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Electrochemistry/Mass Spectrometry as a Tool in Metabolism Studies – a Review

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Highlights

- EC/MS provides information on the oxidative metabolism of drugs.
- LC may be added to provide polarity information of the metabolites.
- Metabolism information may be obtained without animal experiments.

Abstract

The combination of electrochemistry (EC) and mass spectrometry (MS) has become a more and more frequently used approach in metabolism studies in the last decade. This review provides insight into the importance of metabolism studies during the drug development process and gives a short overview about the conventionally used methods since electrochemistry is often intended to substitute or minimize animal-based studies. The optimization of the electrochemical conditions is of great importance for a successful comparison with in vitro approaches. The type of metabolism reactions, which can be simulated by EC, has been extended with new cell types and working electrodes. Although the mechanism differs from the enzyme-catalyzed turnover, electrochemistry can be used to simulate a significant number of the respective reactions.

An expanded set-up consisting of EC, a chromatographic separation and MS allows to distinguish between an electrospray ionization (ESI) in-source and an electrochemical oxidation and provides information on the polarity of the electrogenerated compounds. A main advantage of EC for metabolite generation is the possibility to isolate reactive species because of the purely instrumental approach. Especially when a preparative electrochemical cell with a larger working electrode surface is used, metabolites can be generated in sufficient quantities for their subsequent structure elucidation. Besides, the compounds can also be used for selective trapping experiments with different cell components such as small peptides, proteins or DNA bases. Current and possible future developments and applications of EC are presented and discussed as well.

Abbreviations
ACN = acetonitrile
ADME = adsorption, distribution, metabolism, excretion
Ag/AgCl/Cl = silver/silver chloride, reference electrode
APAP = acetaminophen, paracetamol
APPI = atmospheric pressure photoionization
AQ = amodiaquine
AQQI = amodiaquine quinone imine
AUX = auxiliary electrode, counter electrode
Keywords
Electrochemistry, drug metabolism, mass spectrometry, liquid chromatography

Introduction
Although the combination of electrochemistry and mass spectrometry often is considered to be a very recent combination of methods, it was first described in the scientific literature more than forty years ago: Already in 1971, Bruckenstein and Gadde introduced electrochemistry coupled to mass spectrometry (EC/MS) for the in situ detection of electrochemical oxidation products. They used a porous platinum electrode, which was in contact with a solution to be electrolyzed and with the vacuum inlet of the mass spectrometer.\(^{[1]}\) With this approach, they...
studied gaseous products, e.g. oxygen, generated upon electrochemical oxidation of 0.1 M perchloric acid. With the development of thermospray ionization (TSP), it was possible to detect non-volatile and polar compounds in solution. In 1986, Hambitzer and Heitbaum were the first to study oxidation processes (in this case of N,N-dimethylaniline) with online-EC/TSP-MS. They observed the formation of dimers and trimers depending on the applied oxidation potential. The high flow rates used in TSP-MS (1-2 mL min\(^{-1}\)) adversely affected the conversion efficiency of the electrochemical oxidation. Therefore, the interest in the online coupling of EC and MS declined in the following years. However, after the introduction of the electrospray ionization interface (ESI), a revival of EC/MS was observed, as it was now possible to analyze thermally labile and non-volatile compounds in solution. The direct coupling of EC to ESI-MS was first investigated in detail by Van Berkel et al. with respect to cell design and coupling mode (floated or decoupled from the ESI high voltage). They showed that the direct coupling of EC and MS allowed (1) the electrochemical ionization of neutral compounds, (2) the study of electrode reactions, and (3) the preconcentration of silver ions and the related signal enhancement in ESI-MS using anodic stripping voltammetry. Thereafter, the number and kind of EC applications has significantly increased, especially in the field of metabolism studies. The respective methods and applications including their current limitations and future potential are presented within this review.

**Drug Development and Oxidative Metabolism**

The fate of a drug in the body is determined by many processes, including its absorption, distribution, metabolism and excretion (ADME). To ensure a target-oriented and cost efficient drug development process, pharmacokinetics and the metabolism of the drug candidate must be understood as early as possible, because selected metabolites may be the cause for various adverse effects. 

Most drugs are metabolized in the liver, which contains a large number of metabolizing enzymes. Therefore, it is not surprising that most adverse reactions affect this organ and may result in hepatotoxicity. A simplified scheme of possible metabolic routes for xenobiotics is displayed in Figure 1. The first step of the biotransformation is called phase I metabolism and comprises various reactions such as dehydrogenations, hydrolyses, reductions or oxidations. One of the most important reactions is the The first step of metabolism in the liver involves an oxidative functionalization catalyzed by enzymes of the cytochrome P450 (CYP450) group. The formed phase I metabolites contain functional groups such as hydroxyl, amino or carboxylic groups. This allows their excretion or more frequently a subsequent phase II conjugation to endogenous compounds such as glutathione (GSH), glucuronic acid or sulfate. Conjugation reactions are essential for the detoxification and excretion of phase I metabolites drugs, because the highly polar phase II adducts are prone to be rapidly eliminated from the body (left route in Figure 1). However, some phase I metabolites may be highly reactive and can thus irreversibly bind to cellular macromolecules such as proteins or even DNA before phase II conjugation can take place (right route in Figure 1). The short half-life of these very reactive metabolites -often less than one minute- renders their detection in complex matrices such as plasma or whole blood difficult or even impossible. Therefore, it is merely possible to quantify the formation of such reactive species in humans. Reactive metabolites are often quinoid species, which are susceptible to nucleophilic attack from thiol groups of small molecules, e.g. cysteine or GSH. If these reactive species are formed at concentrations above the concentration of thiols in the liver, they may attach to various liver proteins. As a consequence, the modified proteins may show disturbed functionalities, which may be the reason for the liver toxicity. One of the most well-known
pharmaceuticals regarding the formation of a reactive metabolite during CYP450 metabolism is acetaminophen (APAP).\textsuperscript{[13-15]} APAP is mainly subject to glucuronidation and sulfation and only to a minor extent (2-10%), it is metabolized via the oxidative CYP450 pathway, yielding the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). NAPQI is easily detoxified by conjugation with hepatic GSH. However, if APAP is overdosed, the hepatic GSH level will not be sufficient for a quantitative detoxification. Thus, the metabolite can be covalently bound to functional groups in the body including thiol functions of the liver proteins. A detailed review about drugs, which undergo bioactivation and bind covalently to proteins, can be derived from ref.\textsuperscript{13}

**Conventional Methods for Metabolism Studies**

In order to identify compounds during the drug development process, which undergo undesired metabolic activation to reactive species, their metabolism has to be investigated as early as possible. This is not only the case for pharmaceuticals, but also for other xenobiotics including pesticides, food additives, or fragrances. Various \textit{in vitro} approaches have been developed for this purpose, including supersomes, microsomes, cytosol, S9 fraction, (transgenic) cell lines, transgenic cell lines, primary hepatocytes, liver slices and perfused liver.\textsuperscript{[16]} The aim of all these approaches is to mimic the \textit{in vivo} situation as closely as possible. The assays should also be easy to use, not too laborious and ethically tenable. Figure 2 (adapted from Brandon \textit{et al.}\textsuperscript{[16]}) gives a brief overview about the different approaches, which will be discussed in the following.

