Ferrocene-Based Derivatizing Agents for LC/MS and LC/EC/MS

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FERROCENE-BASED DERIVATIZING AGENTS FOR LC/MS AND LC/EC/MS

DISSERTATION

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on account of the decision of the graduation committee,

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The dissertation has been approved by the promotor:

prof. dr. Uwe Karst

Für meine Eltern

Contents

Abbreviations

General abbreviations

AA	amino acid
AAS	atomic absorption spectroscopy
AATP	4-acetamidothiophenol
AED	atomic emission detector
ACN	acetonitrile
APCI	atmospheric pressure chemical ionization
CDL	curved desolvation line
CEP	cell exit potential
CGE	capillary gel electrophoresis
CID	collision induced dissociation
CV	cyclic voltammetry
$(Cys)_2$	cystine
Cys-Gly	cysteinylglycine
$(Cys\text{-}Gly)_2$	cysteinylglycine disulfide
Da	Dalton
DBA	dibutylamine
DMAP	4-(*N,N*-dimethylamino)pyridine
DNA	deoxyribonucleic acid
DP	declustering potential
DPV	differential pulse voltammetry
DRC	dynamic reaction cell

DTT	dithiothreitole
EC	electrochemistry
ECD	electrochemical detection
ECL	electrochemiluminescence
EI	electron impact
EIC	ethyl isocyanate
EP	entrance potential
ESI	electrospray ionization
FBA	ferroceneboronic acid
FC	ferrocene
FCA	ferrocenecarboxylic acid
FCC	ferrocenecarboxylic acid chloride
Fc-PZ	ferrocenoyl piperazide
FEM	*N*-(2-ferroceneethyl)maleimide
FMEA	ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide
FMTAD	4-ferrocenylmethyl-1,2,4-triazoline-3,5-dione
GC	gas chromatography
GOD	glucose oxidase
GSH	glutathione
GSSG	glutathione disulfide
HCys	homocysteine
$(HCys)_2$	homocystine
HDI	hexamethylene diisocyanate
HPLC	high performance liquid chromatography
HRP	horseradish peroxide

IPDI	isophorone diisocyanate
IR	infrared
LC	liquid chromatography
LC/MS/MS	liquid chromatography tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
m/z	mass-to-charge ratio
MAMA	9-(*N*-methylaminomethyl)anthracene
MALDI	matrix assisted laser desorption ionization
MDI	methylenebis(phenylisocyanate)
MIC	methyl isocyanate
2-MP	1-(2-methoxyphenyl)piperazine
MPG	2-mercaptopropionylglycine
MRM	multiple reaction monitoring
MS	mass spectrometry
Nac	*N*-acetylcysteine
$(Nac)_2$	*N*-actetylcystine
NBDPZ	4-nitro-7-piperazinobenzo-2-oxa-1,3-diazole
NH_4ac	ammonium acetate
NMR	nuclear magnetic resonance
ODN	oligodeoxynucleotides
OPA	*o*-phtalaldehyde
PAH	polyaromatic hydrocarbon
PCR	polymerase chain reaction
PenSH	penicillamine

PhIC	phenyl isocyanate
2-PP	1-(2-pyridyl)piperazine
PQD	pulsed collision induced dissociation
PUR	polyurethane
RSD	relative standard deviation
RP	reversed phase
RT	room temperature
s	second
SIM	single ion monitoring
SFP	3-ferrocenyl-succinimidylpropionat
SWV	square wave voltammetry
t	time
T_m	melting temperature
t_R	retention time
TCEP	tris-(2-carboxyethyl)-phosphine hydrochloride
THF	tetrahydrofuran
TEA	triethylamine
TIC	total ion current
TLC	thin-layer chromatography
TLV	threshold limit value
TOF	time of flight
TMS	trimethylsilane
UV	ultraviolet

Abbreviation of amino acids

Name	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1

Introduction

1.1 Introduction and scope

Ferrocene was discovered accidentally in 1951 by Kealy and Pauson [1], who tried to obtain bis(cyclopentadien), $(C_5H_5)_2$. They reacted cyclopentadienylmagnesium bromide with iron(III) chloride and obtained a stable, orange-colored compound. At the same time, Miller, Tebboth and Tremaine [2] independently obtained bis(cyclopentadienyl)iron by a different route. Both groups proposed a di-σ-complex structure. The compound showed remarkable stability: It was unaffected by water, strong acids and bases and remained stable at high temperatures. Woodward and coworkers [3] discovered the possibility of Friedel-Crafts acylation of ferrocene in 1952. They assumed a sandwich structure with equivalent C-H bonds as well as equivalent Fe-C_5H_5 bonds and suggested the name 'ferrocene' with the 'ene' ending implying aromaticity. Fischer and Pfab confirmed the structure by X-ray experiments [4].

In the next decade, a large number of ferrocene reactions and derivatives were discovered. Yielding the ferrocinium cation at low potentials, ferrocene and its derivatives show a reversible redox behaviour. Due to this and other unique properties, ferrocenes have been frequently used in analytical chemistry. An overview about this topic is given in **Chapter 2**. The analytical methods are linked to the special properties of ferrocene: Beside purely

electrochemical methods, like cyclic voltammetry and differential pulse voltammetry, and the use of electrochemical detection for HPLC separation, ferrocene derivatization was used to enable the detection by atomic spectroscopy methods and electrochemiluminescence. Furthermore, detection limits in mass spectrometry were improved and ferrocene was used as a mass tag to determine the number of functional groups. Additionally, a summary of ferrocene reagents used for the derivatization of various functional groups is presented.

In general, the best sensitivity with ESI-MS is achieved for analytes, which exhibit ionic character in solution. As a result, medium polar compounds like phenols only yield poor mass spectra. Therefore, common derivatizing agents contained either ionic functionalities, such as a quaternary amine groups, or other functionalities, such as amines or carboxylic acids, which can be ionized in solution by proper adjustment of the pH. In ferrocene, a polar group, which can be easily protonated or deprotonated, is missing. Instead, the ESI-MS spectrum of ferrocene is dominated by the intact molecular cation $[M]^+$, for which one-electron oxidation in the electrospray interface is responsible [5]. The ferrocene-based chemical derivatization for MS was introduced by Van Berkel et al. [6]. They made use of the electrochemical processes being inherent to ESI to enhance MS signal intensities. As preionic compounds, ferrocene derivatives are relatively non-polar before their oxidation to the ferrocinium cation. They are perfectly suited for LC/MS measurements, as reversed-phase liquid chromatography provides best results for moderately polar analytes. Consequently, the hyphenation of LC/EC/MS was introduced

by Diehl et al. [7] for ferrocene derivatized phenols and alcohols. For quantitative conversion to the ferrocinium cation, an electrochemical flow-through cell with a porous glassy carbon working electrode possessing a very high surface area is introduced between the column and the mass spectrometer. Additionally, selectivity is gained by using an atmospheric pressure chemical ionization source in the heated nebulizer mode to introduce the sample into the mass spectrometer. Thus, only compounds, which are already ionic in solution, are detected.

The goal of this thesis is to apply LC/EC/MS to the determination of other functional groups than the hydroxyl group. Environmental and bioanalytical topics will be covered in this thesis, which focuses on the determination of isocyanates (**Chapter 3**) and thiols (**Chapter 4, 5** and **6**). Both types of compounds are chemically instable, thus requiring a derivatization step prior to analysis.

In **Chapter 3**, the synthesis of ferrocenoyl piperazide (Fc-Pz) as a new derivatizing agent for isocyanates is presented. Commonly used mono- and diisocyantes are derivatized and detected by LC/EC/MS. The dependence of the mass spectrometric response on the applied cell potential is demonstrated. Analytical figures of merit are obtained by calibration measurements with the synthesized standards. The method is applied to the analysis of air after thermal degradation of polyurethane foam.

In living cells, free thiol groups can be found in proteins and in low molecular mass components. The free cysteines of a protein can participate in substrate binding and catalysis. The formation of disulfide bonds between adjacent or distant cysteine groups contributes to stabilization of the active conformation of polypeptide chains [8]. By labeling with ferrocenes, it is possible to find out which cysteine residues in proteins are involved in disulfide bridges and which of them are free, as is described in **Chapter 4**. *N*-(Ferroceneethyl)maleimide (FEM) is used as a derivatizing agent, which was originally invented for HPLC/ECD measurements by Shimada et al. [9]. The signal intensity of ferrocene-labeled peptides in LC/EC/MS is enhanced compared with LC/MS, and a trypic digest of a model protein is analyzed.

The low molecular mass thiols in living cells act as major endogenous antioxidants and as important regulators of cellular homeostasis [10]. The proportion of free thiols to disulfides of low molecular weight thiols in body fluids is a marker of stress and several diseases. **Chapter 5** describes the simultaneous quantitative determination of cysteine, homocysteine, glutathione, cysteinylglycine and *N*-acetylcysteine and the corresponding disulfides. Differential derivatization of free thiols and disulfide-bound thiols is applied using two different ferrocene-based derivatizing agents, one being the known FEM and the second one the newly developed derivatizing agent ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide (FMEA). Tandem MS experiments enable the detection of unknown thiols. The application of this method is shown for urine samples.

In **Chapter 6,** the differential derivatization is applied to proteins. Three whey proteins (α-lactalbumin, β-lactoglobulin B and β-lactoglobulin A) are selected as model compounds to show the applicability of this method to protein mixtures. The differentiation between free (FEM-labeled) and bound cysteine residues (FMEA-labeled) is performed by LC/MS with precursor ion scan and LC/EC/MS. The assignment of FEM and FMEA-labeled cysteines in the amino acid sequence of a single tryptic digest peptide is possible by fragment ion spectra.

General conclusions and some remarks concerning the advantages and drawbacks of using ferrocene-based derivatizing agents in LC/MS and LC/EC/MS analysis as well as future perspectives on the topic are discussed in **Chapter 7**, which concludes this work.

1.2 References

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[2] Miller, S. A.; Tebboth, J. A.; Tremaine, J. F., *J. Chem. Soc.* **1952**, 632-635.

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[9] Shimada, K.; Oe, T.; Nambara, T., *J. Chromatogr.* **1987**, 419, 17-25.

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Chapter 2

Ferrocene-Based Derivatization in Analytical Chemistry[‡]

Ferrocene-based derivatization has raised considerable interest in many fields of analytical chemistry. This is due to the well established chemistry of ferrocenes, which allows the rapid and easy access to a large number of reagents and derivatives. Furthermore, the electrochemical properties of ferrocenes are attractive with respect to their detection. This chapter summarizes the available reagents, the reaction conditions and the different approaches for detection. Electrochemical detection is still most widely used for ferrocene derivatives, e.g., in the field of DNA analysis. However, the emerging combination of analytical separation methods with electrochemistry, mass spectrometry and atomic spectroscopy allows using ferrocenes more universally and in novel applications, where strongly improved selectivity and limits of detection are required.

[‡] Seiwert, B.; Karst, U. submitted for publication.

2.1 Introduction

Since the discovery of ferrocene in 1951, many of its derivatives have been synthesized and characterized. They are of considerable interest in various areas of research and application, like asymmetric catalysis [1], electrochemistry [2], functional biomaterials [3] and even for medical purposes [4, 5]. A large variety of attractive analytical applications of ferrocene and its derivatives has been published as well.

The present chapter highlights the detection oriented derivatization with ferrocenes in analytical chemistry. The review mainly focuses on the direct covalent derivatization of various functional groups by ferrocenes as well as on the respective separation and detection techniques. Other important analytical applications of ferrocenes, in which no covalent derivatization is carried out, are only mentioned in those cases, where they help to understand general reaction mechanisms or detection strategies. This is particularly valid for the use of ferrocenes as electron transfer reagents (‘mediators’) in electrochemical biosensors, which has already been summarized in reviews by Ali et al. and Hill [6-8]. The mediators serve to facilitate the electron transfer between an enzyme and the working electrode of an amperometric transducer, but are not involved into any covalent bond formation or cleavage and do therefore not fit into the focus of this article. The same is valid for selected approaches for electrochemical DNA detection [9], which have also been subject of recent reviews.

Ferrocene and its derivatives are mostly neutral compounds and, unlike many other organometallics, form stable complexes in the presence of water and air. However, they are pre-ionic compounds, because they are highly reversible redox systems, which may readily be switched electrochemically between the ferrocene and form the respective ferrocinium cation at low potentials. A typical cyclic voltammogram of ferrocene derivatives, in this case the ferrocenoyl piperazide (Fc-Pz) derivative of ethyl isocyanante is shown in Fig. 2.1.

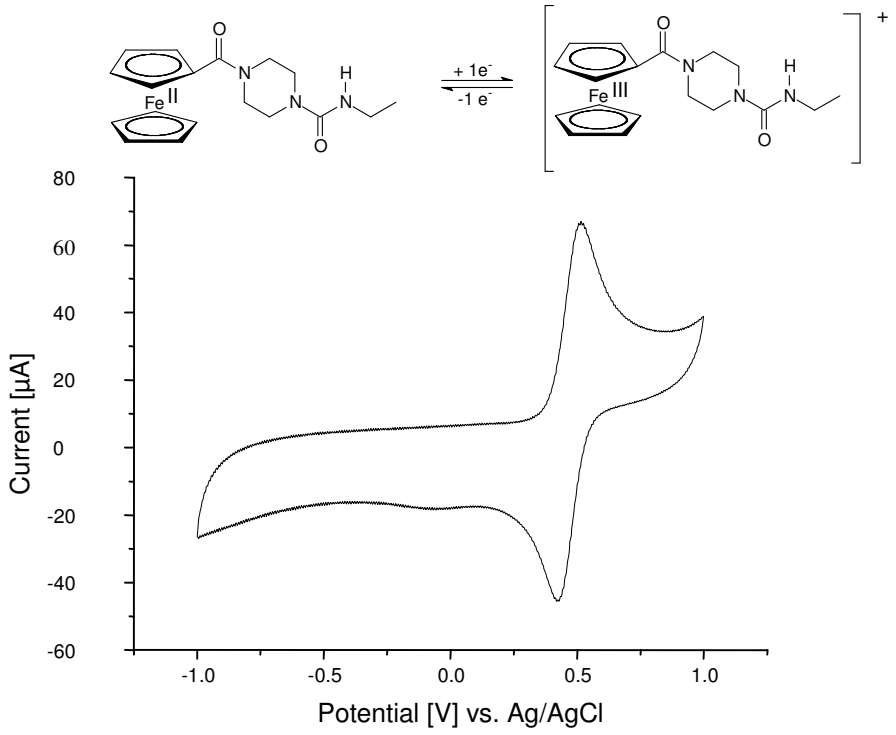


Fig. 2.1 Cyclic voltammogram of the ferrocenoylpiperazide derivative of ethylisocaynante.

Furthermore, the chemistry of ferrocenes is well explored and a large variety of ferrocene derivatives is easily accessible via established synthetic routes. The redox behaviour of ferrocene is sensitive to its covalent or non-covalent binding to other molecules. An unique property of metallocenes is the

possibility of introducing substituents on either one or both of the cyclopentadienyl rings while retaining the properties of a simple one-electron redox couple. The electrochemical oxidation potential is tunable by changing the nature of the substituents. Therefore, ferrocenes allow the use of a large variety of electrochemical detection techniques, including amperometry or voltammetry.

Other detection techniques are possible as well. UV/vis absorbance detection is an option due to the intense color of the ferrocenes. It is well known in literature that the color of the ferrocenes strongly changes upon oxidation, thus allowing spectroelectrochemical measurements [10]. Iron as central ion furthermore allows the use of atomic spectroscopy for ferrocene detection [10]. Atomic absorption spectroscopy as well as inductively coupled plasma excitation with optical emission or mass spectrometry have been used to detect ferrocene derivatives [11-16].

Naturally occurring iron consists of four isotopes: 5.8 % of ^{54}Fe, 91.7 % of ^{56}Fe, 2.2 % of ^{57}Fe and 0.3% of ^{58}Fe [16]. The resulting characteristic isotopic pattern of ferrocene enables the identification of an iron containing compound in complex mixtures when using ‘organic’ mass spectrometry, e.g., electron impact (EI) or electrospray (ESI) MS. The measured as well as the calculated isotopic pattern (dotted line) of the cysteine derivative of FEM are presented in Fig. 2.2.

