have been reviewed by Volk et al.⁶

In 1992, Van Berkel and co–workers showed that radical cations observed for various organic compounds in electrospray ionization mass spectrometry (ESI/MS) originate from electrochemical oxidation in the electrospray source.⁷ A couple of years later, the same group presented a number of different setups for on–line coupling of electrochemistry and electrospray mass spectrometry (ESI/MS).^{8,9} They also described how the electrospray source can be characterized as a controlled current electrolytic cell.^{10,11}

In 1995, Bond et al. showed how a simple flow cell constructed from two lengths of platinum microtubing could be used on–line with ESI/MS in the analysis of copper, nickel and cobalt diethyldithiocarbamates.¹²

In 1996, Baczynskyj used the ESA Coulochem cell coupled on-line with LC/ESI/MS to study the electrochemical oxidation of Delavirdine and other organic compounds.¹³ The same year, Cole and co-workers designed an on-line EC/ESI/MS probe to minimize the time between the electrochemical reaction and the mass spectrometer.^{14,15}

In 1997, Zhou, Hefta and Lee presented a modified version of the source used in 1995 by Zhou and Van Berkel. Electrochemical modifications of 21 phenylthiohydantoin amino acid derivatives in this setup enabled their detection in 50–1000 nM concentrations at low flow rates.¹⁶ The same year, Regino and Brajter-Toth connected a home-made electrochemical cell on-line with TSP/MS and particle beam mass spectrometry (PB/MS) to study the electrochemical oxidation of uric acid and dopamine.^{17,18} The same group also studied the effects of the mobile phase composition on the electrochemical cell conversion efficiency.¹⁹ A couple of years later, the EC/PB/MS-setup was also used for the characterization of intermediate radical cations in non-aqueous media.²⁰ Also in 1997, Stassen and Hambitzer used a thin layer electrochemical cell on-line with TSP/MS to study the electrochemical oxidation of aniline and a number of N-alkyl-anilines.^{21,22} A thorough description of EC/TSP/MS instrumentation was presented a year later.²³

In 1999, Jurva, Wikström and Bruins used the ESA Coulochem cell on-line with ESI/MS to mimic a number of metabolic oxidation reactions performed by cytochrome P450.²⁴⁻²⁶ The same year Iwashi used the ESA Coulochem cell on-line with HPLC/UV/ESI/MS to study the electrochemical oxidation of 3-hydroxyanthranilic acid.²⁷ Also in 1999, Deng and Van Berkel studied the electrochemical oxidation of dopamine in a home made thin layer electrochemical flow cell coupled on-line with ESI/MS.²⁸ The same type of electrochemical cell was also used in a different setup for electrochemically modulated pre-concentration and matrix elimination of tamoxifen and other organic analytes.²⁹

Another interesting approach to EC/ESI/MS was demonstrated by the group of Van Berkel in 2000 when they coupled electrochemically modulated liquid chromatography (EMLC) on-line with ESI/MS.³⁰ This setup allowed the electrochemical oxidation of aniline (on the column) and subsequent separation of the oxidation products prior to the ESI/MS.

In 2001, Diehl, Liesener and Karst used HPLC with post-column electrochemical treatment on-line with ESI/MS for signal enhancement of ferrocenecarboxylic acid esters of various alcohols and phenols.^{31,32} This technique has also been applied in the analysis of gasoline, diesel and mineral oil samples.³³ Hayen and Karst also used the same type of setup for the oxidation of phenothiazine and its derivatives into radical cations or sulfoxides that resulted in lower detection limits.³⁴ Brown, Rollag, Lin and Lee used a similar setup where electrochemical oxidation enabled the detection of a number of compounds that are otherwise difficult to detect by ESI/MS.³⁵ A home made

electrochemical cell was used on-line with electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR/MS) by Zhang, Pali, Eyer and Brajter-Toth for electrochemically enhanced ionization of a number of compounds including triphenylamine and polycyclic aromatic hydrocarbons.^{36,37} Also in 2001, the group of Amster used a home-made electrochemical cell on-line with ESI/MS to study the electrochemical reduction of a number of different metalloproteins.³⁸

Over the last couple of years, the number of publications in the field of EC/ESI/MS has increased. The ease of use and the many advantages that can be gained from adding an electrochemical cell in front of the ESI/MS will probably lead to an extended use in the future. In addition to the obvious use for characterization of oxidation/reduction products and short lived intermediates in various electrochemical reactions, there are several other areas of interest for EC/ESI/MS.

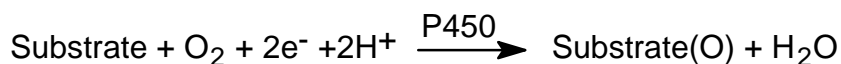
The on-line electrochemical generation of drug metabolites, on which large parts of this thesis are focused, is one field that is expected to find an extended use in the future.

Signal enhancement through electrochemical oxidation of neutral molecules or by addition of electrochemically active tags, such as ferrocene, are areas with great potential to lower detection limits in the analysis of a large range of organic compounds.

Because electrochemical equipment is inexpensive, easy to use and already available in many laboratories, we expect a continued growth for EC/ESI/MS in all of the above mentioned areas in the future.