Intact liver cells (primary hepatocytes) are an ideal example to give an overall picture of possible drug metabolism reactions. However, their isolation is complicated and laborious, since damage of the liver tissue during this process must be avoided. During cultivation of the cells, some enzyme levels may decrease in this artificial surrounding and therefore, a respective assay may suffer from poor conversion rates.\textsuperscript{[17]} In liver slices, intact cell connections are present. Similar to hepatocytes, the preparation of the slices is difficult and the life span of the cells is limited to only a few days.\textsuperscript{[16]} An isolated perfused liver gives the best overview of the \textit{in vivo} situation due to the fact that it comprises many functions of a working liver together with an almost complete range of metabolic enzymes. However, it is only available from animal liver and for each compound to be tested, one animal is needed.\textsuperscript{[16]} Liver cell lines are less popular compared to the other \textit{in vitro} systems, because not all phase I and II enzymes are expressed. Generally, the absolute amount of enzymes in cell lines is low, so that it is hardly possible to differentiate and characterize the individual enzymes.\textsuperscript{[11]} In contrast, the expression of enzymes, especially of CYP and of UGT (UDP glucuronosyltransferase) isozymes in transgenic cell lines or supersomes is much higher, since one specific isofrom is selectively overexpressed. Unfortunately, this material is comparably expensive. In contrast to this, supersomes are more affordable. They also originate from transgenic cell lines, but in this case, the vesicles from the endoplasmic reticulum (comparable to microsomes) are derived from insect cells, which are transfected with a baculovirus. Since these insect cells lack endogenous CYP enzymes, the expression of one isofrom or a specific genotype (e.g. CYP2C9) can be achieved. Thus, they allow the study of the metabolism depending on different polymorphisms of enzymes.\textsuperscript{[16]}

Human liver cytosol is prepared by centrifugation of a whole liver homogenate. Since it mainly contains the soluble phase II enzymes glutathione-S-transferase (GST), N-acetyltransferase (NAT) and sulfotransferase, it is used for phase II \textit{in vitro} studies. Obviously, no information on phase I reactions is obtained.\textsuperscript{[16]} The S9 fraction is the supernatant, which is obtained after centrifugation at 9000g. It contains microsomal and cytosolic enzymes and provides both phase I and II activity, respectively. However, the activity of the S9 fraction compared to cytosol or microsomes is rather low: Some metabolites are formed only to a minor extent and may thus easily be overlooked. A
combination of the three *in vitro* systems S9 fraction, cytosol and microsomes is a good approach for metabolism studies.\(^\text{[16]}\)

Compared to the *in vitro* assays mentioned so far, the application of human (HLM) or rat liver microsomes (RLM) is currently the most popular approach to study the phase I metabolism of a drug. HLMs consist of vesicles of the endoplasmic reticulum of hepatocytes and contain mainly enzymes of the CYP450 family or UGT. Similar to cytosol and the S9 fraction, they are prepared by differential centrifugation from liver preparations, such as liver slices or primary hepatocytes. HLMs are easy to handle and are commercially available at reasonable prices. Studies based on HLMs allow to investigate the influence of specific isoforms being responsible for the metabolism by simply adding targeted enzyme inhibitors to the incubation mixture. Due to the preparation of microsomes, they show an increased level of phase I enzymes. On the one hand, this leads to a beneficial conversion of drugs, which is advantageous for the isolation of phase I metabolites. On the other hand, these increased concentrations complicate a comparison with the *in vivo* situation. Therefore, HLMs are only suited for qualitative screening of metabolites.\(^\text{[16]}\) The isolation of metabolites for further analytical characterization is therefore only reasonable in those *in vitro* assays, which show a good metabolizing activity. Typically, HLMs are incubated with the drug for about 90 min in a buffered solution at a physiological pH of 7.4. Afterwards, an organic solvent as acetonitrile is added in order to quench the enzymatic activity and to extract the metabolites from the incubation solution. The extracted species are subsequently analyzed by means of liquid chromatography/mass spectrometry (LC/MS). The formation of reactive metabolites in the microsomal incubations may lead to covalent binding to macromolecules or other nucleophiles present in the incubation media, thus withholding them from subsequent LC/MS analysis.\(^\text{[18]}\) The addition of a trapping agent such as GSH often allows the indirect detection of these electrophilic species, which can be identified by the formed adducts.\(^\text{[19]}\) Nevertheless, some reactive metabolites may not be trapped efficiently so that they still undergo undesired reactions with matrix constituents like proteins.\(^\text{[20]}\) As EU regulations prohibit the toxicity testing of cosmetics and their ingredients based on animal studies, alternative approaches are needed.\(^\text{[21]}\) As any biological material used in *in vitro* assays may express enzymes in a variable way, reproducible data may better be obtained by using purely instrumental methods under the provision of a good comparability to the results obtained *in vivo*.

**Electrochemical Cells**

In 1981, Shono *et al.* were the first to recognize the usefulness of electrochemical oxidation for the synthesis of oxidative phase I metabolites. With their home-made 50 mL batch electrolysis cell, they were able to generate the *N*-dealkylated species of four neuropharmaceuticals without using any further chemical reagents.\(^\text{[22]}\) Today, several types of electrochemical cells are commercially available, which are currently used in metabolism studies. Since the choice of cell and electrode has a great impact on the generated oxidation products, they shall be discussed in the following. A scheme of the cell types is depicted in Figure 3.

Typically, coulometric flow-through cells (a in Figure 3) and amperometric cells (b in Figure 3) are used for EC/MS experiments. The cells comprise three different electrodes: Working (WE), counter or auxiliary (AUX) and reference electrode (REF). The processes during the anodic oxidation of a compound are described in a simplified manner in the following\(^\text{[23-25]}\): At the WE, electroactive species are oxidized (depending on the applied potential) transferring electrons to the electrode. The current flows between the WE and the auxiliary electrode (AUX), also referred to as counter electrode. However, it is difficult to apply a fixed potential between these two electrodes, since the potential may be strongly affected by the current. Therefore, a reference electrode (REF) with high resistance is needed to determine the potential between WE and REF, while the current flows between WE and AUX. Different
materials are available for the WE, which will be discussed below. The material of the AUX
has only limited influence since it does not affect the behavior of the WE, where the
conversion of interest takes place. This behavior of the WE, where the conversion takes place. The cells described below
comprise an AUX consisting of stainless steel or graphite doped Teflon. However AUX can
also be made out of platinum or palladium.\footnote{26} Regarding the REF, several electrodes can be used: One example is the Ag/AgCl/Cl REF, which has an $E^0$ of +222 mV vs. SHE at 25°C. However, due to its size, it cannot be placed close to the WE, which may result in an unstable potential.\footnote{26} In the electrochemical cells described below, whereas the REF are typically Pd/H$_2$ systems. One advantage, compared to the Ag/AgCl/Cl REF is that they are maintenance free and can be operated at higher backpressure.\footnote{26} However, the potential of the Pd/H$_2$ electrode is depending on the pH of the solution of the analyte, thus leading to the requirement of precise pH control in order to obtain reproducible data. The potential of the calomel electrode (Hg/Hg$_2$Cl$_2$/Cl) depends on the concentration of the co-ion and varies between $E^0$ of +245-336 mV SHE at 25°C. However, it uses toxic salts and is therefore not frequently used.\footnote{26}