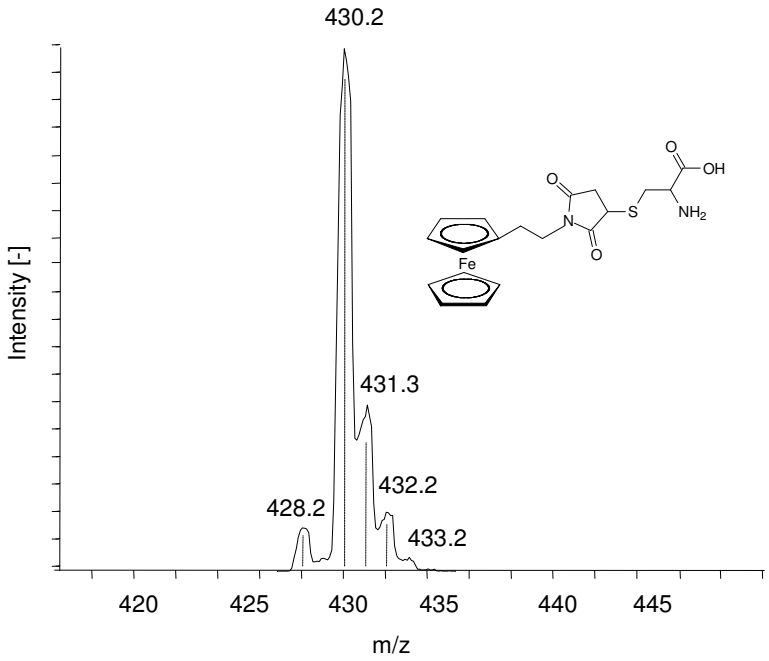


Fig. 2.2 ESI-MS spectrum of the cysteine derivative of FEM. Inserted is the chemical structure of the analyte as well as the calculated isotopic pattern (dotted lines).

2.2 Ferrocene-based derivatizing agents

Derivatization reactions mostly are unpopular in analytical chemistry, because they may involve laborious work protocols and require additional analysis time. However, in many cases, where direct analysis is not possible because chromophoric, fluorophoric or electroactive groups are missing or the analytes are chemically instable, derivatization often is the best option. A suitable derivatization technique may

- convert a chemically labile compound into a stable product,

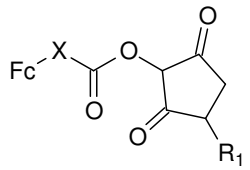
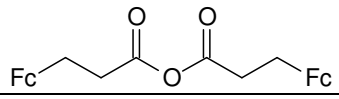
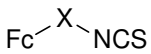
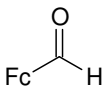
- enable or improve the use of more selective or more sensitive detectors

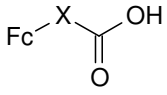
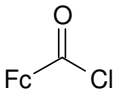
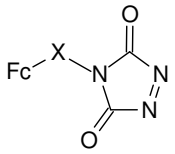
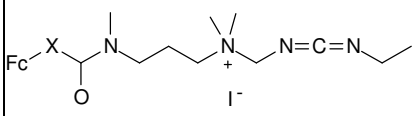
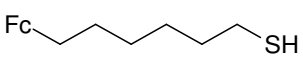
and/or

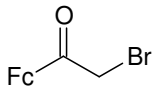
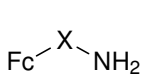
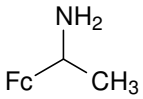
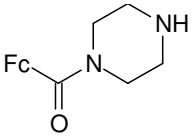
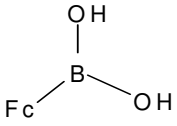
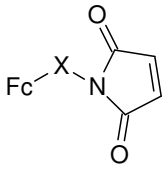
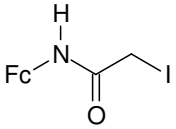
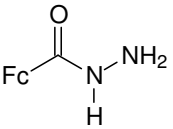
- modify the polarity of a compound to improve its chromatographic separation.

In the past, a large variety of ferrocene-based derivatizing agents was developed for several functional groups. These reagents are listed in Tab. 1, apart from solid phase synthesis approaches and low yield methods, being not suitable for quantitative analysis, that are not considered. From Tab. 1 it is obvious that the largest number of the known ferrocene-based derivatizing agents is dedicated to the analysis of amine functionalities, including small organic molecules and amino acids as well as peptides or proteins. This correlates with the strong demand for powerful analytical methods for proteins and their constituents.

Tab.1.: Ferrocene-based derivatizing agents for different functional groups with appropriate reaction conditions, detection methods and references.

Analyte	Reagent	Reaction conditions	Analytical Method	Refs cited
amine	 X = $(CH_2)_n$ R_1=SO_3Na n = 0, 2 X = $(C{=}O)CH_2CH_2$ X = $CH_2NH(CH_2)_5$	pH 8, 20 min-3 h, RT	HPLC/ECD CGE/ECD on-chip/ECD ICP/MS AAS CV	[75, 76, 79, 80] [89, 87] [94] [12] [13, 16] [29, 49, 50, 23]
amine		triethylamine, RT, 15 min	HPLC/ECD	[75, 76]
amine	 X = $(CH_2)_n$ n = 0, 2	triethylamine, 70°C, 20 or 90 min	HPLC/ECD	[65]
amine		a)1.NaOH, 80°C-100°C, 2 h /10 h 2. $NaBH_3$, 1 h, RT b) EDC, RT, 30 min	HPLC/ECD AAS DPV CV	[74] [13, 14, 51] [21, 22] [13, 14, 51, 52, 61, 98]

Analyte	Reagent	Reaction conditions	Analytical Method	Refs cited
amine	 X = $(CH_2)_n$ n = 0-2 X = CH_2-(NH)-$(CH_2)_5$	a) EDC b) EDC/HOBt, triethylamine, RT, overnight c) isobutyl chloroformate, 2h, RT	CGE/ECD on-chip/ECD AAS MALDI-MS CV	[93] [94, 95] [13] [59] [18, 27, 37, 32, 34, 36, 49, 50, 52]
alcohol, amine		**alcohol** DMAP (catalyst), CH_2Cl_2 5 min **amine** NaOH, 20 min	HPLC/ECD GC/MS GC/AED LC/EC/MS ESI-MS CV	[74, 75, 77, 78] [124] [124, 131, 132] [77, 102, 121-124] [108, 113, 119, 120] [59]
alcohol, amine	NH_2-C-$(CH_2OCH_2Fc)_3$	$(C_6F_5CO)_2$, diisopropylamine or triethylamine RT, 1-2 h	DPV	[40, 41]
diene	 X = $(CH_2)_n$ n = 0, 2	RT, 15 min	LC/ESI-MS	[114, 115]
imino group	 X = $(CH_2)_n$ n =0, 2	pH 9, 6-10 h, 37°C	MALDI-MS SWV	[103, 59]
α,β-unsaturated carbonyl group		RT,12 h	HPLC/ECD	[72]

Analyte	Reagent	Reaction conditions	Analytical Method	Refs cited
carboxyl group		18-crown-6, KF, 80°C, 30 min	HPLC/ECD	[68, 70]
carboxyl group/ aldehyde	  X = $(CH_2)_n$ n = 0, 2, 3	**carboxyl group** a) EDC, HOBt, 37°C, 12h b) diphenylphos-phinylchloride/ triethylamine, 50 min, dark	HPLC/ECD AAS	[25, 69, 70, 71] [11]
		aldehyde $NaCNBH_3$, 24 h, RT	CV	[57]
isocyanate		RT, 30 min	LC/EC/MS	[112]
diol/alkene		**Diol** 70 °C, 10 min	HPLC/ECD GC/AED GC/MS ESI-MS	[73] [132] [125, 126] [113, 116,117]
		Alkene OsO_4 or $KMnO_4$, 70 °C, 10 min	ESI-MS	[111]
thiol	 X = $(CH_2)_n$ n = 0, 2 X = $(C{=}O)NH(CH_2)_2$ X = $(CH_2)_2(NH)(C{=}O)CH_2$	pH = 7, RT, 5 min and 1h	HPLC/ECD ESI-MS LC/MS LC/EC//MS CV DPV	[67] [19] [84] [105, 106] [19, 20] [97]
thiol		pH 7, RT, 24 h	ESI-MS	[24]
aldehyde		acidic pH	HPLC/ECD	[74]

2.3 Analytical methods used in conjunction with ferrocene-based derivatization

2.3.1 Electrochemical detection

2.3.1.1 General aspects of electrochemical detection

The ferrocene/ferrocinium couple shows a simple one-electron redox behaviour. Hence, it is used as reference redox system for organic and, with some limitations, for aqueous systems [26]. The derivatization with ferrocenes yields electroactive products even from non-electroactive analytes. The ferrocene group retains its speed and reversibility of electron transfer in more complex molecules, too. Therefore, ferrocene, many of its derivatives and even ferrocene labeled proteins [14, 27-29] are frequently used as mediators for electrochemical measurements with particular focus on amperometric biosensors. As this aspect was summarized by Hill and Frew [8], it will not be discussed in more detail here.

This part of the chapter therefore focuses on those analytical methods, where the electrochemical signal is based on the attachment, steric shielding or dissociation of ferrocene-based derivatizing agents. These include the electrochemical detection of changes in diffusion coefficients and changes upon reaction of the derivatives. Furthermore, this chapter includes the electrochemical detection based on the recycling of the redox couple.

2.3.1.2 Electrochemical detection of attached ferrocene derivatives

The derivatization with ferrocenes enables the detection of originally not electroactive analytes by electrochemical methods like cyclic voltammetry (CV), differential pulse voltammetry (DPV) and square wave voltammetry (SWV). The detected current is proportional to the concentration of the analyte at the electrode. The attachment method was used to identify derivatized biomolecules. Di Gleria et al. [30] used the attachment of ferrocenes to monitor cysteine-containing biomolecules by CV measurements. Ferrocenecarboxylic acid (FCA) was coupled by carbodiimide to enzymes with the goal to alter the electron transfer with the electrodes [31]. Several ferrocene labeled linkers for amino functionalities in proteins were evaluated by Tranchant et al. [32] with respect to the electrochemical detection by CV.

The attachment of ferrocene was used to monitor enzyme reactions as, for example, those catalyzed by tyrosinase (TR) [33]. TR catalyses the oxidation of tyrosine (or other phenols) to L-DOPA (or other respective catechol derivatives). The formed diol functionalities were derivatized by ferroceneboronic acid (FBA) and this derivatization was monitored by DPV. Furthermore, the ferrocene derivatization is applied to monitor immunological reactions. The biotinylated antigens are immobilized on streptavidin modified gold electrode and the antibody labeled with ferrocene moieties binds in a competitive immunoreaction to the immobilized antigen (Fig. 2.3) The signal is detected by DPV [34]. If nanostructured gold surfaces on silicon chips are used, this approach will reduce non-specific binding of proteins to a native gold surface [35].

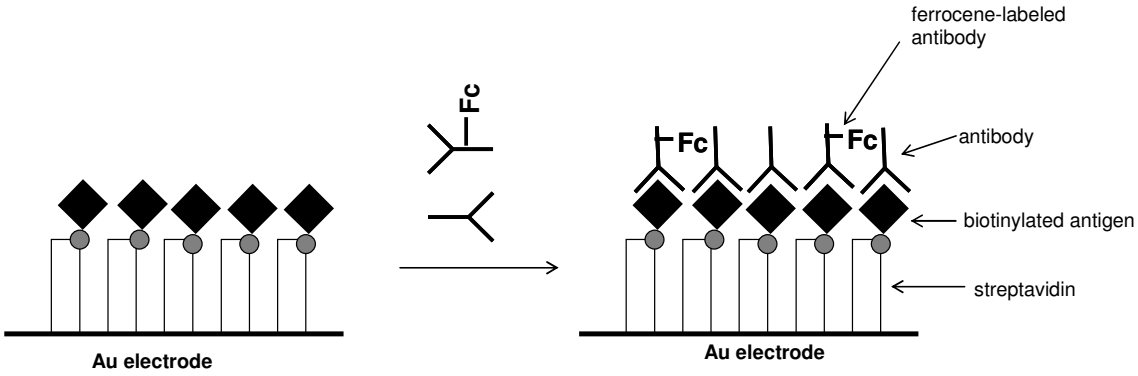


Fig. 2.3 Scheme of a competitive immunoassay with electrochemical detection.

In the sandwich electrochemical immunoassay [36], a capture antibody connected to the electrode reacts with the antigen and in a second step the antigen binds to the signal antibody containing the ferrocene unit as shown in Fig. 2.4.

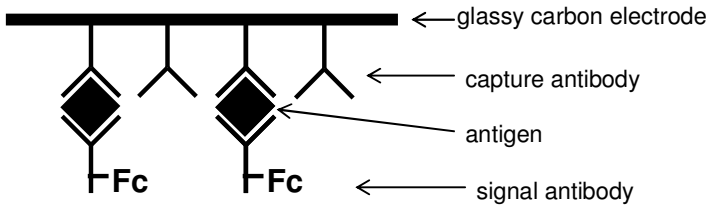


Fig. 2.4 A schematic representing of the molecular architecture of a sandwich electrochemical immunosensor.

The 'sandwich assay' is the most common design for an electrochemical DNA sensor as well (Fig. 2.5). The assay consists of three individual DNA components: an immobilized capture strand, a target strand and a probe strand containing the ferrocene reporter group. If all components are combined, an electrochemical response at the electrode is detected [37, 38].

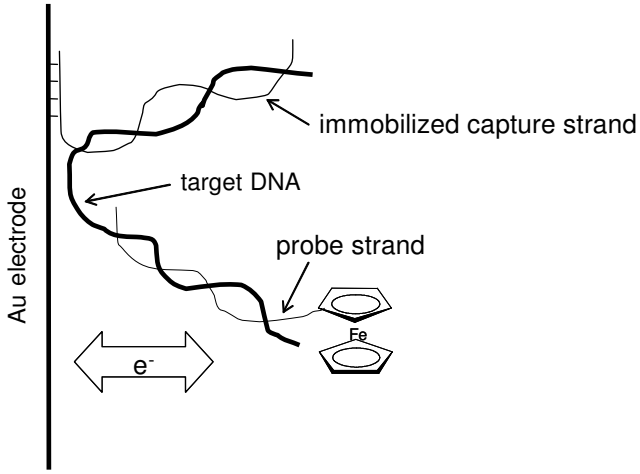


Fig. 2.5 Principle of the 'sandwich assay' for electrochemical DNA sensors.

A further development of this assay is done by using a two-piece reagentless assay for DNA. Therefore, the capture strand is connected via a linker to the probe strand [39]. Dehybridization from the surface-bound strand will occur at temperatures at which a fully matched strand remains hybridized to the surface-bound strand. Using this strategy, it is possible to detect single nucleotide mismatches [38]. However, the background current is very high, thus hampering the limit of detection for these measurements. Bioelectrocatalytic reactions were used to amplify the detection of ferrocene connected to DNA after hybridization [39]. For this purpose, the redox-active DNA replica is coupled to the glucose oxidase-mediated oxidation of glucose.

Furthermore, an amplification of the voltammetric signal may be obtained by the use of labels containing more than one ferrocene unit, like triferrocenyl-tris(hydroxymethyl)aminomethane (Tris-Fc) [40, 41], ferrocene derivatized diblock and triblock copolymers [42] or ferrocene coated nanoparticle/strepta-

vidin conjugates [43-45]. The remarkable sensitivity of the ferrocene-coated nanoparticles may be attributed to the large number of ferrocenes present at the surface (127 per gold nanoparticle) and close proximity with respect to the underlying electrode. The method was used for the determination of sulfhydryl groups in surface bound proteins or peptides [45] and for DNA hybridiztion detection [43, 44] as shown in Fig. 2.6. The block copolymer conjugates were utilized as DNA probes in a three-component sandwich-type electrochemical detection strategy. The single polymer-based DNA probe contains an average of approximately 10 ferrocenyl groups. Multilayering is possible as well and leads to further enhanced signals [42].