1.2 Cytochrome P450

The cytochrome P450 enzymes comprise a large family of heme containing proteins with a cysteine in the active site that acts as an axial ligand to the heme iron. Over 500 forms of cytochrome P450 have been found in different forms of life, including plants, bacteria and mammals.³⁹⁻⁴² All enzymes in the P450 family are monooxygenases i.e. they catalyze the incorporation of one oxygen atom of dioxygen into substrate while the other oxygen atom is reduced by two electrons to give water. NADPH usually provides the two electrons needed for this process. The overall reaction is presented below.



There is a great diversity in the reactivity among different P450 enzymes and a large range of transformations is catalyzed. The most important and commonly encountered

cytochrome P450 catalyzed oxidations are hydroxylations, epoxidations, heteroatom oxidations/dealkylations, dehalogenations and alcohol oxidations.^{43,44}

The reactive site of all P450 enzymes contains an iron protoporphyrin IX (Figure 1.1) with cysteine as the fifth ligand, leaving the sixth coordination site to bind and activate molecular oxygen.

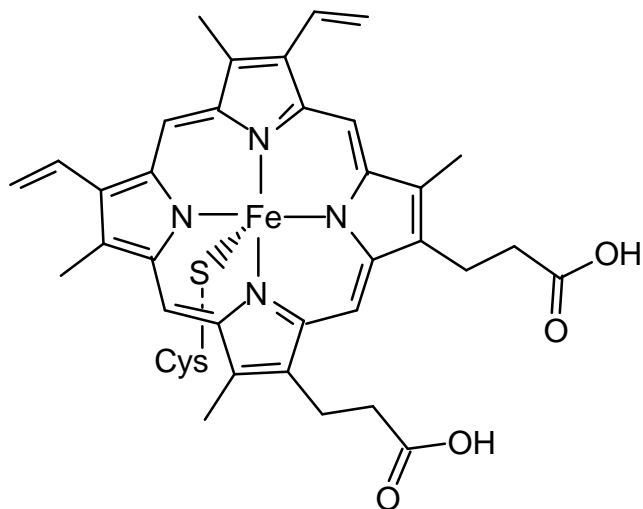


Figure 1.1 Iron protoporphyrin IX with cysteine as the sixth ligand

The catalytic cycle of cytochrome P450 is given in Figure 1.2. The resting form of the enzyme is a six coordinate (low spin) ferric state (**1**) with water as the sixth ligand. The catalytic cycle is initiated as the substrate (R-H) enters the active site and the water molecule is displaced, generating a five coordinate (high spin) ferric state (**2**). The next step is a reduction of the iron(III) center to a ferrous state (**3**). Dioxygen then reacts with **3** to form an oxyferrous complex (**4a,b**). One electron reduction of (**4**), the rate limiting step in all cytochrome P450 catalyzed reactions, is supposed to give a negatively charged iron(III)–peroxo complex (**5**) which upon protonation yields a hydroperoxide complex (**6**). A second protonation followed by heterolytic cleavage of the O–O bond generates water and an electrophilic, high valent iron–oxo species (**7**) generally considered to be the active oxidant in most cytochrome P450 catalyzed oxidations.