Despite their common three electrodes set up, the applied electrochemical cells can be
differentiated by kind and dimension of the electrodes: Coulometric flow through cells
comprise a porous glassy carbon electrode, which is similar to a frit, in which the analyte is
oxidized.\footnote{26} These cells are commercially available and can be operated at flow rates of up to several hundred $\mu$L min$^{-1}$. Therefore, they can easily be integrated into a LC system prior to the column. Due to the porous electrode material, the surface area is very large and despite of the high flow rates, an almost quantitative conversion can be achieved.\footnote{26} One drawback is that the electrode material cannot be cleaned manually because the electrodes are embedded into an electrode block and are not accessible for maintenance. Amperometric thin-layer cells allow to easily exchange the different electrodes and to polish them manually if a loss of performance is observed. For the simulation of the oxidative metabolism, glassy carbon (GC), platinum (Pt) and boron-doped diamond (BDD) are used as typical WEs. Initial EC/MS studies were mainly conducted with GC as WE material. The more recently introduced application of BDD offers some interesting features, since it has been reported that hydroxyl (OH$^-$) radicals are formed at the material’s surface.\footnote{27-29} Their generation during the anodic oxidation is initiated through a one electron loss of water, which leads to the formation of adsorbed hydroxyl radicals (\textit{OH$_{ads}$}) in equation 1.1 at the electrode surface.\footnote{29-30}

\begin{equation}
\text{H}_2\text{O} \rightarrow \text{OH}_{ads} + \text{H}^+ + e^- \\
\end{equation}

The following reactions depend on the applied WE materials. These can be divided into non-active and active electrodes\footnote{29}: On active electrodes such as platinum, the OH$^-$
radical may interact with the electrode material and is chemisorbed on the surface. In this case, the active site of the electrode is transformed which results in a higher oxidation state. In some
publications, this higher oxidation state is referred to as higher oxide (O$_{ads}$ in equation 1.2):

\begin{equation}
\text{OH}_{ads} \rightarrow \text{O}_{ads} + \text{H}^+ + e^- \\
\end{equation}

According to Marselli \textit{et al.}, this higher oxide mediates the (selective) oxidation of organic compounds (R in equation 1.3)\footnote{29}:

\begin{equation}
\text{O}_{ads} + R \rightarrow \text{RO} \\
\end{equation}

The higher oxide can also decompose, which results in the formation of oxygen (equation 1.4):

\begin{equation}
2\text{O}_{ads} \rightarrow \text{O}_2 \\
\end{equation}

On non-active electrodes like BDD, the interaction between the electrode surface and OH$^-$
radicals is much lower. Thus, the oxidation of organic compounds can be mediated through the OH$^-$
radical. These radicals are able to react with functional groups in a molecule, which are not prone to be transformed through electron transfer reactions (direct electrochemical
oxidation). However, as indicated in equation 1.5, the OH$^-$
radical mediated oxidation may
even lead to a complete mineralization of the organic compound under formation of CO₂ and H₂O.

\[
equation 1.5: \quad \cdot \text{OH}_{\text{ads}} + R \rightarrow mH₂O + nCO₂ + H^+ + e^-
\]

This reaction competes -in a similar way as active electrodes do- with the evolution of oxygen

\[
equation 1.6: \quad \cdot \text{OH}_{\text{ads}} \rightarrow 1/2 O₂ + H^+ + e^-
\]

It is necessary to carefully adjust the potential, since gaseous products such as CO₂ and O₂ may be generated otherwise. This may a) result in unstable electrochemical conditions and b) is of course not intended in metabolism studies. However, the application of BDD offers a larger potential window than GC, since oxygen evolution takes place at a potential of 2.2 V vs. SHE, while the standard redox potential for O₂ is 1.23 V vs. SHE.\[^{29}\]

Electrochemical cells integrated into a microfluidic device ((c) in Figure 3) offer various advantages over conventional systems such as a much lower sample and solvent consumption. The cell layout as shown in Figure 3 has been developed by Odijk \textit{et al.} in order to achieve high conversion efficiencies and easy online coupling to ESI-MS for drug metabolism studies.\[^{31}\] Unlike other electrochemical cells, its counter electrode is located in a separate microchannel in order to minimize undesired side reactions. The long-term goal is to produce the chips in large quantities at corresponding low prices, thus becoming suited for a single use. Memory effects and performance limitations caused by adsorption phenomena at the electrode surface can be avoided in this case. Odijk \textit{et al.} have already shown the efficiency of their set-ups by mimicking the oxidative metabolism of amodiaquine\[^{32}\], procainamide\[^{33}\], and mitoxantrone.\[^{34}\] In the latter work, they improved the initial design of the cell and could show a conversion of nearly 100% for mitoxantrone using flow rates thirty times lower than in a comparable study with a commercially available flow-through cell.\[^{19}\]

Set-up (d) in Figure 3 shows a schematic view of an in-source electrochemical cell adapted from Brajter-Toth \textit{et al.}\[^{34}\] It is reasonable that in the electrospray process, electrochemical oxidations may take place due to the high applied potential. This has first been recognized by Kebarle and co-workers in 1991, who used an electrospray capillary with a zinc tip and detected Zn²⁺ in the recorded mass spectra.\[^{35}\] When they used a stainless steel needle with a Zn inlet instead, which was insulated from the electrospray process, no Zn²⁺ ions were observed. Thus, the release of Zn²⁺ from the Zn-tipped needle was not related to corrosion effects but to an electrochemical oxidation. If electrochemical oxidations in the ESI interface can be controlled, it is a useful tool to enhance the ionization of analytes.\[^{36}\] In the modified electrospray by Brajter-Toth \textit{et al.} (shown in Figure 3), the electrospray needle is divided into two parts, which are connected by tubing from polymer material. If the electrospray is operated in high voltage (HV) mode, the second part of this divided electrospray emitter acts as a WE and the curtain plate of the MS as REF/AUX. If a low voltage is applied to the electrochemical cell, the first part of the electrospray emitter acts as a WE and the second part as REF/AUX. Hence, an electrochemical oxidation is accomplished prior to the electrospray process.\[^{34}\] Such in-source oxidations were carried out for several compounds including dopamine, cysteine\[^{34}\] or uric acid.\[^{37}\] Another interesting approach introduced by Girault \textit{et al.} is the tagging of cysteiny1 residues in proteins by electrochemically generated quinones.\[^{38}\]

When the sample solution containing hydroquinones and proteins reaches the ESI interface, the hydroquinones are oxidized electrochemically towards quinoid species, which are strong electrophiles and therefore prone to react with nucleophiles such as cysteine residues of proteins. Thus, the present proteins are modified by these quinones depending on their amount of cysteine residues. The resulting mass difference can then be used for their identification.