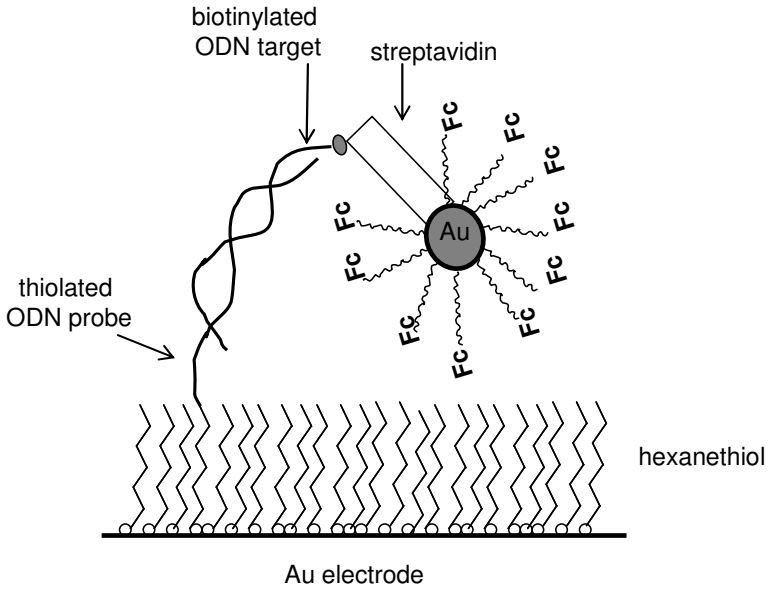


Fig. 2.6 Schemetic representation of the enhanced voltammetric detection of a biotinylated oligodeoxynucleotides (ODN) target.

2.3.1.3 Electrochemical detection of the disappearance of the electrochemical signal by steric hindrance

In another approach, steric reasons cause the disappearance of the electrochemical signal. One example is the change in flexibility and electron-

transfer tunnelling distance of the electroactive tags connected to the electrode. A ferrocene-labeled probe [46, 47] or labeled stem-loop oligonucleotide [48] that alters its flexibility by hybridization may be used to detect complementary target DNA.

This approach was applied to immunosensors as well: The biocatalyzed reaction by the antigen bound to the electrode surface of ferrocene-labeled glucose oxidase (GOD) disappeared when an antibody is bound to the antigen as shown in Fig. 2.7 [49, 50]. An electroactive double-conjugated protein, Fc-BSA-Dig [51] (with 39 molecules of ferrocene and 8 molecules of digoxin bound to BSA) or Fc-GOx-Dig (5 ferrocenes and 6 digoxin) [52] may be used for the same purpose with improved sensitivity. The amplification is due to the combined effect of enzymatic amplification and electrochemical amplification by multiple labeling with ferrocenes.

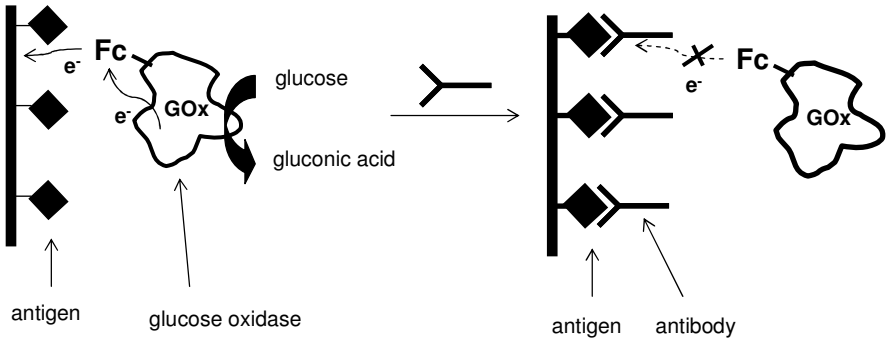


Fig. 2.7 Amperometric analysis of an antibody by an antigen monolayer electrode using a bioelectrocatalyst as redox probe.

The determination of thrombin is another application of this method. A bifunctional derivative of thrombin-binding aptamer with a redox active ferrocene moiety is used for this purpose. The signal in DPV is enhanced by

the change of the aptamer conformation at the electrode from the uncomplexed coil-like form to a quadruplex of the aptamer-thrombin association [53].

2.3.1.4 Electrochemical detection of change in redox response by dissociation of the ferrocene group

Changes in redox response upon dissociation of the ferrocene group may be used for analytical purposes as well: Ferrocene labeled helical peptides with a specific sequence are attached on an electrode. In the presence of matrix metalloproteinases (MMP), the electronic signal decreases because the part of the peptide with the attatched ferrocene is cleaved and the ferrocene as sensing element is not connected to the electrode surface anymore [54]. It is a sensitive route to detect the MMP activity. The dissociation approach was also applied to the detection of complementary oligonucleotides by hybridization with an electrochemically active oligonucleotide probe and subsequent dissociation of the terminally labeled 5' nucleotide by T7 exonuclease [55].

Ligase activity may be monitored by using tethered and ferrocene-terminated DNA hairpins. The exposure to DNA ligase is followed by conditions that denature the hairpin and ferrocene label dissociation is monitored by CV [56].

Another example is the sugar-induced disintegration of a ConA-ferrocene-modified glycogen multilayer film, which can be used for the determination of sugars at a millimolar level [57].

2.3.1.5 Electrochemical monitoring of changes in oxidation potential

The tunability of the ferrocene derivatives can be used to design systems for monitoring several reactions and to investigate catalysts or/and activity of enzymes. This is achieved by monitoring reaction products that have different electrochemical properties compared with the educts. An example is the amperometric assay for aldolase activity, where ferrocenylethylamine and the retro-aldol retro-Michael substrate are monitored at different potentials [58].

Ferrocene-based derivatives having different redox potentials were measured competitively on the electrode. By using ferrocenyl carbodiimide derivatives based on ferrocenecarboxylic acid and ferrocenepropionic acid, the standard and the test sample may be measured by square wave voltammetry within one measurement as shown for RNA and DNA [59].

2.3.1.6 Electrochemical detection based on the reversibility of the oxidation/reduction

The reversibility of oxidation/reduction of ferrocene can be used to detect ferrocene derivatives by a recycling bienzyme electrode. The electrode consists of a lactase-glucose dehydrogenase bienzyme membrane coupled with an oxygen electrode and can be used for the detection of immunoassays [60].

2.3.1.7 Electrochemical detection based on changes in diffusion coefficient

An affinity assay was developed by Mosbach et al. that is based on the modulation of the diffusion coefficient of an electroactive ferrocene label upon complementary recognition leading to an increase of the molecular weight [61]. Using an electroanalytical technique, which is correlated to the diffusion coefficient of the redox species, e.g. cyclic voltammetry, the decrease of the diffusion coefficient can be monitored as a decrease of the diffusion-limited current. The signal is intensified by redox cycling [62].

2.3.2 Electrochemical detection coupled to separations

2.3.2.1 HPLC with electrochemical detection

High-performance liquid chromatography (HPLC) with electrochemical detection (ECD) is a useful method for the determination of trace components in complex matrices because of its excellent selectivity and sensitivity as shown in earlier reviews about this topic [63]. The oxidation potential ranges from 0.2 to 0.6 mV vs. Ag/AgCl for most derivatives and depends on the substituents on the cyclopentadienyl ring. The electroactivity of ferrocene is generally even higher than that of catechols, whose oxidation to the *o*-quinone at 600 mV vs. Ag/AgCl has been used for routine analysis in HPLC/ECD. Ferrocenes can be detected selectively even in the presence of other electroactive aromatic compounds. The ferrocene derivative undergoes facile oxidation and the product can be readily reduced. A dual electrode detection scheme is used. The collection efficiency and thus the reversibility (ratio of the

current at the downstream detector to that at the upstream detector) of the ferrocenyl group is even higher than that of the catechol group [64].

The use of the ferrocenyl group as electrophore for HPLC/ECD measurements was first reported by Tanaka et al. [64]. *N*-Succinimidyl-3-ferrocenylpropionate and later ferrocenylisothiocyanate [65] were used as derivatizing agents for amines. The same group later introduced derivatizing agents for alcohols (ferrocenoyl azide and 3-ferrocenylpropionyl azide) as well [66]. The maximum sensitivity was obtained for the three derivatizing agents at 0.4 mV vs. Ag/AgCl with a detection limit of $3*10^{-8}$ M and $5*10^{-8}$ M, respectively. For thiols, *N*-(ferrocenyl)maleimide (FM) and *N*-(2-ferroceneethyl)maleimide (FEM) [67], for fatty acids 3-bromoacetyl-1,1'-dimethylferrocene [68] and ferrocenylethylamine [69-71] were used with comparable limits of detection. The ferrocenylethylamine derivatives of retinoic acid were detected by coulometric reduction (-100 mV) after on-line coulometric oxidation (+400 mV) with a detection limit of $2*10^{-8}$ M. Microcystin-LR was derivatized at its α,β-unsaturated carbonyl group and selectively detected by HPLC/ECD [72]. Gamoh et al. used ferroceneboronic acid (FBA) to derivatize diols prior to HPLC/ECD [73]. Kubab et al. [74] introduced ferrocenecarboxyhydrazide for aldehyde detection by HPLC/ECD. Other groups reported HPLC/ECD methods for peptides and proteins. In detailed studies by Eckert and Koller [75, 76] and Koppang et al. [77], several ferrocene-based derivatizing agents were investigated for their usefulness in peptide and protein analysis. Recently, the group of Koppang et al. [78] reported on a selective analysis method for secondary amines. The method

uses the selectivity of *o*-phtalaldehyde (OPA) to 'mask' primary amines prior to derivatization of the secondary amines by ferrocenecarboxylic acid chloride (FCC). Dual electrode detection (E_1=0.7 V and E_2=0.2 V vs. Ag/AgCl) was used to selectively detect FCC derivatives showing a complementary oxidation and reduction peak, whereas OPA derivatives show only an oxidation signal at the upstream electrode.

The coupling of HPLC and ECD can furthermore be used to detect the hybridization of a target nucleotide with a complementary oligonucleotide carrying an electrochemically active ferrocene group [79, 80]. The introduction of a ferrocene-labeled terminator by polymerase was measured by HPLC with coulometric detection [81], too. The method is rapid and is characterized by a detection limit comparable to that of radioisotope and enzyme-linked coloumetric assays. The sensitivity of the method depends on the stability of the hybrid complex as measured as its melting temperature (T_m). However, a higher T_m implies decreased mismatch sensitivity. By incorporation of polymerase chain reaction (PCR), the sensitivity of the HPLC/ECD-method can be enhanced at least thousandfold [82, 83] and quantification is possible since DNA amplification proceeds exponentially under low cycles of PCR.

A drawback of LC/ECD is the limitation with respect to isocratic elution. Only if special electrochemical methods that reduce the baseline increase are applied, e.g., with the use of a coulometric array detector, gradient elution may be carried out. LC/ECD with differential labeling with two ferrocene-based derivatizing agents is possible to enhance the selectivity. One possibility for

future applications is the differential derivatization of thiols and disulfides by *N*-(2-ferroceneethyl)maleimide (FEM) and ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide (FMEA), two ferrocene-based maleimides. [84].

2.3.2.2 Electrophoresis with electrochemical detection

In principle, the number of theoretical plates and therefore, the separation power of capillary electrophoresis is higher compared with HPLC.

Capillary gel electrophoresis (CGE) is the method of choice for DNA sequencing and fingerprinting, because it offers short run times, high efficiencies, low sample consumption, and it is compatible with automated sample injection [85]. The chip-based approach adds the ability to multiplex the analysis. Laser-induced fluorescence (LIF) is usually applied for detection. Although the method is very sensitive, it is relatively difficult to miniaturize and expensive due to the use of lasers and special optical components. Advantages of electrochemical methods are sensitivity, compatibility with microfabrication, simplicity and inexpensive instrumentation. As for the fluorescence approach, derivatization is mostly needed to enhance the general applicability of the method [86].

Ferrocene-based electrochemically active labels are particularly useful due to their diversity and tunable potential, which enable the simultaneous detection of different analytes as well. Applying sinusoidal voltammetry, minimal differences in redox potentials can be selectively monitored. This technique coupled to CGE separation was applied to low resolution of four color DNA

sequencing and has been pioneered by Brazill et al. [87]. Discrimination between one tag and all others is accomplished through a ‘phase-nulling’ technique. The signal for each tag is selectively eliminated, while the other three responses remain virtually unchanged. This analysis scheme allows the selective identification of differently tagged oligonucleotides. The same group demonstrated the applicability of the method to single nucleotide polymorphism screening of DNA [88, 89, 90]. The electroactive label is introduced by a primer with ferrocene acetate covalently attached to its 5’ end [89] or by a chain terminator like ferrocene-acycloATP [88, 90], which is incorporated by a thermostable polymerase. The extension product is separated by CGE from any excess terminator or the unextended primer and finally detected by sinusoidal voltammetry.

The first electrochemical capillary flow immunoassay was developed by Lim et al. [91, 92]. The separation is based on differences in the isoelectric points. Cation exchange capillary columns were applied to selectively trap the free ferrocene-labeled antibody, whereas the antibody-antigen complex with human choronic gonadotropin passes the column and can be detected by a three electrode flow-through cell [93]. The approach was later transferred onto a chip [94]. The multichanneled matrix column was functionally coated with cation exchange resin to detect histamine in blood. Another group [95] later developed a chip-based electrochemical immunoassay with two reaction formats: a direct and a competitive mode of operation. The reagents were mixed in a precolumn, followed by an immunochemical reaction. For the direct protocol (Fig. 2.8a), the free labeled antibody and the labeled antigen/antibody

complex were separated by electrophoresis and detected downstream by a gold coated carbon screen-printed anode. For the competitive immunoassay, the labeled antigen bound to the antibody was separated from the free labeled antigen and the internal standard (Fig. 2.8b). Recently, the separation of an immunoassay was performed on-chip by a column packed with boronate-activated agarose beads [96].

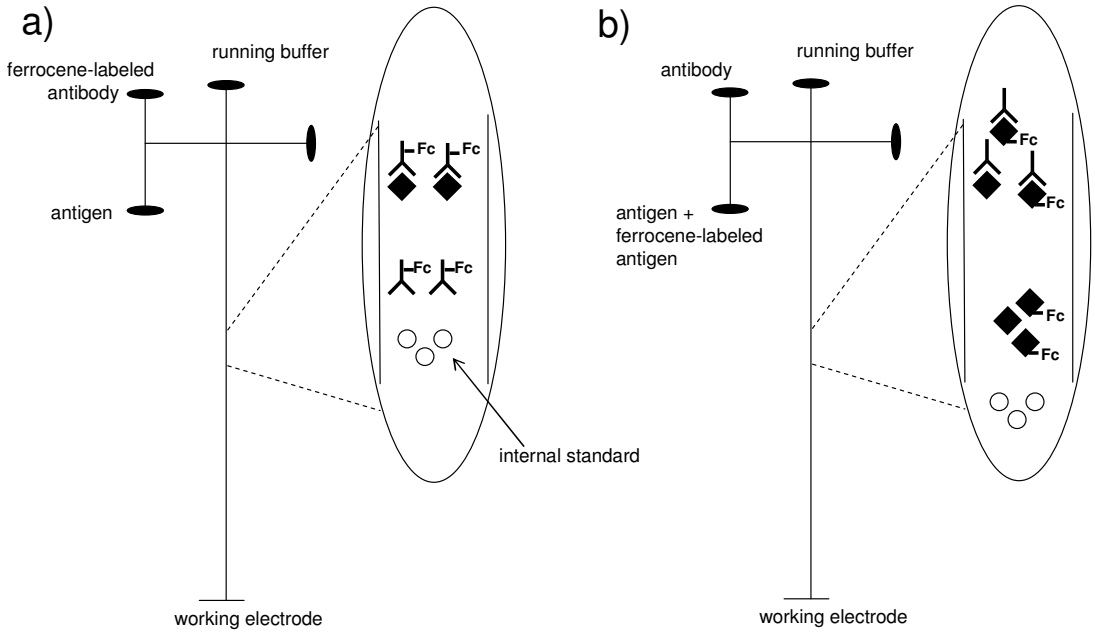


Fig. 2.8 Schematic of the on-chip (a) direct and b) competititve) electrochemical immunoassay protocols.