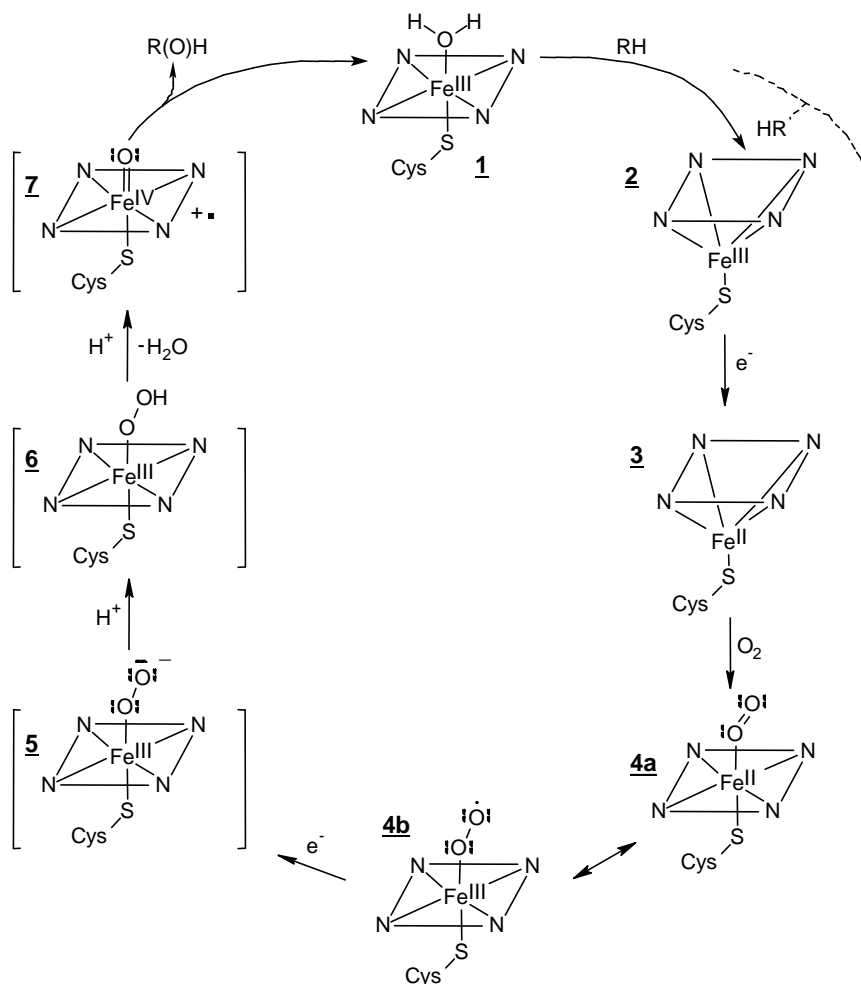


Figure 2.2 The catalytic cycle of cytochrome P450

The first four P450 states in the catalytic cycle are stable enough to allow isolation and characterization by different spectroscopic techniques. Crystal structures have been determined for states 1–4 of bacterial P450cam.⁴⁵ The reactions following the reduction of 4 are very rapid and the structures within brackets in Figure 1.2 have not yet been fully characterized. A crystal structure presumed to correspond to the oxo–ferryl porphyrin radical intermediate 7 has been presented but the crystal structure might also in part be made up from the hydroperoxo–iron species 6.⁴⁵ The debate about what is the actual “reactive oxygen” form of cytochrome P450 is not yet resolved. Newcomb et al. have presented data that suggest that the hydroperoxo–iron intermediate 6 can act as an electrophile and be the actual oxygenating intermediate for many oxidations.⁴⁶ In some P450 enzymes protonation of the hydroperoxo–iron species 5 may be inhibited in the

active site. In such enzymes the peroxy–iron intermediate has an extended lifetime and can take part in oxidations initiated by nucleophilic attack. Examples where such a mechanism is believed to take place are the oxidation of cyclohexane carboxaldehyde catalyzed by P450 2B4 and the conversion of human androgens to estrogens by P450 aromatase.⁴⁴ The oxoferryl porphyrin radical **7** is the most electrophilic species in the catalytic cycle and believed by many to be the reactive species in most P450 catalyzed oxidations. Heterolytic cleavage of the hydroperoxy–iron species **6** would generate a formal $\text{Fe}^{\text{V}}=\text{O}$ species, but extraction of an electron from the porphyrin moiety would give the more stable oxoferryl porphyrin radical **7**. Some possible resonance forms of **7** are presented in Figure 1.3.³⁹ In the discussion about oxygenation mechanisms of different substrates in chapter 2, this species is simplified as $[\text{Fe}=\text{O}]^{3+}$ and is presumed to be the active intermediate in all mechanisms presented.

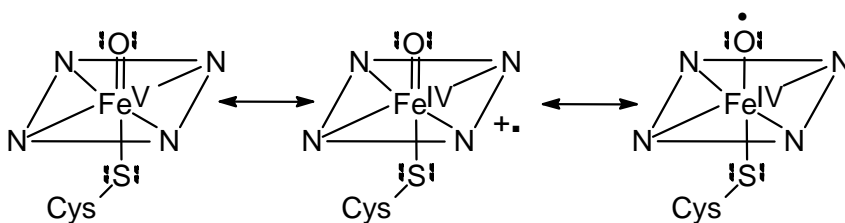


Figure 1.3 Resonance forms of the electrophilic, high valent iron–oxo species, believed by many to be the active intermediate in most cytochrome P450 catalyzed oxidations.

1.3 Reactive oxygen species (ROS)

The importance of reactive oxygen species (ROS) in health and disease has been recognized by every branch of medicine and biological science. Overwhelming evidence indicates that ROS play a role in many major health problems and that inhibition of oxidative damage to molecules, cells, and tissues prevent chronic and degenerative diseases.⁴⁷ Some examples of the many disorders that have been associated with oxidative stress are Parkinson's disease, Alzheimer's disease, diabetes, atherosclerosis, HIV and aging.⁴⁸

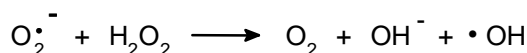
Many ROS present in biological systems have the potential to induce damage. These include hydrogen peroxide (H_2O_2), organic hydroperoxides, hypochlorous acid (HOCl), nitric oxide ($\text{NO}\cdot$), peroxyxynitrite (ONOO^-), superoxide ($\text{O}_2^{\cdot-}$) alkoxy radicals and the hydroxyl radical ($\cdot\text{OH}$).⁴⁹

There are many endogenous sources for ROS including⁵⁰i) normal aerobic respiration, i.e. reduction of O_2 by mitochondria that ultimately results in H_2O_2 production;

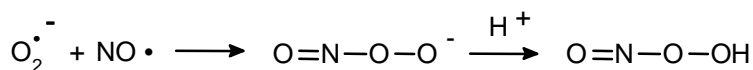
ii) stimulated polymorphonuclear leukocytes and macrophages releasing superoxide, supposedly to protect the body from bacteria;⁵¹ iii) peroxisomes, organelles responsible for degrading fatty acids and other molecules that produce hydrogen peroxide as a by-product; iv) induction of cytochrome P450 enzymes resulting in oxidant by-products.⁵² One example of an enzyme that is very active in the formation of ROS is CYP2E1, a cytochrome P450 isoenzyme that can be induced by chronic alcohol consumption.⁵³ There is also a large range of exogenous sources for reactive oxygen species *in vivo* such as tobacco smoke, ionizing radiation, pollutants, organic solvents, anesthetics, hyperoxic environment and pesticides.