Van Berkel \textit{et al.} developed flow-through electrodes and combined a grounded upstream electrode with the emitter electrode for (1) reducing the cystine group to free thiol groups and (2) oxidizing hydroquinone to the quinone at the same time.\[^{39}\] The main advantage of such in-source oxidations is the detection of reactive (radical) species due to the very short transfer
time to the MS. A survey of electrochemical processes inherent in the electrospray process is given by the review of Van Berkel and Kertesz.\textsuperscript{[36]} Furthermore, further technical modifications of the electrospray emitter have been summarized and reviewed by Prudent and Girault.\textsuperscript{[40]}

The EC/(LC)/MS Set-up

The combination of EC, (LC,) and MS has recently become a popular tool for the phase I metabolism simulation of xenobiotics.\textsuperscript{[41]-[45]} Selected respective technical arrangements are depicted in Figure 4. A continuous flow of the solution of the xenobiotic is transferred through the electrochemical cell, typically under oxidative conditions, and reaches the mass spectrometer. While mass voltammograms are recorded for EC/MS (upper pathway in Figure 4). The collection of the effluent in a six-port valve and subsequent injection into an LC/MS instrument is carried out in EC/LC/MS (lower pathway in Figure 4). The first work regarding the coupling of EC, LC, and MS dates back already to the late 1980s when Volk et al. studied the products of the anodic oxidation of 6-thiopurine via LC/Thermospray MS.\textsuperscript{[46]} With this set-up, they were able to distinguish between reactions, which occurred prior to the MS detection (anodic oxidation) and reactions in the thermospray interface. However, when the EC cell is placed directly in front of the LC column, the separation conditions such as solvent composition and flow rate have to be applied. The set-up was modified by Jurva et al. by adding a six-port valve, in order to decouple electrochemical conditions from LC conditions.\textsuperscript{[47]} This was necessary as they had established an online Fenton reaction, which required a low flow rate of 2 µL min\(^{-1}\) through the cell and was incompatible with the LC flow rate of 200 µL min\(^{-1}\). Also with the implementation of other cell types, like the amperometric thin-layer cell mentioned above, it is necessary to decrease the flow rate to (1-10 µL min\(^{-1}\)) with the goal to obtain sufficient conversion. Furthermore, the direct coupling of EC and MS does not provide information on isomers, which may be formed upon anodic oxidation. By means of reversed-phase LC coupled online to the EC cell, Baumann et al. demonstrated that for the muscle relaxant tetrazepam, six different isomers with one additional oxygen (+O isomers) were electrochemically generated.\textsuperscript{[41]} Furthermore, the high voltage applied in the ESI interface may cause oxidation reactions during the ionization process. Without a separation step, it is hardly possible to distinguish between these reactions. When EC/MS is used, a mixture of different compounds including buffer salts and oxidation products enters the MS at the same time. Depending on the concentration and the ionization behavior of the individual substances, it is possible that the detection of oxidation products suffers from signal suppression. However, if the oxidation products are separated from each other, the ionization can be improved.\textsuperscript{[48]}

To sum up, the major advantage of the direct coupling of EC and MS is the fast and easy screening for possible oxidation products, which allows investigating many compounds in a very short time. If more information with respect to isomers or structures is needed, the implementation of a chromatographic separation is necessary.

Optimization of the Electrochemical Oxidation

One of the most common approaches in EC/MS to optimize the electrochemical conditions is the recording of a mass voltammogram, which is a three-dimensional plot of mass spectra in dependency of the applied WE potential. Mass voltammograms are obtained by coupling the EC cell directly to the interface of a MS. The sample is pumped through the cell while the oxidation potential is ramped in a defined period. During this potential ramp, the mass spectra are continuously recorded. A mass voltammogram for the anti-platelet drug ticlopidine (TCL) is shown in Figure 5.
While at 0 mV vs. Pd/H₂, the [M+H]+ (m/z 264) signal of the TCL is clearly visible, ramping of the potential leads to its decline under formation of the reaction products DHP (m/z 262) and TP (m/z 260). The mass voltammogram shows that the oxidation starts at approximately 1500 mV vs. Pd/H₂ and that TP is generated at higher potentials than DHP. Recording of such a mass voltammogram takes no longer than 5 min, thus it is a suitable method to obtain a fast overview about possible oxidation reactions of the parent compound. Mass voltammograms are particularly useful to identify the optimum potential of particular electrochemical reactions observed, and they are influenced by various experimental parameters including working electrode materials, electrolyte composition and pH of the solution. However, a direct coupling of EC and MS is only recommended when volatile buffers such as ammonium formate or acetate are used.

Another possibility for the optimization of the electrochemical oxidation is the application of square-wave potential pulses as has been shown by Nouri-Nigjeh et al. for the anesthetic drug lidocaine. The oxidation at constant positive potentials yielded only small amounts of an aromatic hydroxylation and an N-dealkylation product. By switching the potential quickly between a negative and positive potential (at a defined cycle time), the product spectrum could be directed towards the N-dealkylation at cycle times below 0.2 s and towards the aromatic hydroxylation using pulses between 0.2 and 12 s. Using pulsed potentials, the electrode surface is (re)activated and the oxidation yield is improved.

Important work on the influence of the cell design and the coupling with ESI-MS with respect to generated oxidation products was performed by the group of Nyholm and co-workers. In 2006, they compared the electrochemical oxidation of a manganese complex by using two different cell types. In the commercially available flow-through cell, the AUX and WE are not separated. Thus, interfering reactions such as reduction at the AUX are possible. With the separation of AUX and WE however, they obtained higher yields of oxidation products since no subsequent reduction was possible any more. Based on these results, it has to be taken into account that –when using the commercial flow-through cells- not all species detected in the mass spectrum must exclusively have been generated through anodic oxidation at the WE.

Mass Spectrometric Detection in EC/MS

As mentioned above, the development of the ESI interface revolutionized the application of MS detection. Since most of the drugs, which are subject to EC/MS studies, are polar, their detection with ESI-MS is easy. In some cases, the parent drug is merely visible in the MS, but upon electrochemical oxidation a more readily ionized species is generated. This has recently been shown by Simon et al. for the seleno-containing compound 9-phenyl-2,3,4,5,6,7,8,9-octahydro-1H-selenoxanthene (Selenox), which is oxidized to a cation showing a significantly improved ionization efficiency.