2.3.2.3 Separation based on the ionic and preionic character of ferrocene derivatives

Selective concentration of positively charged compounds and repulsion of negatively charged compounds is one way of separation. The activity of the protein kinase A can be monitored using this approach [97]. A gold electrode modified with anionic thioctic acid selectively accumulates and detects the positively charged ferrocene-labeled substrate. Protein kinase A introduces a

phosphate group changing the net charge to negative and repulsion takes place.

The polyanionic perfluorosulfonated Nafion polymer is known for its ability to exchange and accumulate cationic or procationic species and to repel anionic compounds. The preionic character enables the enrichment of ferrocene derivatives as ferrocinium salt in Nafion by applying an anodic potential. By cathodic stripping, the accumulated ferrocene derivative is released from the film in its neutral form [98]. By using these electrodes, it is possible to discriminate between species that are oxidized at the same potential. Electrochemical enzyme assays for the detection of alkaline phosphatase [99, 100] were developed based on this method. One of them applies ferrocenylethyl phosphate as substrate. The Nafion film acts as an electrostatic barrier against the ester phosphate (Donnan exclusion) and the enzyme-generated ferroceneethanol is preconcentrated and detected [100]. A Nafion-loaded carbon paste electrode was used to amplify immunoassay signals by a preconcentration step of the derivatives labeled with ferrocene ammonium salts [101].

2.3.3 Mass spectrometric (MS) detection

2.3.3.1 General considerations for MS detection

The derivatization with ferrocene increases the mass of the analyte. Furthermore, an iron atom, an electroactive group and a predetermined fragmentation site may be introduced into the analyte. These new properties facilitate the analysis of several compounds by mass spectrometry. Mass

spectrometric detection may be targeted to either the iron atom (‘inorganic’ mass spectrometry) or the complete derivatizing agent (‘organic’ mass spectrometry).

The mass increase and the amount of iron correlate with the number of ferrocene labels per analyte. If quantitative derivatization takes place, the number of functional groups will correlate with the number of ferrocenes. Therefore, mass spectrometric approaches as inductively coupled plasma mass spectrometry (ICP-MS) [12, 44] or electron impact (EI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), or matrix assisted laser desorption ionization-mass spectrometry (MALDI-MS) may be applied.

2.3.3.2 ICP-MS

For the ICP-MS approach, the concentration of the analyte has to be known to determine the number of labels [44]. The detection of iron by ICP-MS may lead to erroneous results due to various polyatomic interferences such as $^{38}Ar^{16}O^{+}$, $^{40}Ar^{14}N^{+}$ on $^{54}Fe^{+}$, and $^{40}Ar^{16}O^{+}$ on $^{56}Fe^{+}$ and $^{40}Ar^{18}O^{+}$ on $^{58}Fe^{+}$. An effective method for the reduction of spectroscopic interferences is the use of a dynamic reaction cell (DRC) and/or collision cell technique or the application of high resolution ICP-MS with a double focusing sector field instrument. Ammonia as reaction gas is used to eliminate argon-based spectral interferences for the analysis of iron [102]. Under the conditions used by Deng et al. [12], the detection limit was 3.2 ng/L for Fe. Furthermore, ICP-MS may be used for the quantification of ferrocene-labeled analytes. Ferrocene

tethered hydroxysuccinimide ester was either directly or via horseradish peroxidase (HRP) as a bridge coupled to a monoclonal antibody of 2,4-dichlorophenoxyacetic acid. Competitive immunoreactions were carried out on a microplate. The bound conjugate was dissolved in 1% (v/v) nitric acid with In as internal standard and the signal of ^{56}Fe was detected by DRC-ICP-MS [12].

2.3.3.3 MALDI-MS

The mass of a ferrocene-based mass tag typically exceeds 200 Da. The mass tag can therefore easily be determined even with low resolution mass spectrometers. Especially MALDI-TOF-MS may be used for that purpose, leading to the singly protonated molecule even for proteins and other large molecules. The technique was used to verify the performance of labeling methods for proteins, DNA [38,59,88-90,103,104] and for the detection of single based extension products utilizing a 5' ferrocene labeled primer [89].

2.3.3.4 ESI-MS

Peptides, proteins and other large molecules lead to a charge state distribution in ESI-MS. By deconvolution, the mass of the analyte may be determined. ESI-MS was applied to determine the number of thiol and disulfide groups in proteins. The analyte was labeled stepwise [105] with *N*-(2-ferroceneethyl)maleimide (FEM) and differentially labeled [106] by FEM and ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide (FMEA).

However, the detection of the mass increase is not the main reason why derivatization by ferrocenes is performed for MS measurements. ESI has

been reported to show excellent results for the determination of ionic and polar analytes, since they are either already ionized or can easily be ionized under comparably soft conditions. Analytes of lower polarity are less accessible for protonation or deprotonation, resulting in losses of sensitivity. Although being non-polar, ferrocenes are preionic molecules: Due to the electroactivity at relative low potential, the ESI-MS spectrum of ferrocene is dominated by the intact molecular cation $[M]^+$. An one-electron oxidation mechanism, which takes place in the electrospray interface is responsible for this effect [107]. The ferrocene-based chemical derivatization for MS was introduced by Van Berkel et al. [108]. This method has proven to be an effective tool for transforming analytes that are neither ionic nor readily ionized in solution into products, which are amenable for ESI-MS detection. In general, signal levels for ferrocenes in ESI-MS are significantly improved using this approach. This expands the applicability of ESI-MS to a broader range of (non-polar) compounds and adds detection selectivity as well, because only one specific functional group is derivatized. The one-electron oxidation is performed with excellent results by either a specially designed interface [109] or by an electrochemical cell positioned before the interface [110]. A further advantage of the electrochemical cell approach is that the potential can be adjusted precisely to allow selective oxidation. A nice approach is the use of a heated nebulizer as interface, for which a commercial APCI interface with the corona discharge switched off may be used most conveniently [110]. By this method, only pre-ionized substances were detected. The spectra of most derivatives are very simple, usually consisting of the molecular cation with no or only little fragmentation. The applicability of

the method was demonstrated for alcohols and phenols [110], alkenes [111], isocyanates [112], neutral steroids [113] and 1α-hydroxyvitamin D [114, 115]. The detection limits are in the nanomolar range. The ferrocene derivatives show a characteristic isotopic pattern due to the natural isotopic distribution of iron. This facilitates the determination of ferrocenes in complex mixtures.

Furthermore, derivatization has been used to direct the fragmentation. Ferrocene derivatization of carbohydrates was employed to obtain more information out of the tandem MS spectra, which are dominated by the loss of water molecules for the underivatized carbohydrates. The distinction between the diasteromers of isomeric low molecular weight mono- and disaccharides may be achieved this way [116-118]. Each epimer shows the diagnostic ions derived from the cyclic boronate at *m/z* 254 (Fig. 2.9). The other fragment ions represent the successive loss of formaldehyde (30 Da) and water (18 Da).

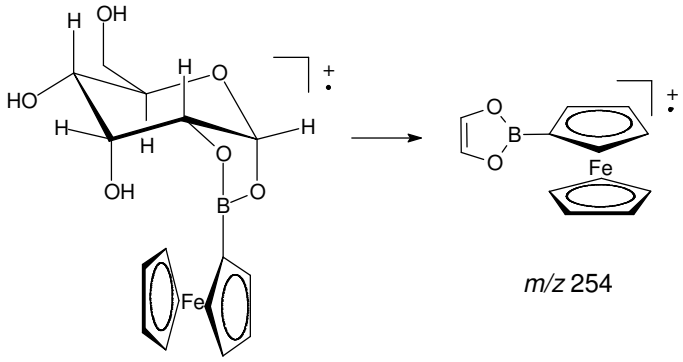


Fig. 2.9 Fragmentation scheme for FBA derivatives.

Most ferrocene-based derivatizing agents undergo a characteristic fragmentation pathway in ESI-MS. A loss of 65 Da, corresponding to the symmetrically coordinated ligand Cp (leaving as a radical), is the most abundant fragment of ferrocene in ESI-MS experiments. This fragment occurs as well for ferrocene derivatives, which are directly connected to an alkyl

chain. It was observed for amine derivatives of 3-ferrocenyl-succinimidylpropionat (SFP) and thiol derivatives of *N*-(2-ferrocene)ethylmaleimide (FEM) (Fig. 2.10).

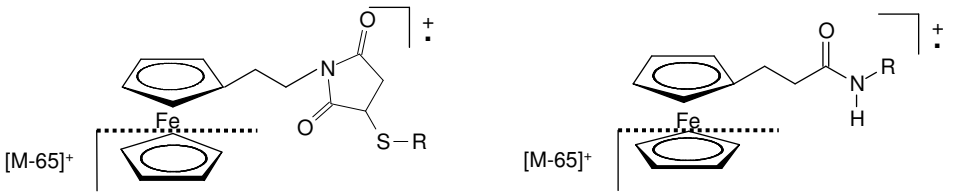


Fig. 2.10 Fragmentation scheme for FEM and SFP derivatives.

Thus, detection selectivity for these derivatives is enhanced by using the neutral loss mode. Furthermore, several fragment ions yield high intensities, which enable their sensitive detection in the multiple reaction monitoring (MRM) mode.

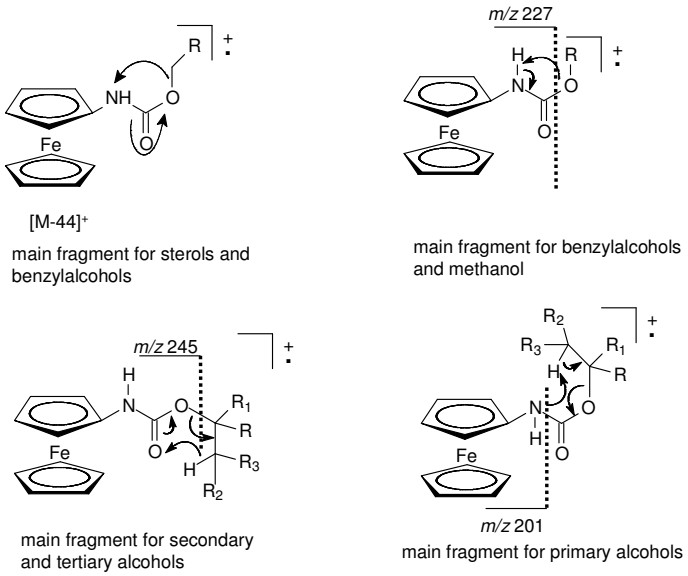


Fig. 2.11 Fragmentation scheme for ferrocenoyl azide derivatives.

Van Berkel and co-workers investigated the fragmentation pathways for ferrocenoyl azide derivatives of phenols and primary, secondary and tertiary alcohols. By using tandem mass spectrometry, including both precursor ion

scan (*m/z* 201, *m/z* 227, *m/z* 245) and neutral loss scan mode ([M-44]$^+$) (Fig. 2.11), it is possible to distinguish between these groups of compounds [108, 119]. Furthermore, the use of energy resolved product ion spectra leads to information about the molecular structure, including positional isomers [120]. The differentiation between alcohols and phenols is possible for FCC derivatives by the presence or absence of *m/z* 213 and *m/z* 230. The fragment ions *m/z* 213 and *m/z* 185 are also observed for FMEA derivatives of thiols [84] and iscocyanates derivatized by ferrocenoyl piperazide (Fc-Pz) [112] (Fig. 2.12).

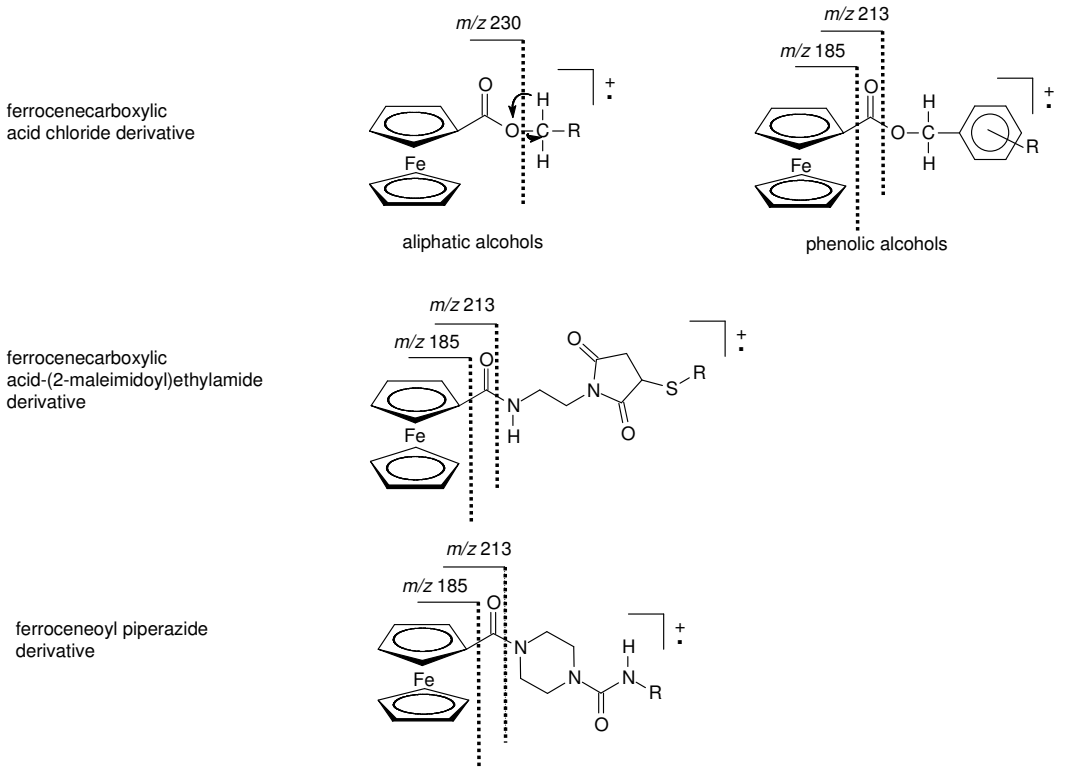


Fig. 2.12 Fragmentation scheme for FCC, FMEA, Fc-PZ derivatives.

The 4-ferrocenylmethyl-1,2,4-triazoline-3,5-dione (FMTAD) derivative of Vitamin D shows *m/z* 199 as main fragment [113] (Fig. 2.13). The transition was used for the sensitive monitoring of the analyte. The MRM mode was not

very sensitive for the underivatized analyte because the fragment ions, produced by the neutral loss of water molecules, only show low abundance.

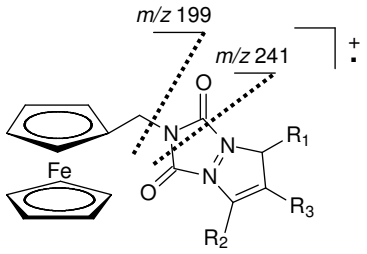


Fig. 2.13 Fragmentation scheme for FMTAD derivatives.

2.3.3.5 LC/MS and LC/EC/MS

Analytes of low polarity are best suited for RP-HPLC separations. Ferrocenes add a relatively large non-polar group to the analyte and thus improve the RP-HPLC separation. Because of the preionic character of ferrocenes, they can be easily oxidized post-column in an electrochemical cell and detected by MS very sensitively. This combination was introduced by Diehl et al. for the determination of phenols and alcohols [121-123], and was later applied for the analysis of isocyanates as well [112].