The most reactive species of the ROS is the hydroxyl radical on which this brief introduction will be focused. Hydroxyl radicals are produced in living organisms in various ways, often as a result of degradation of other reactive oxygen species. Some *in vivo* sources of hydroxyl radicals are given below.

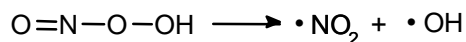
1) The Haber Weiss reaction.⁵⁴



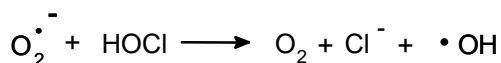
2) Homolysis of peroxyntirite⁵⁰ which is generated *in vivo* from the reaction between $\text{O}_2^{\bullet -}$ and $\text{NO}\cdot$.



The protonated product is unstable and is cleaved homolytically.

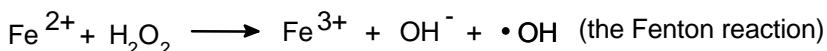
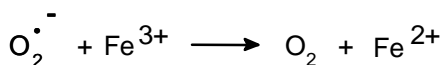


3) Reaction between $\text{O}_2^{\bullet -}$ and HOCl .⁵⁵

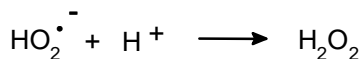
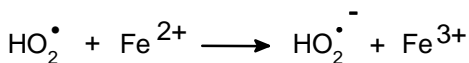
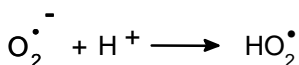


The most important source of hydroxyl radicals in a cellular environment is probably the Haber–Weiss reaction. The overall reaction presented above is somewhat more complex

and a more thorough description of the iron catalyzed Haber–Weiss reaction is presented below.⁴⁹



Superoxide is produced *in vivo* through reduction of molecular oxygen by various processes as discussed earlier. Hydrogen peroxide is essential for the last step of the Haber–Weiss reaction, the Fenton reaction. A number of enzymes including amino acid oxidase, amine oxidase and glucose oxidase generate hydrogen peroxide directly.⁵⁶ Other enzymes such as xanthine oxidase, aldehyde oxygenase and NADPH oxygenase generate superoxide that can be converted to hydrogen peroxide as follows.⁵⁷



A transition metal ion in its lower valence state is needed for the last step in the Haber Weiss reaction, the Fenton reaction. A number of ions, such as Fe^{2+} , Cu^+ , Ti^{3+} , Cr^{2+} and Co^{2+} , can donate an electron to hydrogen peroxide.⁵⁵ *In vivo*, only iron and copper ions are present in high enough concentrations to play a major role in the generation of hydroxyl radicals. Iron is the most abundant transition metal *in vivo*, and thereby biologically most relevant. In the mammalian cell nucleus, however, the concentration of Cu^{2+} is high and, therefore, copper is believed to be largely responsible for oxidative damage to DNA and membranes in the nucleus.⁵⁸ The location of transition metal ions *in vivo* is often a crucial parameter that decides where oxidative damage takes place.^{59,60}

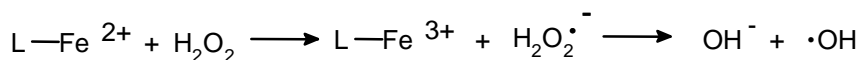
Biological systems are very complex and in order to gain information about the reactivity of different molecules towards hydroxyl radicals, several *in vitro* systems have been developed for hydroxyl radical generation. The most common ways of generating hydroxyl radicals *in vitro* are presented below.⁶¹

1) Radiolysis of aqueous solutions in the presence of N_2O or H_2O_2 is the cleanest way of generating hydroxyl radicals and is often the method of choice for kinetic studies.⁶²

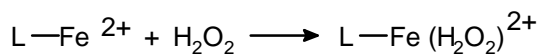
2) Photolysis of hydrogen peroxide.⁶³

3) Chemical reactions, mainly from the reaction between hydrogen peroxide and transition metals in their lower valence states as for example Fe^{2+} and Cu^+ . This reaction is known as the Fenton reaction and is by far the most used chemical method.⁶⁴

Although it has been over 100 years since Fenton performed his experiments, the mechanism of the reaction is still debated. It is clear that the net Fenton reaction is a simplification of a more complex process. The easiest way to explain the reaction would be a single electron donation from Fe^{2+} to hydrogen peroxide (outer sphere mechanism).

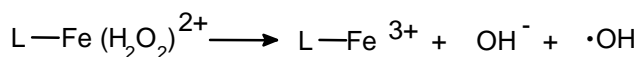


On thermodynamic grounds this mechanism is unfavorable and an inner sphere mechanism involving a transient ferrous peroxide complex is more likely.^{65,66}

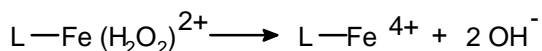


The complexing ligand, represented by (L) in the above scheme, might be any chemical or biological chelator and is of great importance to the outcome of the reaction. The iron-hydrogen peroxide complex could then break down in a number of ways:

1) to give a hydroxide ion and a hydroxyl radical



2) to give two hydroxide ions⁶⁵



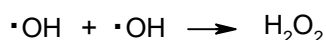
3) direct reaction with a substrate



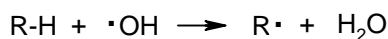
The choice of the ligand, pH and the type of solvent used are of great importance for the outcome of the reaction. In a chemical Fenton system most variables are easy to control but in a biological system, the iron will form complexes with numerous different biological chelators, and the type of ligand will decide the fate of the reaction. Ferrous ions are stable in acidic solutions but in neutral or basic aqueous solutions a ligand is generally added to a chemical Fenton system to prevent precipitation of ferrous and ferric hydroxides. For the experiments described in chapters 3, 4 and 5 ethylenediaminetetraacetic acid (EDTA) was used. Under these conditions, formation of hydroxyl radicals via break-down of the iron-hydrogen peroxide complex to give a hydroxide ion and a hydroxyl radical (pathway 1) is expected to be the main reaction pathway. Regardless if the Fenton reaction takes place via “free” hydroxyl radicals or via direct reaction with a transient $\text{Fe}(\text{H}_2\text{O}_2)^{2+}$ species, the Fenton reaction remains an excellent system for the incorporation of oxygen into organic molecules.

The hydroxyl radical has a half-life of about one nanosecond and reacts very quickly with most organic and biological molecules, generally with rate constants above $10^7 \text{M}^{-1} \text{s}^{-1}$.⁶¹ The reactions can be divided into four different types:

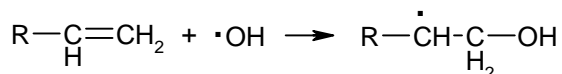
1) radical-radical reactions



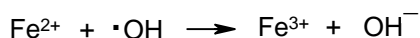
2) hydrogen atom abstraction; the hydroxyl radical can extract a hydrogen atom from various organic compounds and thereby generating a new radical



3) addition reactions; the hydroxyl radical is electrophilic and adds readily to aromatic rings and double bonds⁶⁷



4) electron transfer reactions; the hydroxyl radical is a powerful oxidant and commonly reacts with inorganic compounds by abstracting an electron.



In complex systems various combinations of the above described reactions often take place. In reactions with organic compounds, hydrogen atom abstraction from aliphatic

groups and addition to unsaturated π -systems are the predominant reactions. Since both these processes generate a new radical, the end products of reactions between hydroxyl radicals and organic molecules are dependent on the molecular structure of the organic compound and the nature of the surrounding media.

1.4 Antioxidants

Antioxidant activity can be defined as the protection against oxidative damage.⁴⁷ There are three major ways for an antioxidant to protect biomolecules from damage induced by oxidative stress *in vivo*: i) suppression of the formation of ROS i.e. compounds that chelate iron in such a way that it becomes less active in Fenton chemistry, ii) scavenging of radicals and iii) repairing oxidative damage.⁶⁸

When it comes to the hydroxyl radical, the high reactivity excludes radical scavenging as an option unless the radical scavenger is added in extremely large amounts on the basis of competition kinetics.⁶⁹ If the radical scavenger on the other hand also acts as an iron chelating agent, it will be very close to the place of radical formation and may be effective in scavenging hydroxyl radicals even at low concentrations.

To guard against damage by ROS, organisms have developed a number of antioxidant enzymes including superoxide dismutase (SOD), catalase and glutathione peroxidase.⁷⁰ The main non-enzyme antioxidants in the human body are the water soluble ascorbic acid (vitamin C) and uric acid and α -tocopherol (vitamin E) in membranes.⁷¹ Free methionine and surface exposed methionine residues of all proteins in a cell may also constitute an important mechanism for the intracellular scavenging of ROS.⁷²

1.5 Scope of the thesis

The introduction of combinatorial chemistry has resulted in a dramatic increase in the number of new chemical entities that support drug discovery efforts. To limit the cost of drug development, it is vital to try to eliminate as many compounds as possible at the early stages of the discovery process. One important consideration in high-throughput screening is metabolic stability, and, in case of instability, identification of the metabolites. The main goal of our studies has been the development of electrochemical techniques on-line with electrospray mass spectrometry for *in vitro* generation and characterization of drug metabolites. These purely instrumental methods may have advantages over, or may be complementary to, the existing methods of screening, i.e., *in vitro* studies with purified enzymes or organ fractions e.g. microsomes, hepatocytes and liver slices.