ESI-MS works best with highly polar compounds, which are easily protonated or deprotonated. Although oxidation reactions will most frequently lead to products with increased polarity, some exceptions are known. These include quinoid products resulting from oxidation of dihydroxyaromatics or their analogous amines. The most prominent examples may be the dehydrogenation product of paracetamol, NAPQI (N-acetyl-p-benzoquinone imine) and its hydrolysis product, p-benzoquinone. For this purpose, atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI) may be superior. Nouri-Nigjeh et al. investigated the electrochemical oxidation of phenacetin and used an APCI source in the negative ion mode for the ionization of p-quinone. Another interesting alternative comprises atmospheric pressure photoionization. For some compounds, such as ethinylestradiol or the anti-inflammatory drug ciclesonide, the LOQ can be improved significantly using APPI-MS, either in the positive or even in the negative ion mode. However, if an electrochemical cell should be coupled
directly to For APCI or APPI sources, these ionization sources, an increase of the flow rate from 10-20 µL min⁻¹ for ESI-MS may be required for stable operation. If the EC cell is directly coupled to the MS without any separation step, the application of a high resolution instrument such as a time-of-flight (ToF) or an Orbitrap Fourier Transform (FT)-mass spectrometer will allow to assign molecular formulae calculated from exact masses with only small mass deviations. Actually this is not always trivial as can be concluded from Figure 65. Mass spectra of ticlopidine are shown at an oxidation potential of 2500 mV vs. Pd/H₂ recorded on a ToF and an Orbitrap FT-MS, respectively. Ticlopidine is dehydrogenated upon anodic oxidation thus yielding TP (thienopyridinium ion m/z 260: C₁₄H₁₁NClS) and DHP (dihydropyridinium ion m/z 262: C₁₄H₁₃ClNS). Due to the isotope pattern of chlorine, the peak of DHP is overlaid by the peak of ³⁷Cl-TP in the ToF mass spectrum (right spectrum in Figure 65). The use of an Orbitrap FT-MS allows the resolution of the two peaks as displayed in the left spectrum in Figure 65.

It has to be expected that a significant fraction of the products formed upon electrochemical oxidation first has to be identified. Several mass analyzers are well suited for this purpose, and those with an excellent full scan sensitivity and the possibility to provide accurate mass information deliver the most valuable results. Only in rare cases, all products of an electrochemical conversion can be detected best in either the positive or the negative ion mode. Therefore, an option for rapid polarity switching is helpful to detect most products. Possibilities for fragmentation experiments with the goal to further elucidate the molecular structure of the products are highly valuable as well. Despite their excellent capabilities for quantification, quadrupole (Q) mass analyzers are technically limited by their scanning speed and duty cycle. Ion trap mass analyzers are preferable for EC/MS due to their high sensitivity in the full scan mode and their excellent fragmentation characteristics. Time of flight (ToF) and OrbitrapFT-MS instruments, while providing accurate mass information and rapid scanning, are limited with respect to fragmentation. Although more expensive, hybrid mass analyzers as Q-ToF or Q-Orbitrap FT-MS combine accurate mass information and the option to gather structural information by fragmentation and are therefore excellent instruments for EC/MS experiments.

**Adduct Formation of Metabolites with Glutathione**

Besides the electrochemical simulation of the oxidative phase I metabolism, selected phase II reactions can be simulated as well. Therefore, a second flow transported by syringe pump and tubing is added downstream of the EC cell, delivering a suitable trapping agent like glutathione (GSH) or cysteine (CYS) via a mixing T-piece. Reactive species that are formed upon anodic oxidation are frequently electrophilic and can then react with nucleophiles, e.g., thiol groups in small peptides. The reaction products can then be detected mass spectrometrically. The first work in this field was carried out by Getek et al. already in 1989.[57] The authors generated GSH and CYS adducts of NAPQI. The quantitative conversion of NAPQI to the respective GSH conjugate was verified by recording cyclic voltammograms, which, in presence of the trapping agent, lacked a reduction peak for NAPQI. The online conjugation of the reactive metabolite NAPQI with GSH, recorded on an ESI-MS is displayed in Figure 7.

APAP is detected at an m/z ratio of 152. The signal intensity for APAP decreases as the potential is increased. In contrast to thermospray MS, the reactive oxidation product NAPQI cannot be detected in ESI-MS. However, if GSH (m/z 308) is added, NAPQI will be transformed to a polar GSH adduct being amenable to electrospray ionization and yielding an m/z of 457.

Madsen et al. used EC for the simulation of the phase I and II metabolism of diclofenac.[45] The electrochemically generated oxidation products were trapped with GSH, which resulted in several GSH adducts. The purely instrumental set-up allowed the thorough characterization
of the adducts by means of LC/MS/MS, which was beneficial for the subsequent analysis of more complex biological samples. From the MS/MS spectra obtained by means of EC/LC/MS/MS, selective reaction monitoring (SRM) transitions were defined for the triple quadrupole instrument which allowed the detection of several GSH adducts in *in vitro* and *in vivo* samples.

### Adduct Formation of Metabolites with Proteins

The trapping of electrochemically generated reactive species with larger molecules as proteins requires the consideration of some additional facts: As ion suppression resulting from high salt contents frequently hampers the mass spectrometric detection of proteins a chromatographic separation should be carried out prior to ESI-MS with the goal to improve the signal to noise ratio. If dedicated reversed phase columns with wider pores are used, most salts will elute within the void time, thus avoiding ion suppression, which may only occur upon coelution of protein (adducts) and salts. One good option to investigate protein adduct formation of reactive metabolites by means of EC/LC/MS is to use the protein β-lactoglobulin A (LGA). Due to its structural homogeneity and comparably low molecular mass as well as one reactive free thiol group, LGA adducts can be detected particularly well even in case of a limited mass gain. This has been demonstrated for the LGA adduct formation of various electrogeneated reactive metabolites based on quinoid structures but also for platinum containing drugs such as Cisplatin. As an example, the adduct formation of electrochemically generated NAPQI with LGA is presented in Figure 8. The upper part depicts ESI-MS data of the native and unmodified protein. Its raw spectrum is displayed on the left side. LGA shows the commonly observed charge distribution for proteins. After deconvolution (spectrum on the right) the neutral mass of LGA is obtained (18363 Da). It is obvious that the charge states indicate a shift towards larger m/z (left mass spectrum below) upon formation of the adduct between electrogenerated NAPQI and LGA. The almost quantitative conversion can best be observed in the deconvoluted spectrum on the right. It is likely that the modification of LGA through NAPQI occurred at the free thiol group. However, if the binding site has to be localized more precisely, a tryptic digest has to be carried out.