The degree of polarity of derivatizing agents can further be tuned by functional groups at the alkyl side chains. This possibility was used for the development of FMEA. It is slightly more polar than FEM and the separation of the derivatives of both reagents can easily be performed. The method was applied to the differential labeling of disulfides and thiols. While the FMEA derivatives elute first, the FEM derivatives elute later [84]. Precursor ion scan and neutral loss scan may, in addition to the discrimination by retention time, be used to distinguish between both groups of derivatives.

The combination with electrochemistry enables the selective detection of ferrocene-labeled analytes. The charge state distribution in ESI-MS spectra of multiply charged analytes is broadened and shifted to higher charges. In many cases, the signal intensity will be increased if the electrochemical potential is applied due to oxidation of the ferrocenes to ferrocinium ions. The performance of the method was shown for a tryptic peptide mixture with the goal to selectively determine the cysteine containing peptides [105].

2.3.4 GC/MS

Although readily subliming at temperatures exceeding 100°C, ferrocenes are remarkably stable, thus enabling their detection also by gas chromatography (GC). Peaks of secondary alcohols tend to broaden and tail on different capillary columns, which makes their direct analysis difficult and inaccurate. As derivatizing agent for the GC-MS analysis of phenols and alcohols FCC was used [124]. The volatility of the FCC esters is high. With a temperature program, alcohols up to a chain length of 34 may be eluted. The MS may be used to assign alcohols and phenols. The mass spectra of alcohols show a fragment of *m/z* 230 corresponding to the ferrocenecarboxylic acid cation. Phenol ethers show a base peak of *m/z* 213 corresponding to the ferrocenecarbonyl cation and *m/z* 230 is absent. Thus, alcohol and phenol selective chromatograms can be recorded. In all cases, the $[M]^+$, which may be used to identify the compound, is present in the mass spectra as well [124]. The GC-MS enables the separation and identification of structural isomers due to its high resolution. In LC/MS, the chromatographic resolution is lower, thus leading to a more difficult separation of the lower alkylphenol

derivatives. For the higher molecular weight derivatives, both GC/MS and LC/MS will not allow to completely separate the very large number of possible structural isomers. The LC/MS method, however, is applicable to alkylphenols with higher masses.

The derivatization by FBA and thus the large increase in mass and in gas chromatographic retention time is an advantage for analysing low molecular mass diols [125, 126]. The mass spectra of electron impact ionization are dominated by molecular ions $[M]^+$. The ferrocenyl group largely inhibits fragmentation modes within the substrate moiety. The characteristic isotopic pattern reflects the presence of ^{10}B, ^{54}Fe and ^{57}Fe as minor natural isotopes and gives rise to distinctive isotopic patterns. Electron impact spectra show several reagent-derived ions (e.g. *m/z* 230 $[FcB(OH)_2]^+$, *m/z* 212 $[FcBO]^+$, *m/z* 186 $[FcH]^+$ and *m/z* 121 $[C_5H_5Fe]^+$). Thus, the selected ion monitoring mode enables the detection of individual boronates. All spectra of the derivatives contain a strong and characteristic ion at *m/z* 212 [125]. Furthermore, it is possible to distinguish between isomers as for example pinacol and 2-methylpentane-2,4-diol.

2.3.5 Atomic spectroscopy methods

2.3.5.1 General considerations for atomic spectroscopy

The ferrocene label contains an iron atom, which enables the determination of analytes by spectroscopic methods. Atomic spectroscopy is a powerful tool, because the calibration is in principle only dependent on the element, but not dependent on the individual compound.

2.3.5.2 Atomic absorption spectroscopy

The average number of ferrocene moieties bound to an analyte can be determined by using atomic absorption spectroscopy (AAS) [13]. The iron in the ferrocene derivatives is analyzed after direct aspiration of the aqueous solution using an air-acetylene flame and detection of the absorbance at 248.3 nm. The detection limit observed is 0.06 mg/L for iron [13].

Two concentrations have to be measured to determine the number of protein labels: The protein concentration is determined by a bicinchoninic acid protein assay [127], which is, however, associated with the same large uncertainty as many other methods for protein quantification. The iron content is determined after digestion with nitric acid at 100°C [16,14]. With these two concentrations, the average number of labels is determined.

An immunoassay based on the labeling with organometalic compounds was described by using the quantitative measurements of the ferrocene-labeled antigens by AAS [15]. The antibody was bound to a water-insoluble polymer. Upon centrifugation, the antibody was precipitated, carrying with it the

antibody-ferrocene-metallohapten-complex. Aliquots of the supernatant containing the residual ferrocene-labeled antigens were injected into a graphite furnace AAS [11].

2.3.5.3 Atomic emission detection

The idea to use GC analysis coupled to atomic spectroscopy after derivatization of chemical functionalities with element tags has been introduced by Hagen et al. [128].

Iron has excellent detection characteristics in AED: it may be detected at 302 nm down to 50 fg/s and exhibits a selectivity versus carbon of 4.6×10^6 [129]. Because volatile iron compounds are not naturally present, underivatized matrix compounds do not interfere. Thus, a simple and sensitive determination of alkylphenols after derivatization by FCC was developed. The method was shown to be useful for the quantification of 20 C_0-C_3-alkylpenols in crude oil [130, 131] and *o*-phenylphenol in citrus fruits [124].

Furthermore, atomic emission spectroscopy was used to quantify ferroceneboronate derivatives of 1,2- and 1,3-diols [132]. For ferroceneboronic acid derivatives, boron can be detected at 249 nm in addition to iron at 259 nm.

GC-AED is limited to volatile samples. The detection limit is much better than that of GC-MS. However, no information about possible coelution and about the mass and nature (phenol or alcohol) of the analyte is available. Therefore,

GC/MS and GC/AED may be considered as excellent complementary tools for ferrocene-based derivatization approaches with volatile analytes.

2.3.6 Electrochemiluminescence

Electrochemiluminescence (ECL) is a form of chemiluminescence, in which the chemiluminescence reaction is preceded by an electrochemical redox reaction. The oxidation of luminol by hydrogen peroxide as a classical chemiluminescence reaction is catalyzed by Fe(III), ferricyanide, horseradish peroxidase and other iron-containing compounds. Ferrocene and its derivatives catalyze the above mentioned system, too [133]. Due to this reaction, ferrocinium as well as oxidized ferrocene-labeled proteins can be detected down to subnanomolar amounts by a simple fluorimeter [16]. The ferrocinium cation, generated at the electrode reacts with luminol to an excited species which is deactivated by light emission (Fig. 2.14).

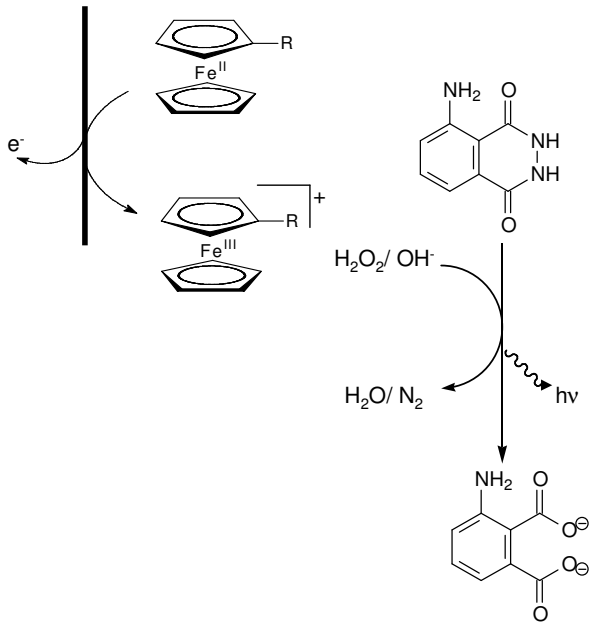


Fig. 2.14 Electrochemiluminescence reaction of luminol with catalytic effect of the ferrocinium cation**.**

The intensity of the light may be further enhanced by suitable substituents at the cyclopentadienyl ring [134]. This detection method can be applied to immunoassays. As long as the labeled antigen is free, luminol oxidation is catalyzed and chemiluminescence is observed. The ferrocene loses its catalytic activity when embedded into an antibody, thus resulting in a signal reduction [133]. The selectivity of the method will be limited if other catalysts are present and if the ferrocinium cation is unstable.

In another approach, ferrocene is used to quench ECL. Tris(2,2'-bipyridine)ruthenium(II) shows electrochemiluminescence in the presence of tripropylamine. The ferrocinium cation quenches the reaction, because it oxidizes the tris(2,2'-bipyridine)ruthenium(II). Using ferrocene as a quencher label on a complementary DNA sequence, an intramolecular ECL quenching in hybridized oligonucleotide strands has been realized [104].

The major advantage of the chemiluminescence approaches is the relatively simple assembly compared to other optical methods, because no light source is needed. A combination of chemiluminescence detector with an electrochemical cell could thus lead to a new detection method for ferrocenes in HPLC.

2.3.7 UV/vis absorbance

Due to the low molar absorptivity of ferrocene, a sensitive detection of ferrocene derivatives by UV/vis absorption is not possible. Thus, the methods using UV detection are not particularly promising for trace analysis. The ferrocene-ferricinium redox couple has significantly different UV/vis absorption

spectra. In ethanol, ferrocene is yellow and exhibits two absorption bands at 325 nm and 440 nm, whereas ferrocinium solutions exhibit a characteristic absorption peak at 620 nm [10]. Thus, ferrocene was used as a colourimetric dye for determining concentrations of glucose, glutamate, lactate and others by measuring the absorbance of the formed ferrocinium cation at 620 nm. Due to the improved stability and water solubility inclusion complexes of ferrocene and β-cyclodextrin were applied instead of pure ferrocene [135].

Spectrophotometric determination of the ferrocene content may be rapidly performed after release of iron from the ferrocene complex by trichloroacetic acid. Iron (III) in the acidic filtrate is reduced to iron(II) and after complexation with ferrozine [3-(2'-pyridyl)-5,6-bis(4'-phenylsulfonicacid)-1,2,4-triazine], spectrophotometric analysis at 564 nm is possible. The method was applied to determine the number of labeled functional groups in proteins. The detection limit was ten times lower compared with AAS, especially when a protein matrix was present [136].

2.4 Conclusions

It is evident that the role of ferrocene derivatization in analytical chemistry does by far exceed their function as mediators in biosensors. By derivatization, an electroactive label is introduced. The reversible redox behaviour of the ferrocene/ferrocinium couple at low potentials is a unique property, which finds widespread application. The electrochemical detection of ferrocenes is cheap and sensitive, and it is possible to detect minor differences in electrode potentials, which allow the analysis of differentially

labeled analytes. Several ferrocene labels can be determined within one measurement. The application of ferrocenes in HPLC/ECD is well established. However, the limitation to isocratic elution and problems with the limited separation efficiency of LC and identification of coeluting analytes restrict the general use. Capillary electrophoresis techniques coupled to electrochemical detection are promising due to their large separation efficiency. This is particularly useful for DNA and RNA detection as well as for immunoassays, which are to date the main applications of electrochemical detection combined with separations subsequent to ferrocene-based derivatization. Electrochemical detection methods on microchips have some significant advantages over fluorescence detection: They offer sensitivity, compatibility with microfabrication, simplicity and inexpensive instrumentation. As for the fluorescence approach, derivatization is mostly needed to enhance the general applicability of the method. Ferrocenes are readily available for this purpose. However, strong research activities are still required to develop new and improved ferrocene-based derivatizing agents for all of these applications.

The electrochemical ionization of ferrocene derivatized analytes is a useful tool to determine non-polar compounds, but a functional group has to be present in the molecule that undergoes facile reaction in order to introduce a ferrocene label. For more polar functionalities, the improvement in LC separation is important. Furthermore, the possibility of selective enhancement in LC/EC/MS compared to LC/MS is an option. Dedicated fragmentation is an additional opportunity that should be investigated in the future for other

functionalities. Ferrocene derivatives of nucleic acids have not been investigated with LC/EC/MS yet, although this appears to be promising.

The detection of ferrocene derivatives by ICP/MS and ICP/OES seems to be promising as well, especially combined with other metal complexes, thus using differential derivatization. The hyphenation with LC, CE or GC adds selectivity. The reduction of interferences with Ar species and reduction of operating costs are important issues in this area. GC/MS and GC/AED are only options for volatile derivatives and the applications are therefore limited.

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Chapter 3

Ferrocenoyl Piperazide as Derivatizing Agent for the Analysis of Isocyanates and Related Compounds Using Liquid Chromatography/Electrochemistry/Mass Spectrometry (LC/EC/MS)‡

Ferrocenoyl piperazide is introduced as a new pre-column derivatizing agent for the analysis of various isocyanates in air samples using reversed-phase liquid chromatographic separation, electrochemical oxidation/ionization and mass spectrometry. The non-polar derivatives can be separated using a phenyl-modified stationary phase and a formic acid/ammonium formate buffer of pH 3, which yields excellent separations especially for one crucial group of isocyanates consisting of 2,4- and 2,6-toluylenediisocyanate (2,4- and 2,6-TDI) and hexamethylenediisocyanate (HDI). Electrochemical oxidation at low potentials (0.5 V vs. Pd/H_2) leads to formation of charged products, which are nebulized in a commercial atmospheric pressure chemical ionization (APCI) source, with the corona discharge operated only at low voltage. Limits of detection between 6 and 20 nmol/L are obtained for the isocyanate derivatives, and calibration is linear over at least two decades of concentration. The method is applied for the analysis of air after thermal degradation of a polyurethane foam, and it is demonstrated that it is suitable as well for the analysis of carboxylic acid chlorides and of isothiocyanates.

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3.1 Introduction

Isocyanates (R-N=C=O) nowadays find widespread application in the manufacture of pharmaceuticals, pesticides and polyurethanes (PUR). Isocyanate-based polymers have found many applications, e.g. as paints, adhesives, insulations, sealants, textile fibers, lacquers and finishes. The industrial use of isocyanates is mainly based on their high reactivity towards nucleophilic agents, e.g. alcohols or amines, often showing quantitative reaction yields without any side reactions [1]. However, this reactivity causes a high toxicity. When inhaled, isocyanates can bind to human tissues, proteins and DNA, forming toxic adducts and metabolites which may cause adverse health effects especially to the respiratory system, including acute pulmonary oedema, chronic obstructive pulmonary diseases and bronchial asthma [2,3].

Occupational exposure can occur at many workplaces while handling the native compounds, during spraying isocyanate-based paints or when heating and processing polyurethane (PUR) or related products [4]. It is known that not only those diisocyanates are released that were used during manufacture, but complex mixtures containing also lower isocyanates as degradation products [5].

In order to comply with the 5 ppb threshold limit value (TLV), identification and sensitive quantification of mono- and diisocyanates in workplace air is very important. As the analytes are very reactive, direct spectroscopic methods are not applicable to the analysis of individual isocyanates and derivatization methods must be used. Early methods were based on colourimetric

techniques with restricted selectivity and sensitivity [6], while modern analytical approaches focus on derivatization with nucleophilic reagents, followed by liquid chromatographic (LC) separation and photometric or fluorimetric detection. In the past decades, compounds containing amine functionalities, such as 1-(2-methoxyphenyl)piperazine (2-MP) [7-12], 9-(N-methylaminomethyl)anthracene (MAMA) [13,14], 1-(9-anthracenylmethyl) piperazine (MAP) [15,16], 1-(2-pyridyl)piperazine (2-PP) [17] and 4-nitro-7-piperazinobenzo-2-oxa-1,3-diazole (NBDPZ) [18] were introduced as derivatizing reagents. In recent years, the use of mass spectrometry as detection technique became more and more important because of its high selectivity and low limits of detection. A sensitive MS method makes use of dibutylamine (DBA) as derivatizing reagent [4, 5, 12, 19]. While DBA is only suited for MS detection due to the lack of chromophoric or fluorophoric groups, other reagents that were previously used with photometric detection are now applied with increased sensitivity in tandem MS detection [20, 21].