References

1. S. Bruckenstein; R. R. Gadde, "Use of a porous electrode for in situ mass spectrometric determination of volatile electrode reaction products", *J. Am. Chem. Soc.*, **1971**, 93, 793-794.
2. G. Hambitzer; J. Heitbaum, "Electrochemical thermospray mass spectrometry", *Anal. Chem.*, **1986**, 58, 1067-1070.
3. T. A. Getek, "The development of thermospray mass spectrometry combined with electrochemical redox processes", Proceedings of the 34th ASMS Annual Conference on Mass Spectrometry and Allied Topics, Cincinnati, **1986**.
4. K. J. Volk; R. A. Yost; A. Brajter-Toth, "On-line electrochemistry/thermospray/tandem mass spectrometry as a new approach to the study of redox reactions: The oxidation of uric acid", *Anal. Chem.*, **1989**, 61, 1709-1717.
5. K. J. Volk; R. A. Yost; A. Brajter-Toth; J. A. Freeman, "A dynamic approach for studying redox reactions: electrochemistry on-line with mass spectrometry", *Anal. Chem.*, **1992**, 64, 421-425.
6. K. J. Volk; R. A. Yost; A. Brajter-Toth, "Electrochemistry On Line with Mass Spectrometry, Insite into Biological Redox Reactions", *Anal. Chem.*, **1992**, 64, 21A-33A.
7. G. J. V. Berkel; S. A. McLuckey; G. L. Glish, "Electrochemical origin of radical cations observed in electrospray ionization mass spectra", *Anal. Chem.*, **1992**, 64, 1586-1593.
8. F. Zhou; G. J. V. Berkel, "Electrochemically enhanced electrospray ionization mass spectrometry", Proceedings of the 42nd ASMS Conference on Mass Spectrometry and Allied Topics, Chicago, Illinois, **1994**.
9. F. Zhou; G. J. V. Berkel, "Electrochemistry Combined On-Line with Electrospray Mass Spectrometry", *Anal. Chem.*, **1995**, 67, 3643-3649.
10. G. J. V. Berkel; F. Zhou, "Electrospray as a controlled-current electrolytic cell: Electrochemical ionization of neutral analytes for detection by electrospray mass spectrometry", *Anal. Chem.*, **1995**, 67, 3958-3964.
11. G. J. V. Berkel; F. Zhou, "Characterization of an electrospray ion source as a controlled-current electrolytic cell", *Anal. Chem.*, **1995**, 67, 2916-2923.
12. A. M. Bond; R. Colton; A. D'Agostino; A. J. Downard; J. C. Treager, "A role for electrospray mass spectrometry in electrochemical studies", *Anal. Chem.*, **1995**, 67, 1691-1695.
13. L. Baczynskyj, "EC-LC-ESI MS: Electrochemistry Combined with Liquid Chromatography and Electrospray Mass Spectrometry", Proceedings of the 44th ASMS Conference on Mass Spectrometry and Allied Topics, Portland, Oregon, **1996**.
14. W. Lu; X. Xu; R. B. Cole, "On-line linear sweep voltammetry-electrospray mass spectrometry", *Anal. Chem.*, **1997**, 69, 2478-2484.

15. X. Xu; W. Lu; R. B. Cole, "On-Line Probe for Fast Electrochemistry/Electrospray Mass Spectrometry. Investigation of Polycyclic Aromatic Hydrocarbons", *Anal. Chem.*, **1996**, 68, 4244-4253.
16. J. Zhou; S. Hefta; T. D. Lee, "High sensitivity analysis of phenylthiohydantoin amino acid derivatives by electrospray mass spectrometry.", *J. Am. Soc. Mass. Spectrom.*, **1997**, 8, 1165-1174.
17. M. C. S. Regino; A. Brajter-Toth, "An Electrochemical Cell for On-Line Electrochemistry/Mass Spectrometry", *Anal. Chem.*, **1997**, 69, 5067-5072.
18. M. C. S. Regino; A. Brajter-Toth, "Real time characterization of catalysis by on-line electrochemistry/mass spectrometry. Investigation of quinone electrocatalysis of amine oxidation", *Electroanalysis*, **1998**, 11, 374-379.
19. M. Regino; C. Weston; A. Brajter-Toth, "Effect of mobile phase composition on the electrochemical cell conversion efficiency in electrochemistry/mass spectrometry", *Anal. Chim. Acta*, **1998**, 369, 253-262.
20. T. Zhang; A. Brajter-Toth, "On-line investigation of the generation of nonaqueous intermediate radical cations by electrochemistry/mass spectrometry", *Anal. Chem.*, **2000**, 72, 2533-2540.
21. G. Hambitzer; J. Heitbaum; I. Stassen, "Electrochemical thermospray mass spectrometry-anodic oxidations of *N,N*-dialkylanilines", *J. Electroanal. Chem.*, **1998**, 447, 117-124.
22. I. Stassen; G. Hambitzer, "Anodic oxidation of aniline and *N*-alkylanilines in aqueous sulphuric acid studied by electrochemical thermospray mass spectrometry", *J. Electroanal. Chem.*, **1997**, 440, 219-228.
23. G. Hambitzer; J. Heitbaum; I. Stassen, "Electrochemical thermospray mass spectrometry instrumentation for coupling electrochemistry to mass spectrometry", *Anal. Chem.*, **1998**, 70, 838-842.
24. U. Jurva; A. P. Bruins; H. V. Wikström, "The ESA electrochemical cell coupled with electrospray ionization mass spectrometry", *Proceedings of the 47th ASMS Conference on Mass Spectrometry and Allied Topics*, Dallas, Texas, **1999**.
25. U. Jurva; A. P. Bruins; H. V. Wikström, "In vitro mimicry of metabolic oxidation reactions by electrochemistry/mass spectrometry", *Rapid commun. mass spec.*, **2000**, 14, 529-533.
26. U. Jurva; A. P. Bruins; H. V. Wikström, "In vitro mimicry of metabolic oxidation reactions by electrochemistry/mass spectrometry", Presented at the 15th International Mass Spectrometry conference, Barcelona, Spain, **2000**.
27. H. Iwahashi, "3-Hydroxyanthranilic acid-derived compounds formed through electrochemical oxidation", *J. Chrom. B*, **1999**, 736, 237-245.
28. H. Deng; G. J. V. Berkel, "A Thin-Layer Electrochemical Flow Cell Coupled On-Line with Electrospray-Mass Spectrometry for the Study of Biological Redox Reactions.", *Electroanalysis*, **1999**, 11, 857-865.