Figure 9 shows the workflow, which is used for investigations of drug-protein adducts using EC. After the generation of the adduct, trypsin is added and the mixture is incubated for at least 90 min. Trypsin is a protease cleaving peptide bonds selectively at the C-termini of arginine and lysine residues. Afterwards, the mixture of the resulting peptides is analyzed by LC/ESI-MS and the peptides can be identified based on data base matching. The tryptic digest of LGA, for example, results in the formation of 18 different peptides and the T13 peptide bears the free thiol group. In previous work, this peptide has already been identified as binding site for the EC generated diclofenac quinone imine, which was not surprising, because the reactivity of quinoid compounds towards thiol groups is commonly known. However, in some cases, more complex protein adducts are generated. In a publication by Lohmann *et al.*, two and threefold adduct formation of the nitrenium ion of clozapine with LGA has been described. Hence, it is possible that reactive species do not bind exclusively to the free thiol group of LGA, but also to other functional groups in this protein, for example to amino groups of the N-terminus or of lysine residues.

While LGA is an excellent model protein for initial studies on the one hand, its biological relevance is limited. Human serum albumin (HSA), on the other hand, is the most abundant protein in human blood and is synthesized in the liver. Since reactive metabolites are likely to be formed in the liver, HSA is one of the important potential target proteins *in vivo*. However, its mass spectrum is much more complex because it is larger (66 kDa) and much more structurally heterogeneous than LGA.
Another important protein for studies regarding drug-protein adducts is carbonic anhydrase (CA), which is present in red blood cells and responsible for the transport of carbon dioxide. Similar to LGA, it has one free thiol group that is susceptible for a modification through electrophilic metabolites. Drug-protein adducts of CA have already been identified, for example in rat liver cytosol incubated with bromobenzene, by Koen et al. in 2000.\cite{62} However, the identification of these adducts was accompanied by laborious sample preparation, including the isolation of proteins from the complex reaction mixture, the tryptic digest and the subsequent analysis with LC/MS. Concluding, a good approach to investigate protein adduct formation of reactive metabolites is to start with the model protein LGA and, if successful, to continue the studies with more relevant proteins.

**Comparison with Established Methods**

It is important to evaluate if the data obtained by EC/MS are comparable to the commonly used in vitro studies and thus are valuable to predict the in vivo situation. As mentioned above, liver cell microsomes belong to the most popular in vitro approaches for studying phase I metabolism. Therefore, it is reasonable to compare the results of EC approaches with those obtained by in vitro methods, although of course, the oxidation mechanism is quite different. Nevertheless, in many publications, a good correlation between EC/MS and HLM/RLM has been described.\cite{18-19, 41-42, 63-64} Already in the early 1980s, Shono et al. recognized that the enzyme-catalyzed N-dealkylation of four neuropharmaceuticals, observed in microsomal incubations, could be simulated by the electrochemical oxidation.\cite{65} In the following, many other groups showed that EC might be useful in order to obtain additional information in biological redox reactions. A review including the early applications of EC/MS in the simulation of metabolism reactions has been published by Volk et al. in 1992.\cite{66} The interest in EC/MS renewed with the increased use of ESI, which enabled the easy detection of oxidation products. However, it lacked a systematic investigation regarding the comparison of EC/MS with other in vitro approaches. Pioneering work in this field has been accomplished by Bruins and co-workers.\cite{43, 67} One of the most cited publications in this field is their systematic work published in 2003, in which they oxidized a range of pharmaceuticals comprising different structural moieties in a flow-through cell with a porous graphite electrode and compared the oxidation products to metabolites known from CYP450 enzyme reactions.\cite{43} They deduced that CYP450 reactions, which are initiated by an electron transfer, can be mimicked by the EC/MS system. This includes N-dealkylation, S- and P-oxidation, alcohol oxidation and dehydrogenation reactions. Reactions initiated by hydrogen atom transfer may not be imitated with direct electrochemical oxidations, Reactions requiring a hydrogen abstraction could not be mimicked, e.g., the hydroxylation of unsubstituted aromatic rings or O-dealkylation. Further reactions that were not mimicked by EC were the epoxidation of benzo[a]pyrene, S-dealkylation, the oxidative deamination of primary amines, the oxidation of aldehydes and the formation of N-oxides. Furthermore, it is important to note that enzyme-catalyzed reactions are regioselective, whereas electrochemical oxidations yield products resulting from the most labile sites, they can be described as chemoselective. in a molecule without stereochemical considerations.

With new working electrode materials emerging, the reactions occurring in vivo and in vitro, which could be simulated by EC, were extended. In a publication by Nouri-Nigjeh et al., the hydroxylation of the anesthetic lidocaine was investigated in presence of H$_2$O$_2$. The authors explain that lidocaine is oxidized through a mechanism `reminiscent of the reaction of oxoferryl radical cations during the aromatic hydroxylation of substrates by P450.` This reaction is driven by platinum-oxo species, which are formed at the platinum WE.\cite{68} The formation of N-oxides, which constitutes an important metabolic pathway in vivo, was also reported for lidocaine by the same authors for some drugs such as the anesthetic lidocaine.\cite{49} In this publication by Nouri Nigjeh et al., the formation of the N-oxide was
observed by applying a reductive potential at a gold working electrode. The authors describe that dissolved oxygen is reduced to the superoxide anion, which is then subject to disproportionation and follow-up reactions with water (1% H₂O content) under formation of reactive oxygen species (ROS) including hydrogen peroxide. Reaction of the compound with hydrogen peroxide leads to the formation of the respective N-oxide. Johannson et al. were also able to generate lidocaine-N-oxide applying an electrochemically assisted Fenton reaction. The xenobiotic was altered through indirect oxidation with hydroxyl radicals. In addition to the usually observed reactions in direct EC, an aliphatic hydroxylation, in this case of testosterone, a hydroxylation of inactivated aromatic rings like mephenytoin, a benzylic hydroxylation and O-dealkylation of metoprolol were mimicked successfully. The application of WE materials such as Pt or BDD in combination with a highly positive oxidation potential enables the generation of hydroxyl radicals as already mentioned above. These radicals can then undergo further reactions yielding oxidation products which cannot be observed in direct EC. Baumann et al. for example, achieved an aliphatic hydroxylation of the muscle relaxant tetrazepam by applying an oxidation potential of 2000 mV vs. Pd/H₂ at a Pt WE. Compared with HLMs and urine samples from patients showed that the entire oxidative metabolism could be simulated by the electrochemical approach. In this case, the transformation was most likely achieved by a combination of electron transfer reactions (direct electrochemical oxidation) and indirect oxidation mediated through electrochemically generated hydroxyl radicals. Compared with approaches based on any biological systems, EC/MS based metabolism studies allow to detect reactive metabolites directly. In contrast, reactive metabolites generated on HLM or RLM studies will rapidly bind to constituents of the biological matrix and can therefore only be detected by LC/MS indirectly as their adducts, if at all. One respective example is the electrochemical oxidation of the antimalarial agent amodiaquine (AQ), which yields the corresponding quinone imine (AQQI). In ref. AQQI was the major species that was electrochemically generated at 700 mV vs. Pd/H₂, whereas it could be detected in in vitro incubations only as the respective adduct after trapping with GSH.