Ferrocene-based derivatizing agents have been used in combination with chromatographic separations and selective detectors since long. Gas chromatography (GC) of ferroceneboronates of diols and related compounds with electron impact mass spectrometric detection was described by Brooks and Cole [22]. Rolfes and Andersson derivatized phenols with ferrocenecarboxylic acid chloride, separated the derivatives by GC and used the highly selective atomic emission detector for quantification [23, 24]. The majority of publications on ferrocene-based derivatizing agents, however, describes the use of LC with electrochemical detection [25-33]. Shimada et al.

[25] introduced *N*-substituted ferrocene-containing maleimides for the derivatization of thiols with subsequent LC and dual-electrode coulometric detection. Fatty acids were determined by the same group based on derivatization with a bromoacetylferrocene, LC and electrochemical detection [26]. Similar approaches were introduced for the determination of brassosteroids using ferroceneboronic acid by Gamoh et al. [27], and for amino acids, peptides and proteins by Eckert and Koller [28, 29], Cox et al. [30] and Shimada et al. [31, 32]. Lo et al. recently presented ferrocenehexanethiol for the pre-column derivatization of microcystins [33].

Van Berkel et al. introduced the use of the electrospray interface itself as electrochemical reactor after derivatization of various groups of analytes with ferrocenes [34-37]. Electrochemical oxidation of the derivatives leads to formation of the respective ferrocinium ions, which are detected in the mass spectrometer with excellent limits of detection, as demonstrated for the analysis of alcohols in food extracts [34] and plant oils [35]. The fragmentation pathways of the derivatives were elucidated in further work of the same group [36]. Ferroceneboronic acid was used by Van Berkel et al. to analyze alkenes after their oxidation to diols [37], and by Williams et al. to determine several neutral mono- and disaccharides [38] and various estrogens [39]. While all of these methods do not involve a separation, Karst et al. used the combination of pre-column derivatization with ferrocenoyl azide, LC separation, electrochemical conversion and MS detection for the determination of alcohols and phenols [40]. The method could be combined with rapid separations on

very short LC columns [41] and was applied for the analysis of alcohols and phenols in mineral oils [42].

Based on this work, it was considered to develop a dedicated ferrocene-based method for the analysis of isocyanates with reversed-phase liquid chromatography, electrochemical conversion and mass spectrometry. The respective data are presented within this chapter.

3.2 Experimental part

3.2.1 Chemicals

Ferrocenecarboxylic acid, 4-(*N,N*-dimethylamino)pyridine (DMAP), piperazine, acetyl chloride, most isocyanates, propyl isothiocyanate, formic acid and ammonium formate were purchased from Aldrich Chemie (Steinheim, Germany) in the highest purity available. Methyl isocyanate was obtained from Chem Service (West Chester, PA, USA). For the preparation of the mobile phases for HPLC, acetonitrile and water (both HPLC gradient grade) were obtained from Biosolve (Valkenswaard, The Netherlands).

3.2.2 Instrumentation

All HPLC/MS-experiments were performed on a LCMS QP8000 single quadrupole mass spectrometer (Shimadzu, Duisburg, Germany) equipped with an atmospheric pressure chemical ionization (APCI) source connected to a SCL-10Avp controller unit, DGU-14A degasser, two LC-10 ADvp pumps, SUS mixing chamber (0.5 mL), SIL-10A autosampler and a SPD-10AV UV/vis detector. The equipment used for on-line electrochemical oxidation was

obtained from ESA (Chelmsford, MA, U.S.A.) and comprised a Coulochem II electrochemical detector and a model 5021 conditioning cell. For the protection of the working electrode, a PEEK in-line filter (ESA) was mounted between column and electrode.

3.2.3 LC conditions

A binary gradient of acetonitrile and a formic acid/ammonium formate buffer (20 mM, pH = 3) was used for the liquid chromatographic separation. The column employed for the MS measurements was a NC-03 Prontosil 120-5-PHENYL (Bischoff chromatography, Leonberg, Germany) with 5 µm particle size, 120 Å pore size, 250 mm length and 3.0 mm i.d.. Injection volume was 10 µL. As flow rate, 0.6 mL/min was selected. The following gradient profile was applied for all measurements:

t [min]	0.03	15	23	25	34	36	37	39
c (CH_3CN) [%]	20	50	55	60	90	90	20	stop

3.2.4 MS conditions

The APCI interface was used as heated nebulizer. Therefore, an APCI probe voltage of only 0.1 kV was applied. The nebulizer flow rate was set to 2.5 mL/min, the APCI temperature was set to the highest value possible (500 °C) and the curved desolvation line (CDL) temperature to 300 °C. The CDL voltage was set to -5 V, the deflector voltages to +35 V and the detector voltage to 1.7 kV.

3.2.5 Conditions for cyclic voltammograms

Fc-PZ or the derivatized isocyanates were dissolved in a mixture of 10 mL ammonium formate buffer (0.1 M) and 10 mL acetonitrile to form a 0.32 to 0.37 mM solution. After 5 min of stirring under a nitrogen atmosphere, the stirrer was turned off and cyclic voltammograms were recorded in a potential range from -1000 to 1000 mV with a scan rate of 50 mV/s.

3.2.6 Synthesis of the derivatizing agent

In order to derivatize isocyanates, ferrocenoyl piperazide (M = 298 Da) was synthesized. Ferrocenecarboxylic acid chloride was prepared according to literature [23, 24, 40]. At room temperature, a solution of 1.43 mL (16.6 mmol) of oxalyl chloride in 30 mL of toluene was added to a stirred suspension of 3.0 g (13.0 mmol) of ferrocene carboxylic acid and catalytic amounts (3.5 mg) of DMAP in 35 mL of toluene. The reaction mixture was stirred for 1 h at room temperature. The color changed from orange to dark red. The solution was heated to 80°C to complete the reaction. After evaporation of the solvent, the residue was extracted several times using warm pentane (T = 30°C). Ferrocenecarboxylic acid chloride precipitated as dark red crystals and was used without further purification. A solution of ferrocenecarboxylic acid chloride (500 mg, 2 mmol) in 60 mL dry dichloromethane was added dropwise to a solution of piperazine (680 mg, 8 mmol) in 20 mL dry dichloromethane over a period of half an hour under cooling in an ice bath. Afterwards, it was stirred for 1 h at room temperature, before a part of the solvent was evaporated and the solution was filtered in order to remove the excess of piperazine. Subsequently, the solution was evaporated to dryness. As a side

product, bis(ferrocenoyl)piperazide may be obtained, which is only poorly soluble in ether. Therefore, the crude product was suspended in ether and the solution was filtered and evaporated. The obtained yellow to orange substance was a relatively clean derivatization reagent with only small amounts of side product. Further cleaning of the substance by preparative column liquid chromatography in portions of 500 mg on a stationary phase of silica gel 100 from Fluka (Buchs, Switzerland) with only methanol as eluent yielded 720 mg (25 %) of pure derivatization reagent.

3.2.7 Synthesis of the derivatives

The derivatives of monoisocyanates and isothiocyanates were prepared as follows: An excess of the respective monoisocyanate or isothiocyanate (0.4 mmol) was dissolved in 15 mL of anhydrous toluene and the derivatizing reagent (60 mg, 0.2 mmol) in anhydrous toluene (30 mL) was added. The mixture was stirred at room temperature for 30 min. Afterwards, 2 mL methanol were added to destroy the remaining isocyanates. The solvent was removed by evaporation. By this method, the derivatives of methyl isocyanate, ethyl isocyanate, phenyl isocyanate and Fc-PZ-propyl isothiocyanate were synthesized.

For the synthesis of Acetyl-Fc-PZ, 125 mg (0.42 mmol) Fc-PZ in 30 mL toluene and 23 µL (32 mg, 0.42 mmol) acetyl chloride were reacted. A catalytic amount of 3.5 mg DMAP was added. The mixture was stirred for 4 h to complete the reaction. The further procedure was performed as in case of the monoisocyanates.

For the derivatization of diisocyanates, equimolar amounts of ferrocenoylpiperazide in respect of the isocyanate functionalities were dissolved in anhydrous toluene, mixed with the dissolved isocyanate and stirred at room temperature for 30 min. The solvent was evaporated evaporation and orange solids were obtained. According to this procedure, the derivatives of 2,4-TDI, 2,6-TDI, HDI, MDI and IPDI were synthesized.

3.2.8 Characterization of the synthesized substances

Ferrocenoyl piperazide: ^{1}H-NMR (δ/ppm, 60 MHz, $CDCl_3$): 1.73 (s, 1H, NH); 2.85 (t, 4H); 3.71 (t, 4H); 4.24 (s, 5H); 4.26 (dd, 2H); 4.54 (dd, 2H); IR (cm^{-1}, KBr): 3306 (m), 3074 (w), 3001 (w), 2974 (w), 2859 (w),1617 (s), 1540 (s), 1470 (m), 1410 (m), 1285 (s), 1263 (w), 1237 (w), 1173 (w), 1006 (m), 824 (m); ESI-MS: *m/z* 299 $[M+H]^+$.

Fc-PZ-MIC: ^{1}H-NMR (δ/ppm, 60 MHz, $CDCl_3$): 2.84 (d, 2H); 3.49 (m, 4H); 3.67 (m, 4H); 4.23 (s, 5H); 4.31 (m, 2H); 4.56 (m, 2H); IR (cm^{-1}, KBr): 3366 (m), 3083 (w), 2905 (w), 1608 (s), 1549 (s), 1470 (s), 1414 (s), 1262 (s), 1172 (w), 1147 (w), 1105 (w), 1001 (m), 816 (m); ESI-MS: *m/z* 356 $[M+H]^+$.

Fc-PZ-EIC: ^{1}H-NMR (δ/ppm, 60 MHz, $CDCl_3$): 1.15 (t, 3H); 3.48 (m, 6H); 3.67 (m, 4H); 4.32 (s, 5H); 4.41 (dd, 2H); 4.56 (dd, 2H); IR (cm^{-1}, KBr): 3359 (m), 3090 (w), 2975 (w), 2859 (w), 1616 (s), 1539 (s), 1470 (m), 1410 (m), 1265 (s), 1173 (w), 1141 (s), 1105 (w), 1006 (m), 824 (m); ESI-MS: *m/z* 370 $[M+H]^+$.

Fc-PZ-PIC: ^{1}H-NMR (δ/ppm, 60 MHz, DMSO): 3.54 (m, 4H); 3.66 (m, 4H); 4.27 (s, 5H); 4.40 (m, 2H); 4.60 (m, 2H); 7.26 –7.44 (m, 5H); IR (cm^{-1}, KBr): 3316 (m), 3069 (w), 2921 (w), 2856 (w), 1637 (s), 1594 (s), 1537 (s), 1470 (w), 1444 (s) 1404 (w), 1244 (s), 1171 (w), 1147 (w), 1105 (w), 993 (m), 821 (m), 754 (s); ESI-MS: *m/z* 418 $[M+H]^+$.

Fc-PZ-MDI: ^{1}H-NMR (δ/ppm, 60 MHz, $CDCl_3$): 3.52 (m, 8H); 3.65 (m, 8H); 3.84 (m, 2H); 4.23 (s, 10H); 4.34 (m, 4H); 4.55 (m, 4H); 6.88 – 7.21 (m, 8H); IR (cm^{-1}, KBr): 3433 (m), 3097 (w), 2915 (w), 2853 (w),1645 (s), 1595 (s), 1513 (s), 1471 (s), 1413 (s), 1242 (s), 1172 (w), 1105 (w), 993 (m),991 (w),820 (m), 728 (m); ESI-MS: *m/z* 847 $[M+H]^+$.

Fc-PZ-IPDI: ^{1}H-NMR (δ/ppm, 60 MHz, $CDCl_3$): 0.96 –1.10 (m, 15H); 1.7 (m, 1H); 3.46 (m, 8H); 3.71 (m, 8H); 4.24 (s, 10H); 4.33 (m, 4H); 4.57 (m, 4H); IR (cm^{-1}, KBr): 3355 (m), 3086 (w),2900 (m),1622 (s),1538 (s),1471 (s), 410 (m), 251 (s),1171 (w),1105 (w), 1003 (m), 821 (w); ESI-MS: *m/z* 819 $[M+H]^+$.

Fc-PZ-HDI: ^{1}H-NMR (δ/ppm, 60 MHz, $CDCl_3$): 1.01 – 1.41 (m, 8H); 3.34 (m, 4H); 3.47 (m, 8H); 3.68 (m, 8H); 4.25 (s, 10H); 4.34 (m, 4H); 4.55 (m, 4H); IR (cm^{-1}, KBr): 3333 (m), 3085 (w), 2922 (w), 2856 (w), 1617 (s), 1549 (s), 1465 (s), 1409 (s), 1254 (s), 1170 (m), 1105 (s), 823 (m), 757 (m), 731 (m); ESI-MS: *m/z* 765 $[M+H]^+$.

Fc-PZ-2,4 TDI: ^{1}H-NMR (δ/ppm, 60 MHz, DMSO): 2.02 (s, 3H); 3.58 (m, 8H); 3.68 (m, 8H); 4.26 (s, 10H); 4.39 (m, 4H); 4.60 (m, 4H); 7.05 (m, 3H); IR (cm^{-1},

KBr): 3254 (w), 3084 (w), 2909 (w), 2855 (w), 1622 (s), 1507 (s), 1471 (s), 1411 (s), 1249 (s), 1170 (m), 1105 (w), 1003 (m); 822 (w), 781 (w), 757 (w); ESI-MS: *m/z* 771 $[M+H]^+$.

Fc-PZ-2,6 TDI: ^{1}H-NMR (δ/ppm, 60 MHz, DMSO-d_6): 2.36 (s, 3H); 3.53 (m, 8H); 3.70 (m, 8H); 4.26 (s, 10H); 4.39 (m, 4H); 4.57 (m, 4H); 7.10 (m, 3H); IR (cm^{-1}, KBr): 3324 (w), 3095 (w), 2909 (w), 2857 (w), 1669 (s), 1646 (s), 1598 (s), 1516 (s), 1472 (s), 1416 (s), 1254 (s), 1174 (w), 1105 (w), 1003 (m), 819 (m), 760 (m); ESI-MS: *m/z* 771 $[M+H]^+$.

Fc-PZ-propyl isothiocyanate: ^{1}H-NMR (δ/ppm, 60 MHz, $CDCl_3$): 0.98 (t, 3H); 1.75 (m, 2H); 3.54–3.91 (m, 10H); 4.26 (s, 5H); 4.36 (m, 2H); 4.58 (m, 2H); IR (cm^{-1}, KBr): 3313 (m), 3084 (w), 2955 (w), 2922 (w), 2862 (w), 1600 (s), 1542 (s), 1467 (s), 1411 (m), 1389 (m), 1348 (m), 1222 (w), 1105 (w), 1076 (w), 1008 (m), 809 (w), 761 (w); ESI-MS: *m/z* 400 $[M+H]^+$.

Acetyl-Fc-PZ: ^{1}H-NMR (δ/ppm, 60 MHz, $CDCl_3$): 2.14 (s, 3H); 3.69 (m, 8H); 4.25 (s, 5H); 4.35 (dd,2H); 4.54 (dd, 2H); IR (cm^{-1}, KBr): 3445 (m), 3073 (w), 2919 (w), 1626 (s), 1603 (s), 1451 (s), 1434 (s), 1360 (m), 1282 (m), 1282 (m), 1249 (s), 1171 (m), 1105 (w), 1002 (m), 862 (w), 816 (w), ESI-MS: *m/z* 341 $[M+H]^+$.

Air sampling was performed using a two-channel air sampler pump (model 1067) from Supelco (Bellefonte, PA, USA). Flow rates were determined using a DryCal DC-lite flow calibrator from Bios (Butler, NJ, USA).

The generation of an isocyanate-containing atmosphere was performed in dry glassware to avoid hydrolysis of the reactive compounds. A 500 mL round bottom flask was partly filled with a commercially available MDI based PUR foam (E-Coll from E/D/E GmbH, Wuppertal, Germany). After letting the foam harden and dry for 30 min, the flask was connected to an impinger containing 24.3 mg (0.08 mmol) Fc-PZ in 50 mL of acetonitrile. For 20 min, the flask was heated with a Bunsen-type burner to decompose the PUR material. This procedure is simulating a process where heat is applied to PUR foams, such as welding of insulated water pipes. During this time period, air samples of 200 mL/min were continuously taken from the flask and bubbled through the impinger.