29. J. R. Pretty; H. Deng; D. E. Goeringer; G. J. v. Berkel, "*Electrochemically modulated preconcentration and matrix elimination for organic analytes coupled on-line with electrospray mass spectrometry*", *Anal. Chem.*, **2000**, 72, 2066-2074.
30. H. Deng; G. J. v. Berkel; H. Takano; D. Gazda; M. D. Porter, "*Electrochemically modulated liquid chromatography coupled on-line with electrospray mass spectrometry*", *Anal. Chem.*, **2000**, 72, 2641-2647.
31. G. Diehl; A. Liesener; U. Karst, "*Liquid chromatography with post-column electrochemical treatment and mass spectrometric detection of non-polar compounds*", *Analyst*, **2001**, 126, 288-290.
32. G. Diehl; U. Karst, "*Combining HPLC/MS with electrochemistry for sensitive detection of Ferrocene-labeled alcohols and phenols*", *Proceedings of the 49th ASMS Conference on Mass Spectrometry and Allied Topics*, Chicago, Illinois, **2001**.
33. G. Diehl, PhD Thesis, University of Twente, the Netherlands, **2002**.
34. H. Hayen; U. Karst, "*Determination of phenothiazine and its derivatives by means of on-line HPLC/electrochemistry/mass spectrometry*", *Proceedings of the 49th ASMS Conference on Mass Spectrometry and Allied Topics*, Chicago, Illinois, **2001**.
35. P. Brown; J. Rollag; P. Lin; J. Lee, "*Oxidation and reduction of compounds using electrochemical detector to enhance LC/MS quantification.*", *Proceedings of the 49th ASMS Conference on Mass Spectrometry and Allied Topics*, Chicago, Illinois, **2001**.
36. S. P. Palii; T. Zhang; A. Brajter-Toth; J. R. Eyler, "*Investigation of electrochemically assisted ionization (ECAI) in ESI FT-ICR mass spectrometry*", *Proceedings of the 49th ASMS Conference on Mass Spectrometry and Allied Topics*, Chicago, Illinois, **2001**.
37. T. Zhang; S. P. Palii; J. R. Eyler; A. Brajter-Toth, "*Enhancement of Ionization efficiency by electrochemical reaction products in on-line electrochemistry/electrospray ionization Fourier transform cyclotron resonance mass spectrometry*", *Anal. Chem.*, **2002**, 74, 1097-1103.
38. K. A. Johnson; B. A. Shira; J. L. Anderson; I. J. Amster, "*Chemical and on-line electrochemical reduction of metalloproteins with high resolution electrospray ionization mass spectrometry detection*", *Anal. Chem.*, **2001**, 73, 803-808.
39. B. Meunier; J. Bernadou, "*Active Iron-Oxo and Iron-Peroxo Species in Cytochrome P450 and Peroxidases; Oxo-hydroxo Tautomerism with Water-Soluble Metalloporphyrins*", *Struct. Bond.*, **2000**, 97, 1-35.
40. P. R. O. d. Montellano "*Cytochrome P450, Structure, Mechanism, and Biochemistry*" second ed.; Plenum Press, **1995**.
41. D. F. V. Lewis; J. M. Pratt, "*The P450 catalytic cycle and oxygenation mechanism*", *Drug Metab. Rev.*, **1998**, 30, 739-786.
42. M. Sono; M. P. Roach; E. D. Coulter; J. H. Dawson, "*Heme-Containing Oxygenases*", *Chem. Rev.*, **1996**, 96, 2841-2887.