Structure Elucidation of Metabolites
As nuclear magnetic resonance (NMR) spectroscopy is the most powerful method for structural elucidation of newly generated compounds, it also is of potential use as complementary method to EC/MS experiments. Major disadvantage is its limited concentration sensitivity, leading to limits of detection in the high µg to low mg range, depending on individual compound and information required. In contrast, structure elucidation by means of LC/MS/MS requires lower amounts of analyte and is easy to carry out. The matrix-free surrounding, when using EC, allows the detection and fragmentation even of reactive species. The usefulness of EC for fragmentation experiments was demonstrated by Jahn et al., when they simulated the phase I metabolism of the calcium channel blocker verapamil. They detected a large variety of more than 20 electrochemically generated products. These were thoroughly characterized by fragmentation experiments, which allowed the deduction of structures. With respect to the combination of EC and NMR for structure elucidation, there are several approaches reported in literature. In 1975, Richards and Evans developed an EC cell for in situ NMR, in order to study short lived species in real time during electrolysis. They concluded that for more stable oxidation products, it would be sufficient to couple an external EC cell to a flow-through probe of an NMR. This approach was described by Simon et al. in 2012 for the electrochemical oxidation of paracetamol. They were able to detect the oxidation products NAPQI and benzoquinone and, by integrating the signals, they calculated a conversion of 15% for the parent drug. Very recently, Bussy et al. published a work describing the oxidation of phenacetin and the application of in situ NMR for the elucidation
of the electrochemical reaction pathway. However, for more complex oxidation mixtures, a chromatographic separation prior NMR analysis is required to avoid that the NMR spectrum will be too complex. For this purpose, it is useful to select an offline electrochemical approach with subsequent product purification by preparative LC. Such an approach was used by Madsen et al. who identified the reactive metabolite O-quinone methide from troglitazone. Another example is given by Khera et al., who oxidized the statines simvastatin and lovastatin. They separated the oxidation mixture and collected several fractions which were then analyzed by MS, MS/MS and 1D- and 2D-NMR. With the emerging combination of LC, online solid phase extraction and NMR (LC/SPE-NMR) it is expected that the online combination of EC/LC/SPE-NMR will gain more importance. The application of LC/SPE-NMR will offer the opportunity to use optimum conditions for oxidation, separation and NMR analysis.

Quantification of Metabolites
Quantification of reactive metabolites is a challenging task. As already mentioned above, they normally have a short lifetime, thus preventing their direct detection in blood or urine. If reactive metabolites are conjugated in the liver to endogenous compounds, they can be detected, e.g., as mercapturic acids or glucuronides in urine. Furthermore, if a drug is transformed through phase I reactions, reactive metabolites will not necessarily be the major metabolites, so that the concentrations of these reactive species are often rather low. Two examples are the pain reliever paracetamol with its minor but very reactive metabolite NAPQI and diclofenac, which is transformed to 5-OH-diclofenac to only a small extent, but which is known to be more reactive than its 4'-hydroxylated isomer. The most accurate way of metabolite quantification with LC/ESI MS requires isotope labeled internal standards that allow to compensate signal suppression in the electrospray interface. However, these standards are often not commercially available or their synthesis is expensive and extremely time-consuming.

The combination of EC/MS with an element selective detector opens another road for metabolite quantification. If the xenobiotic compound and its metabolites contain at least one atom of an element not being the ubiquitous elements (C, H, N, O), the noble gases or the most highly electronegative elements (F, Cl), inductively coupled plasma-mass spectrometry (ICP-MS) offers the unique possibility for substance-independent quantification due to atomization of all analytes in the plasma. One example of EC used in combination with LC/ICP-MS is provided in the reference by Lohmann et al. They achieved the relative quantification of electrochemically generated oxidation products of the iodine-containing drug amiodarone (Figure 10). After electrochemical oxidation, the products were separated isocratically by reversed phase HPLC. If an iodine-containing compound eluted from the column, a peak was observed in the mass trace for iodine (m/z 127). Its peak area was directly proportional to its concentration. In case of the parent compound amiodarone, this concentration had to be divided by two, since amiodarone contains two iodine atoms per molecule.

For elements such as sulfur, the detection by means of ICP-MS (isotopes $^{32}$S and $^{34}$S) requires further optimization due to spectral interferences ($^{16}$O$^{16}$O or $^{16}$O$^{18}$O). One example is the detection of clozapine and metabolites by Vanhaecke et al. The simultaneous detection of $^{35}$Cl$^+$ and $^{32}$S$^+$/$^{34}$S$^+$ with a sector-field ICP-MS allowed to distinguish between unreactive metabolites and species, which reacted with the trapping agent GSH, thus showing also a signal in the sulfur trace. The quantification of GSH conjugates of clozapine was reported in 2011 by Wilson and co-workers, who used the sulfur-containing drug omeprazole for external calibration. A similar approach was used for the detection of diclofenac metabolites in rat urine by Corcoran et al. Problems when using LC/ICP-MS are related to the fact that the signal for the analyte is influenced by different plasma conditions caused by a varying content...
of organic mobile phase. For this reason, isocratic LC elution allows LC/ICP-MS detection with particular ease. If it is not possible to achieve an isocratic elution of all compounds in an acceptable time, a step-like gradient can be applied. Baumann et al. have recently used ICP-MS for the analysis of the arsenic-containing drug melarsoprol. The solvent composition was changed after 4.5 min to enable the separation of all compounds. In order to quantify under these circumstances, it is necessary to add an arsenic-containing compound for each gradient step. Another possibility for the compensation of solvent effects is the application of a countergradient. By the implementation of a second LC pump into the system, a second gradient flow is added after the LC separation which compensates for the first gradient. Thus, a constant solvent composition is provided and the plasma conditions remain stable.

**Future Trends**

Although there is still an increasing number of publications dealing with the application of EC for metabolism studies, it has not been established yet for routine analysis. Even though the recording of a mass voltammogram itself does not take long, data evaluation is more laborious and time consuming. As a fast screening method, the application of EC should be as easy as possible. Therefore, it is expected that the automation of the whole EC/MS system, including the generation of mass voltammograms, will be an issue in the future.

Besides, it is expected that EC will expand to other areas as well, for instance, environmental research. One example is given by the group of Küppers et al. in ref. Among other things, they compare the electrochemical oxidation of three different xenobiotics and conclude that EC/MS seems to predict the environmental persistence of these compounds. Another example concerning the application of EC/MS in environmental research is the elucidation of oxidation products occurring after oxidative water treatment. Similar to metabolism studies, the fate of a compound during such treatment processes has to be elucidated in order to exclude the formation of more toxic products.