3.3 Results and discussion

3.3.1 Synthesis

Ferrocenoyl piperazide (Fc-PZ) was synthesized as a new derivatizing agent for the analysis of isocyanates following a two-step route, which is presented in Fig. 3.1. The first step consisted of the reaction of the commercially available ferrocenecarboxylic acid to the respective ferrocenecarboxylic acid chloride (FCC) by using oxalyl chloride and catalytic amounts of *N,N* dimethylaminopyridine. This procedure was introduced by Rolfes and Andersson [23, 24], but for this work, a slightly modified version by Karst et al. [40] was applied. FCC was then reacted with an excess of piperazine under formation of Fc-PZ. Despite the excess of piperazine, small amounts of bis(ferrocenoyl)piperazide are formed as side product. Therefore, purification of the reagent was carried out by preparative column liquid chromatography.

The reagent was characterized by means of ^{1}H-NMR and IR spectroscopy as well as electrospray mass spectrometry (ESI-MS). The respective data are listed in the Experimental Part.

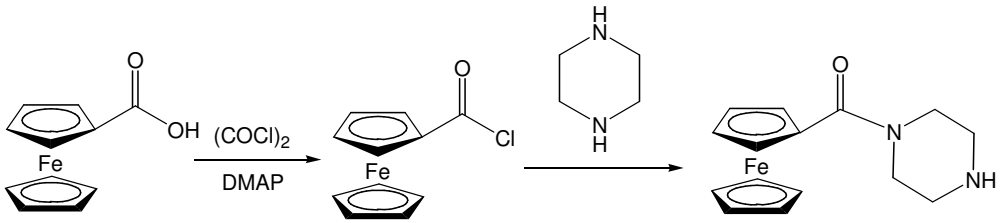

Fig. 3.1 Synthesis of Fc-PZ.

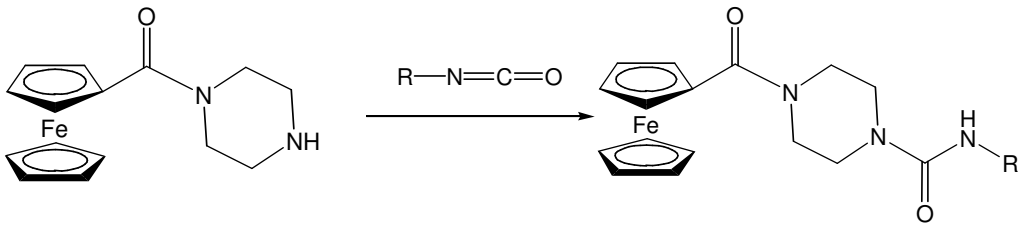

Fig. 3.2 Synthesis of the isocyanate derivatives of Fc-PZ.

For calibration purposes, a series of isocyanate derivatives was synthesized according to Fig. 3.2. The reaction between equimolar amounts of Fc-PZ and isocyanates rapidly yielded the desired products, which were characterized as in case of the reagent. The derivatives of methyl isocyanate (MIC), ethyl isocyanate (EIC), phenyl isocyanate (PhIC), methylenebis(4,4´-phenylisocyanate) (MDI), isophorone diisocyanate (IPDI), hexamethylene diisocyanate (HDI) and the 2,4- and 2,6-isomers of toluylene diisocyanate (2,4-TDI and 2,6-TDI) were obtained this way.

3.3.2 Chromatographic separation

The chromatographic separation of the derivatives was optimized in the following. For the planned electrochemical conversion of the derivatives after separation, it is important to use a conductive mobile phase, which contains volatile buffer components. From earlier work on the analysis of ferrocene-derivatized alcohols and phenols, it was known that a binary gradient consisting of acetonitrile and an aqueous formic acid/ammonium formate buffer with pH 3 fullfills these requirements [40-42]. Initial tests confirmed that this mobile phase is also suitable as well for the separation of the derivatized isocyanates. Various stationary phases were tested, and it became evident that the separation of the derivatives of the aliphatic isocyanates can easily be performed on different reversed-phase columns. However, the separation of the two TDI derivatives and the HDI derivative was difficult, as was reported earlier for the separation of NBDPZ-derivatives of these isocyanates [18]. As in this case, a phenyl-modified stationary phase proved to be better suited for the baseline separation of all three derivatives than C_{18}-modified columns. The chromatogram of a standard mixture consisting of the derivatives of MIC, EIC, PhIC, IPDI, 2,4-TDI, HDI, 2,6-TDI and MDI using UV/vis and electrochemistry/MS detection is provided in Fig. 3.3. The optimization of the electrochemistry/MS detection is described in detail below. When comparing the relative signal intensities in UV/vis and MS detection, it becomes evident that in most cases, a good correlation is obtained.

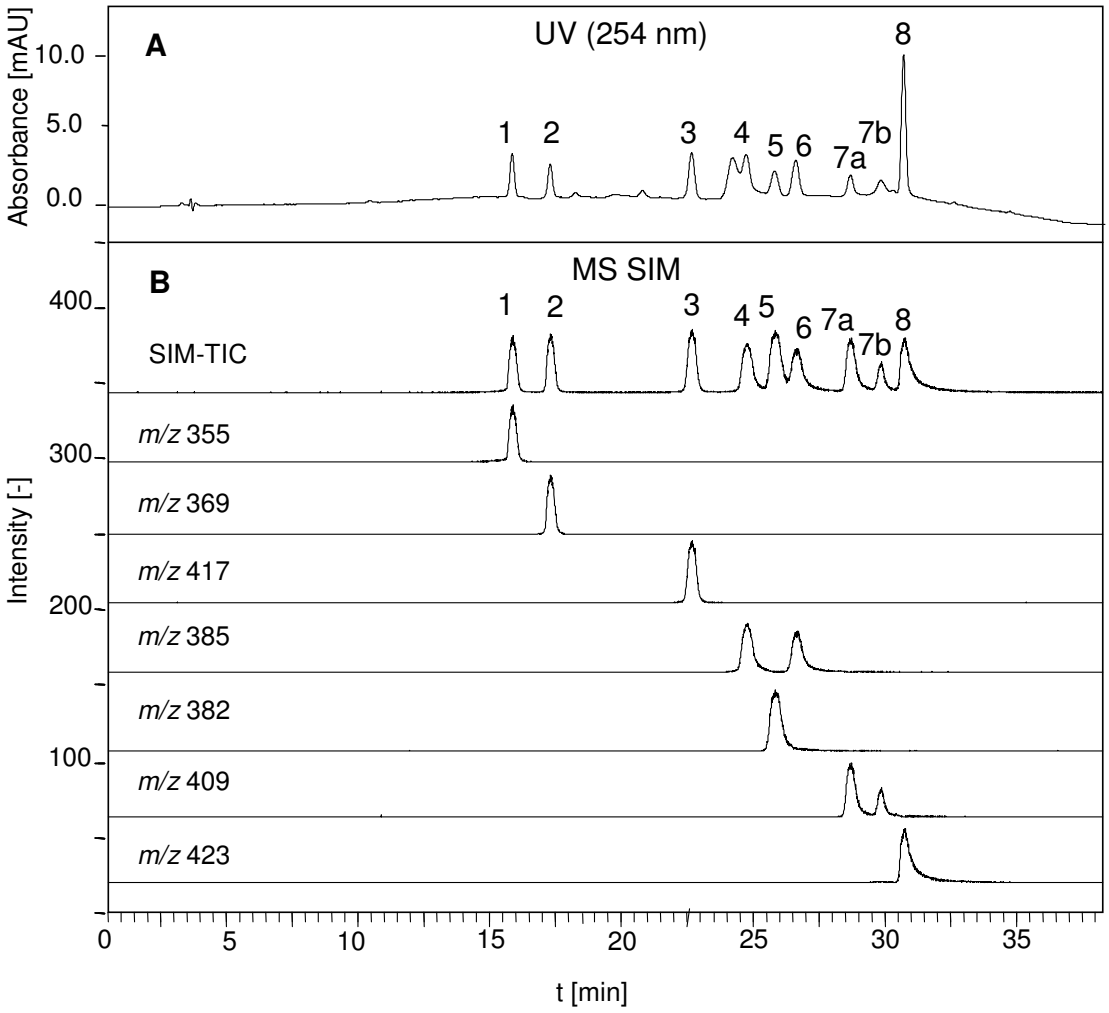

Fig. 3.3 Separation of a mixture of Fc-PZ isocyanate derivatives, concentrations between $1.4*10^{-5}$ and $2.0*10^{-5}$ M for monoisocyanate derivatives and between $8.0*10^{-6}$ M and $9.2*10^{-6}$ M for the derivatives of diisocyanates. Peak assignment: Fc-Pz-MIC (1), Fc-PZ-EIC (2), Fc-PZ-PIC (3), Fc-PZ-2,6-TDI (4), Fc-PZ-HDI (5), Fc-PZ-2,4-TDI (6), Fc-PZ-IPDI (7 a,b), Fc-PZ-MDI (8). (A): UV chromatogram recorded at 254 nm; (B): MS chromatogram, selected ion monitoring (SIM) mode, potential of the electrochemical flow cell 0.5 V vs. Pd/H_2.

However, for the derivatives of the aromatic isocyanates (2,4-TDI, 2,6-TDI and MDI), the relative intensity of the UV absorbance signal is larger due to the strong absorbance of the aromatic rings at the detection wavelength. The chromatogram proves that all of the investigated derivatives can be well separated under the selected conditions. The derivative of HDI elutes between the two TDI isomers. For IPDI, two structural isomers are observed

at slightly different retention times. With this separation, the analysis of real samples should be possible. This was investigated in detail as described later in this chapter.

3.3.3 LC/EC/MS

When analyzing the derivatives with LC/ESI-MS or LC/APCI-MS, but without electrochemical pretreatment in the positive ion mode, the $[M+H]^+$ pseudomolecular ions were observed in all cases. Signal intensities were poor due to the limited polarity of the analytes. For electrochemistry/MS measurements, a porous glassy carbon cell with very large surface area was used with the goal to obtain a high conversion rate of the ferrocene derivatives. This electrochemical cell uses Pd as counter electrode, and a Pd/H_2 reference system. All potentials provided in the following were determined against this reference electrode, with the mobile phase used for the LC separations (see above). The commercial APCI interface was used without any technical modifications, but with the corona voltage switched to only 0.1 kV, in the 'heated nebulizer' ('thermospray') mode. This allows improving the selectivity, as no further unselective ionization process increases the background signal.

The mass spectra for selected derivatives using LC/electrochemistry/MS under these conditions were recorded in the following. Fig. 3.4 shows the mass spectrum of the EIC derivative, the structure of which is inserted.

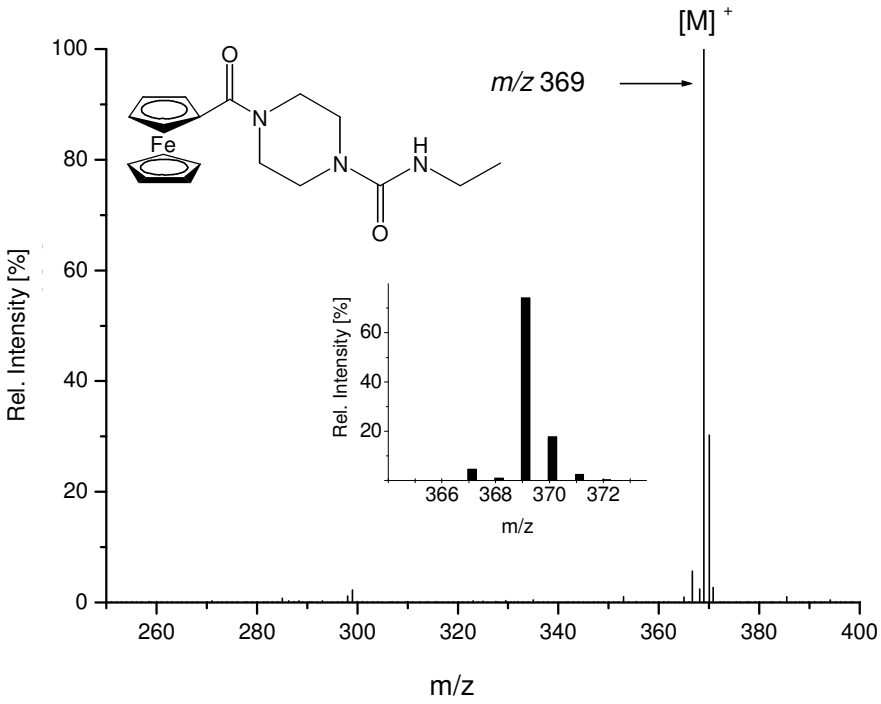

Fig. 3.4 Mass spectrum of Fc-PZ-EIC obtained with electrochemical oxidation. Inserted is the chemical structure of the analyte as well as the calculated isotopic pattern.

Due to the electrochemical conversion, the ferrocinium ion is formed, which leads to the $[M]^+$ signal as base peak. Compared with the approach without electrochemical conversion, signal intensities are much higher, typically by a factor of 50. Under these conditions, the $[M+H]^+$ ion does not contribute significantly to the signal. The inserted mass spectrum shows the calculated abundance of the masses under consideration of the isotopic pattern of the elements. This correlates well with the measured isotopic pattern.

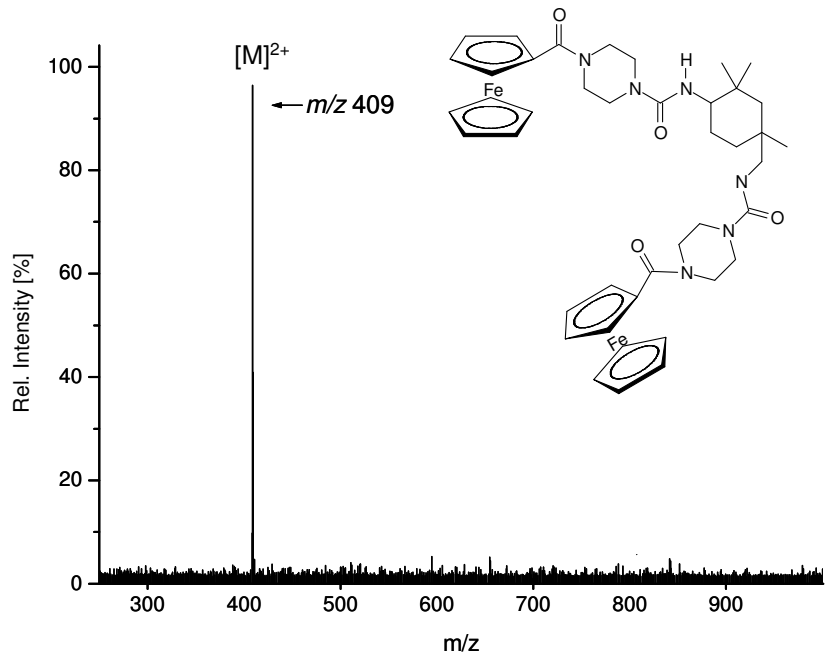

Fig. 3.5 Mass spectrum of Fc-PZ-IPDI obtained with electrochemical oxidation. Inserted is the chemical structure of the analyte.

For all diisocyanate derivatives, $[M]^{2+}$ ions are predominant, while $[M]^{+}$ ions are not detected at all. The mass spectrum of the IPDI derivative is presented in Fig. 3.5, in which its chemical structure is inserted. The dual charge of the derivative is proven by the isotopic pattern of the peak (not shown). Obviously, both ferrocene groups are oxidized during this experiment, as no peak is observed for the singly charged oxidation product at *m/z* 818.

To study the electrochemical behaviour of the ferrocenoyl piperazides, cyclic voltammetry is performed in a mixture of acetonitrile and ammonium formate buffer. The redox system shows a reversible one-electron oxidation of the ferrocene derivative to the ferrocinium cation (Fig. 3.6).