43. J. T. Groves; Y. Z. Han, "Models and Mechanisms of Cytochrome P450 Action", In "Cytochrome P450, Structure, Mechanism, and Biochemistry" second ed.; Montellano, P. R. O. d., Ed.; Plenum Press., **1995**.
44. D. L. Wertz; J. S. Valentine, "Nucleophilicity of Iron-Peroxo Porphyrin Complexes", *Struct. Bond.*, **2000**, *97*, 37-60.
45. I. Schlichting; J. Berendzen; K. Chu; A. M. Stock; S. A. Maves; D. E. Benson; R. M. Sweet; D. Ringe; G. A. Petsko; S. G. Sligar, "The Catalytic Pathway of Cytochrome P450cam at Atomic Resolution", *Science*, **2000**, *287*, 1615-1622.
46. M. Newcomb; R. Shen; S. Y. Choi; P. H. Toy; P. F. Hollenberg; A. D. N. Vaz; M. J. Coon, "Cytochrome P450-Catalyzed Hydroxylations of Mechanistic Probes that Distinguish between Radicals and Cations. Evidence for Cationic but not for Radical Intermediates.", *J. Am. Chem. Soc.*, **2000**, *122*, 2677-2686.
47. L. Packer (Ed.) "Oxidants and Antioxidants, Part A" ; Academic Press, **1999**.
48. D. Armstrong (Ed.) "Free Radical and Antioxidant Protocols" ; Humana Press, **1998**.
49. J. P. Kehrer, "The Haber-Weiss reaction and mechanism of toxicity", *Toxicol.*, **2000**, *149*, 43-50.
50. H. Alho; J. Leinonen, "Total Antioxidant Activity Measured by Chemiluminescence Methods", In "Oxidants and Antioxidants, Part A" ; Packer, L., Ed.; Academic Press., **1999**; Vol. 299.
51. N. Gotho; E. Niki, "Measurement of Superoxide Reaction by Chemiluminescence", In "Oxygen Radicals in Biological Systems, Part C" ; Packer, L., Ed.; Academic Press., **1994**; Vol. 233.
52. M. S. Paller; H. S. Jacob, "Cytochrome P-450 mediates tissue-damaging hydroxyl radical formation during reoxygenation of the kidney", *Proc. Natl. Acad. Sci. USA*, **1994**, *91*, 7002-7006.
53. E. Albano; A. Tomasi; M. Ingelman-Sundberg, "Spin Trapping of Alcohol-derived Radicals in Microsomes and Reconstructed Systems", In "Oxygen Radicals in Biological Systems, Part C" ; Packer, L., Ed.; Academic Press., **1994**; Vol. 233.
54. F. Haber; J. Weiss, "The catalytic decomposition of hydrogen peroxide by iron salts", *Proc. R. Soc. London [Biol]*, **1934**, *A147*, 332-351.
55. P. Wardman; L. P. Candeias, "Fenton Chemistry: An Introduction", *Radiat. Res.*, **1996**, *145*, 523-531.
56. H. Yokoyama, "In vivo measurement of hydrogen peroxide by microelectrode", In "Oxidants and Antioxidants, Part B" ; Packer, L., Ed.; Academic Press., **1999**; Vol. 300.
57. A. L. Feig; S. J. Lippard, "Reactions of non-heme iron(II) centers with dioxygen in biology and chemistry", *Chem. Rev.*, **1994**, *94*, 759-805.
58. M. J. Burkitt, "Copper-DNA Adducts", In "Oxygen Radicals in Biological Systems, Part D" ; Packer, L., Ed.; Academic Press., **1994**; Vol. 234.

59. C. Demougeot; C. Marie; A. Beley, "Importance of iron location in iron-induced hydroxyl radical production by brain slices", *Life Sciences*, **2000**, 67, 399-410.
60. D. R. Lloyd; D. H. Phillips, "Oxidative DNA damage mediated by copper(II), iron(II) and nickel(II) Fenton reactions: evidence for site-specific mechanisms in the formation of double strand breaks, 8-hydroxydeoxyguanosine and putative intrastrand cross-links", *Mutat. Res.*, **1999**, 424, 23-36.
61. G. Czapski, "Reaction of OH radical", In "Oxygen Radicals in Biological Systems, Part A" ; Packer, L., Ed.; Academic Press:, **1984**; Vol. 105.
62. K. P. Madden; H. Taniguchi, "The role of the DMPO-hydrated electron spin adduct in DMPO-OH spin trapping", *Free Rad. Biol. Med.*, **2001**, 30, 1374-1380.
63. R. V. Lloyd; P. M. Hanna; R. P. Mason, "The origin of the hydroxyl radical oxygen in the Fenton reaction", *Free rad. biol. med.*, **1997**, 22, 885-888.
64. H. J. H. Fenton, "Oxidation of tartaric acid in presence of iron", *J. Chem. Soc.*, **1894**, 65, 899-910.
65. C. C. Winterbourn, "Toxicity of iron and hydrogen peroxide: the Fenton reaction", *Toxicol. Lett.*, **1995**, 82/83, 969-974.
66. S. Goldstein; D. Meyerstein; G. Czapski, "The Fenton Reagents", *Free Rad. Biol. Med.*, **1993**, 15, 435-445.
67. C. v. Sonntag; H.-P. Schuchmann, "Pulse radiolysis", In "Oxygen Radicals in Biological Systems, Part C" ; Packer, L., Ed.; Academic Press:, **1994**; Vol. 233.
68. L. C. Bourne; C. A. Rice-Evans, "Detecting and Measuring Bioavailability of Phenolics and Flavanoids in Humans: Pharmacokinetics of Urinary Excretion of Dietary Ferulic acid.", In "Oxidants and Antioxidants, Part A" ; Packer, L., Ed.; Academic Press:, **1999**; Vol. 299.
69. C. v. Sonntag; H.-P. Schuchmann, "Suppression of hydroxyl radicals in biological systems: Considerations based on competition kinetics", In "Oxygen Radicals in Biological Systems, Part C" ; Packer, L., Ed.; Academic Press:, **1994**; Vol. 233.
70. H. Z. Chae; S. W. Kang; S. G. Rhee, "Isoforms of Mammalian Peroxiredoxin that reduce Peroxides in Presence of Thioredoxin", In "Oxidants and Antioxidants, Part B" ; Packer, L., Ed.; Academic Press:, **1999**; Vol. 300.
71. J. P.A. Glasscott; J. L. Farber, "Assessment of Physiological Interaction between Vitamin E and Vitamin C.", In "Oxidants and Antioxidants, Part B" ; Packer, L., Ed.; Academic Press:, **1999**; Vol. 300.
72. J. Moskovitz; B. S. Berlett; J. M. Poston; E. R. Stadtman, "Methionine Sulfoxide Reductase in Antioxidant Defense", In "Oxidants and Antioxidants, Part B" ; Packer, L., Ed.; Academic Press:, **1999**; Vol. 300.