Another interesting application of EC/MS is its upcoming use in protein research. Several publications have already highlighted the possibility of peptide and protein cleavage as well as the reduction of disulfide bonds prior their MS detection. In the latter publications this disulfide reduction had a positive effect on the fragmentation and thus on the sequence analysis of the respective proteins. Very recently Mysling et al. used electrochemical reduction as an alternative for a chemical reduction step for the study of hydrogen/deuterium exchange, monitored by MS. They concluded that EC can substitute a chemical reduction step, but also identified some challenges such as an impaired reduction by a high salt content.

**Conclusion/Summary**

Although electrochemistry (EC) finds widespread use in analytical chemistry, the application of EC for drug metabolism studies for the simulation of oxidative liver metabolism has gained more interest in the last decades. The purely instrumental approach is useful for the prediction of many phase I and phase II metabolic reactions, including, e.g., the hydroxylation of activated aromatics and the adduct formation with small biogenic thiols such as glutathione or thiol-containing proteins. The mechanisms taking place in EC and enzyme-catalyzed approaches are different and therefore the metabolism of a compound cannot be completely mimicked. However, the application of EC has several advantages over conventional in vitro metabolism studies. The detection of reactive species generated by the purely instrumental approach is possible due to the absence of a biological matrix. This allows follow-up investigations with respect to the assessment of the reactivity by using different trapping
agents. Second, it is also possible to carry out a small scale synthesis of standards, which is beneficial for further structure elucidation by means of LC/MS/MS or NMR, respectively. Although this technique will not replace the conventional in vitro or in vivo studies, it may provide additional valuable information, which will otherwise not be available. Thus, under carefully optimized electrochemical conditions, it is possible to benefit from this complementary approach.

References

Author biographies

Helene Faber studied Food Chemistry at the University of Münster, Germany and finished her final thesis in the group of U. Karst on HPLC with biochemical detection. After obtaining her 2nd state exam for certified food chemists in 2010, she re-joined the group of U. Karst for her Ph.D. thesis in the field of electrochemistry coupled to mass spectrometry, which she finished in 2013. Her research interests are in the field of LC/MS and EC/LC/MS with particular focus on pharmaceutical, food chemistry and environmental chemistry applications.

Martin Vogel studied Chemistry at the University of Münster, Germany and earned his Ph.D. in 2001 in the field of derivatizing techniques for LC and LC/MS. He took over a position as Lecturer at the University of Twente, The Netherlands in 2001. In 2006, he accepted a position as Lecturer at the University of Münster and was promoted to Senior Lecturer in 2012. He is currently Chairman of the Working Party of Analytical Chemistry of the German Chemical Society GDCh and has organized and co-organized several international meetings in the fields of separation sciences and hyphenated techniques. His main research interests focus on hyphenated techniques based on chromatography, including LC/MS and EC/LC/MS.

Uwe Karst obtained his Ph.D. in Analytical Chemistry at the University of Münster, Germany in 1993. He moved to the University of Colorado in Boulder, CO, USA as a postdoctoral research associate. After his return to the University of Münster, he finished his Habilitation in 1998. In 2001, he took over a position as Full Professor of Chemical Analysis at the University of Twente, The Netherlands. He accepted his current position as Chair of Analytical Chemistry at the University of Münster in 2005. His research interests focus on hyphenated analytical techniques and their (bio)medical and pharmaceutical applications, including elemental speciation analysis, metallomics, mass spectrometric imaging and electrochemistry/MS.
Figure Captions

Figure 1: Simplified scheme for the metabolism of xenobiotics. CYP450: Cytochrome P450, GSH: glutathione, Gluc: glucuronic acid, GST: Glutathione-S-transferase.

Figure 2: Overview of in vivo and in vitro assays, listed in order of their in vivo resemblance, adapted from Brandon et al.\cite{16}

Figure 3: Schematic overview of different cell types for electrochemical oxidation: (a) coloumnetic flow through cell\cite{88}, (b) amperometric thin-layer cell\cite{88}, (c) microchip cell\cite{31}, (d) electrochemical oxidation inside an electrospray(ES) emitter\cite{34}, REF: reference electrode, AUX: counter or auxiliary electrode, WE: working electrode, HV: high voltage, LV: low voltage, GC: glassy carbon, BDD: boron doped diamond. All pictures are based on the references cited herein.

Figure 4: General set-ups used for EC(/LC)/MS: Mass voltammograms are recorded after direct connection of the EC cell effluent to the MS (upper route). The lower route is used to separate electrogenerated products prior to MS by collection of the EC cell effluent in an injection valve and subsequent injection of the analyte filled in the loop.

Figure 5: Mass voltammogram of the electrochemical oxidation of ticlopidine (TCL, 1x10^{-5} M in 10 mM formic acid pH 3/ACN 50/50 v/v), which was recorded by applying a potential ramp from 0-2500 mV at a scan rate of 10 mV/s using a thin-layer cell (analytical cell 5040, ESA Biosciences, Chelmsford, MA, USA) equipped with a BDD working electrode.

Figure 6: Mass spectra of ticlopidine recorded at an oxidation potential of 2500 mV (1x10^{-5} M ticlopidine in 10 mM FA/ACN 50/50 v/v, analytical thin-layer cell with BDD electrode, flow: 10 µL min^{-1}). Left: FT-mass spectrum, right: time-of-flight mass spectrum.

Figure 7: Adduct formation of reactive metabolites with glutathione (GSH). Paracetamol (APAP, m/z 152, 5x10^{-3} M) was pumped through the electrochemical cell and the potential was ramped from 0-2500 mV within 250 s. GSH (m/z 308, 1x10^{-4} M) was added with a second syringe pump to the effluent via a mixing T-piece. The resulting NAPQI-GSH adduct (m/z 457) was detected by means of ESI-MS in the positive ionization mode.

Figure 8: Adduct formation of reactive metabolites with LGA. APAP (1x10^{-4} M) was oxidized at a constant potential of 1500 mV and the effluent of the EC cell was incubated with LGA (1x10^{-5} M). The mixture was analyzed by means of LC/ESI-ToF-MS. Above: unmodified LGA, below: LGA after reaction with electrochemically generated NAPQI. Figure 9: Simplified scheme of studying drug-protein adducts using EC. First, the reactive metabolite is generated by EC and trapped by the protein. A more precise localization of the binding site can then be carried out by a tryptic digest. The resulting tryptic peptides are analyzed by LC/ESI-MS and identified based on data base matching.

Figure 10: Structure of the iodine-containing drug amiodarone.
Figure 5

[Diagram showing mass spectrometry results with peaks labeled with m/z values and intensity on the y-axis.]
Figure 6

TP
C₁₄H₁₀N₃⁷CIS
m/z = 262.0771

DHP
C₁₄H₁₀N₃⁵CIS
m/z = 262.0457

m/z = 262.0433
Figure 7

[Diagram with mass spectra and molecular structures]

[APAP+H]^+  
m/z 152

[GSH+H]^+  
m/z 308

[NAPQI-GSH+H]^+  
m/z 457

Intensity [×10^6]

Mass-to-charge (m/z)

Oxidation Potential  
vs. Pt/H_2 (mV)
Figure 8