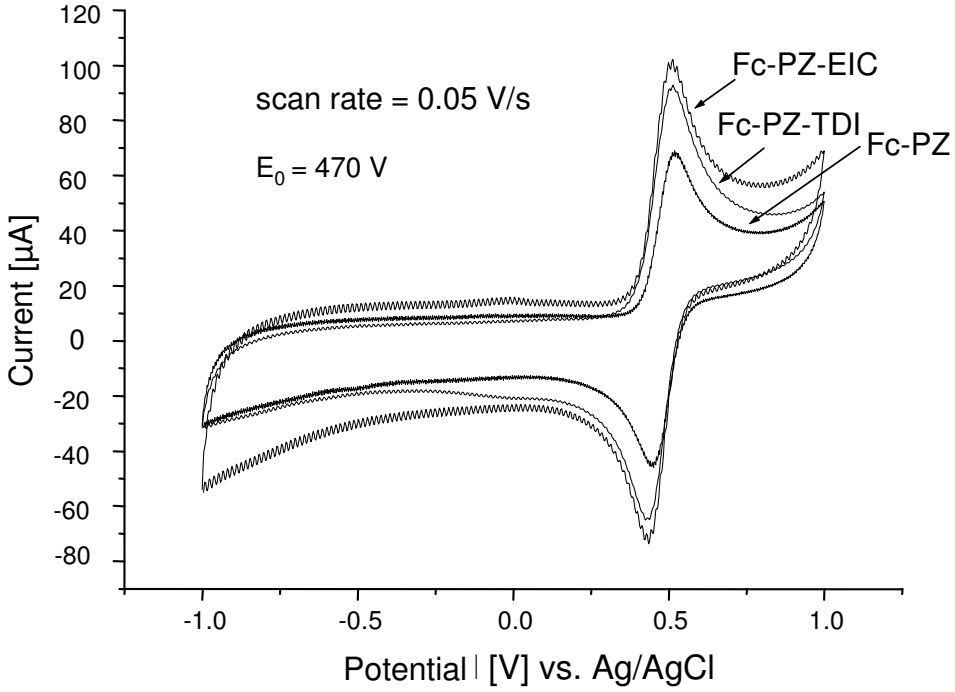

Fig. 3.6 Cyclic voltammogram of Fc-PZ (0.35 mM), Fc-PZ-EIC (0.37 mM), and Fc-PZ-2,6-TDI (0.32 mM) in an 1:1 mixture of acetonitrile and ammonium formate buffer.

The half-wave potential for the derivatization reagent is found to be 480 mV vs. Ag/AgCl. As expected, the influence of the individual isocyanate derivatives was neglectable and the potentials were found to be in the same range. The dependency of the obtained signal on the applied potential is presented in Fig. 3.7. Small signals are already obtained for the monoisocyanates at a potential of 0.3 V vs. Pd/H_2, while the diisocyanates are not detected at all under these conditions. The highest signals for all compounds were observed at 0.5 V, and this potential was therefore selected for all further measurements. This is consistent with cyclic voltammetric measurements. In the range up to 0.7 V, only a minor decrease of the signal is observed for all derivatives. With further increase of the potential, however, the derivatives of aromatic isocyanates rapidly lose signal intensity, while the derivatives of the aliphatic isocyanates (MIC, EIC and, to a lesser extent HDI

and IPDI), still can be detected at 1.0 V. This indicates that the aromatic ring is involved in further oxidation processes of the derivatives.

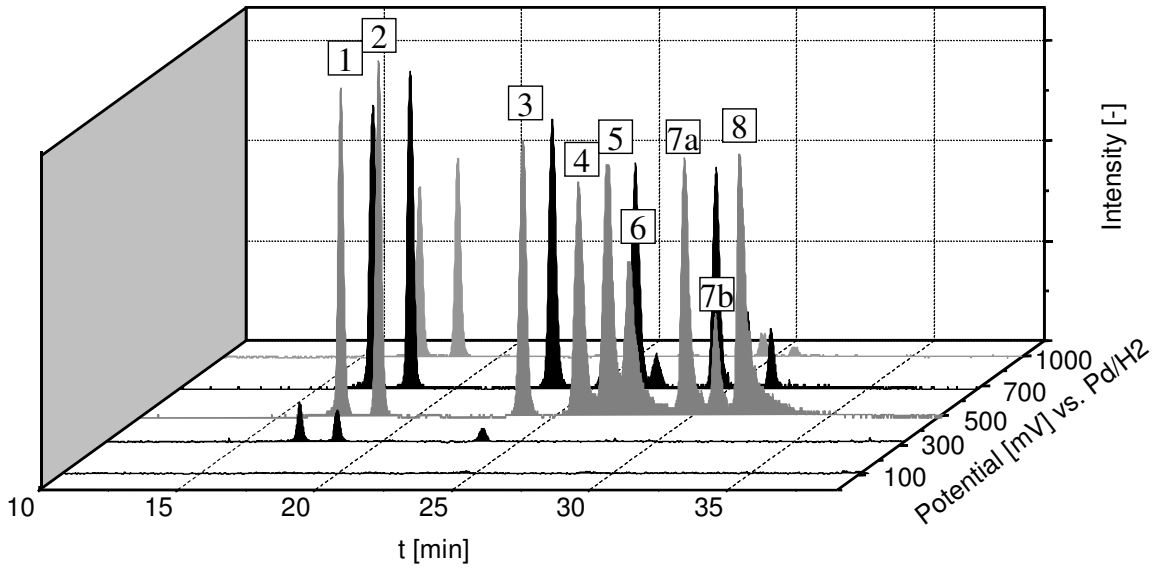

Fig. 3.7 Dependency of the signal intensity on the applied electrochemical potential varied between 0.1 to 1.0 V vs. Pd/H_2, selected ion monitoring (SIM) mode, peak assignment: Fc-PZ-MIC (1), Fc-PZ-EIC (2), Fc-PZ-PIC (3), Fc-PZ-2,6-TDI (4), Fc PZ-HDI (5), Fc-PZ-2,4-TDI (6), Fc-PZ-IPDI (7 a,b), Fc-PZ-MDI (8).

The analytical figures of merit were determined for these derivatives. The respective data are listed in Tab. 3.1. Limits of detection range from 6-20 nmol/L, limits of quantification from 20-50 nmol/L. The linear ranges extend over two decades for all derivatives.

Tab. 3.1 Analytical figures of merit for the investigated isocyanates using the Fc-PZ method.

Analyte	LOD [nM]	LOQ [nM]	RSD [%] (n = 3; c = 0.2 µM)
Fc-PZ-MIC	20	50	2.2
Fc-PZ-EIC	16	50	4.0
Fc-PZ-PIC	13	40	1.1
Fc-PZ-2,6 TDI	7	20	5.9
Fc-PZ-HDI	12	40	2.1
Fc-PZ-2,4 TDI	10	30	4.5
Fc-PZ-IPDI	13	40	4.3
Fc-PZ-MDI	6	20	7.1

3.3.4 Air sampling application

To investigate the suitability of the reagent for the analysis of real samples, the thermal degradation of MDI-based polyurethane foam was studied. It is known from literature that, besides the isocyanate, which was used for synthesizing the polymer, lower isocyanates may be observed during this procedure [43]. This simulates a welding process at a metal pipe, which is insulated with polyurethane material. Sampling was performed with an impinger, which was filled with 50 mL of a 1.6 mM solution of Fc-PZ in acetonitrile. The air samples were pumped through the impinger at a flow rate of 300 mL/min. Initial experiments with known amounts of nebulized isocyanates proved that the recovery was quantitative under these conditions. In Fig. 3.8, the results of the mass spectrometric analysis of the sampling impinger are presented. As a very large amount of MDI was released, the sample was diluted for the determination of MDI by a factor of 1000 prior to injection into the LC/MS system. It should be noted that some peak tailing is obtained for MDI in this experiment. It is assumed that this is due to

absorption or precipitation of the derivative on the cell material and subsequent slow release by oxidation at these high concentrations. This effect was only observed for very high concentrations of those derivatives with the lowest polarity.

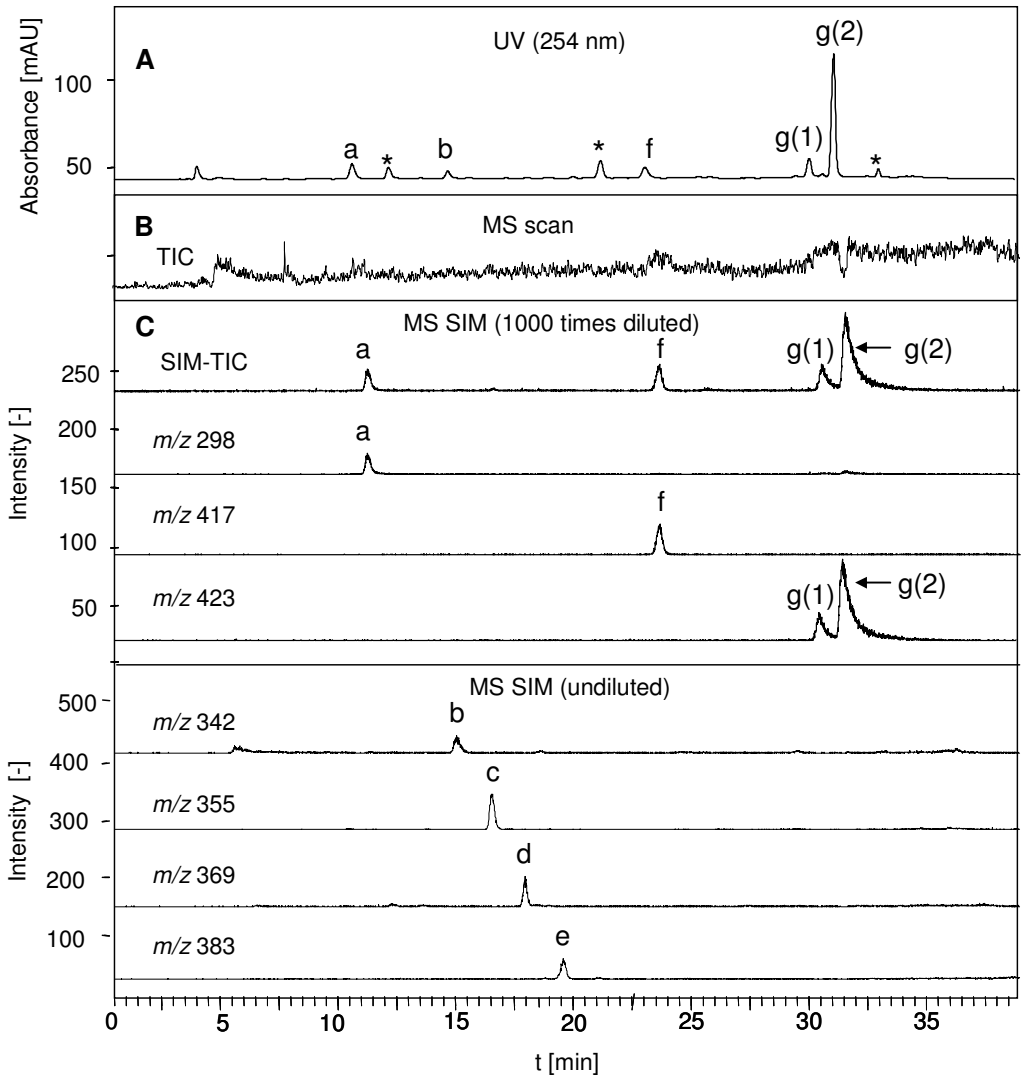

Fig. 3.8 Chromatogram of a thermally treated PUR sample. Peak assignment: Fc-PZ (a), Fc-PZ-ICA (b), Fc-PZ-MIC (c), Fc-PZ-EIC (d), Fc-PZ-PrIC (e), Fc-PZ-PIC (e), Fc-PZ-MDI (g(1), g(2)), (A): UV chromatogram recorded at 254 nm; (B) MS chromatogram scan mode *m/z* 200 – *m/z* 2000 (C): MS chromatogram, selected ion monitoring (SIM) mode, electrochemical flow cell 0.5 V vs. Pd/H_2. The asterisks indicate unidentified compounds, which occured in the UV trace, but which could not be detected by mass spectrometry.

PhIC was found in very high concentrations as well. As was expected from literature data [43], isocyanic acid (ICA), MIC, EIC and propyl isocyanate are all detected as degradation products at low concentrations in the undiluted impinger solution. However, the selected sampling arrangement does not allow a quantitative evaluation of these data. The asterisks indicate unidentified compounds, which occurred in the UV trace, but which could not be detected by mass spectrometry under the conditions used for this work. Therefore, it can be excluded that these peaks are isocyanate derivatives.

3.3.5 Derivatization of isothiocyanates and acid chlorides

It is obvious that a piperazine function is also capable of reacting with isothiocyanates or acid chlorides. To investigate the possibility to apply Fc-PZ for this purpose as well, the respective derivatives of propyl isothiocyanate and acetyl chloride were synthesized and characterized (see above). The mass spectrum of the propyl isothiocyanate derivative of Fc-PZ after electrochemical oxidation is presented in Fig. 3.9.

The mass spectrum of the Fc-PZ derivative of acetyl chloride after electrochemical oxidation is presented in Fig. 3.10, with the inserted chemical structure and calculated isotopic pattern. As in the previous cases, the $[M]^+$ peak dominates. As in case of the isocyanate derivatives, but in contrast to the isothiocyanate derivative, no fragmentation is observed. It is therefore evident that the reagent is suitable to be used for the analysis of this group of compounds as well.

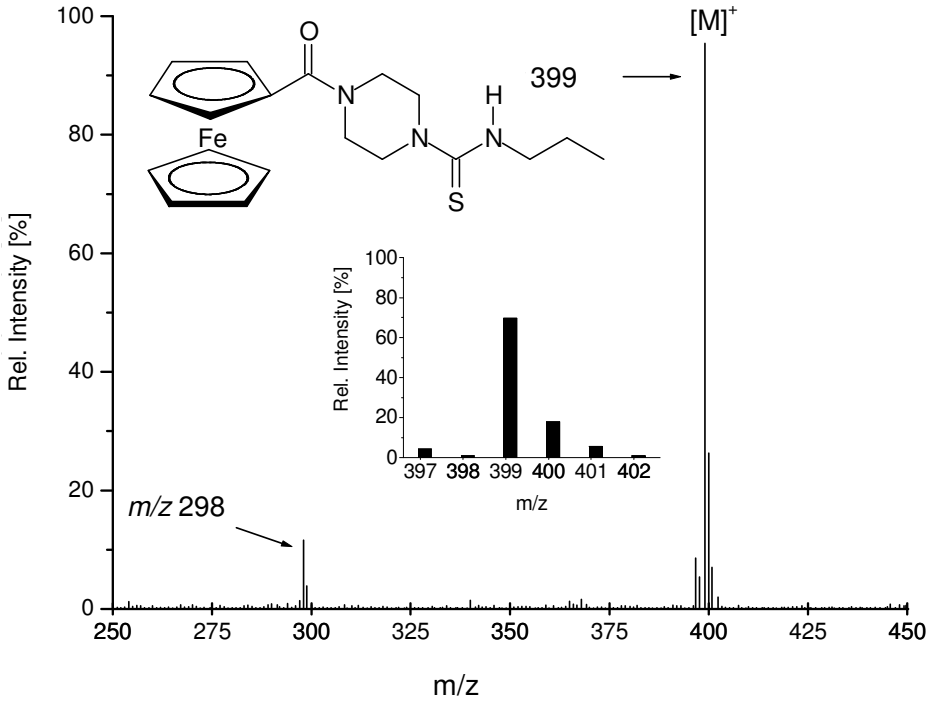

Fig. 3.9 Mass spectrum of Fc-PZ-propyl isothiocyanate obtained with electrochemical oxidation. Inserted are the chemical structure of the analyte and the calculated isotopic pattern.

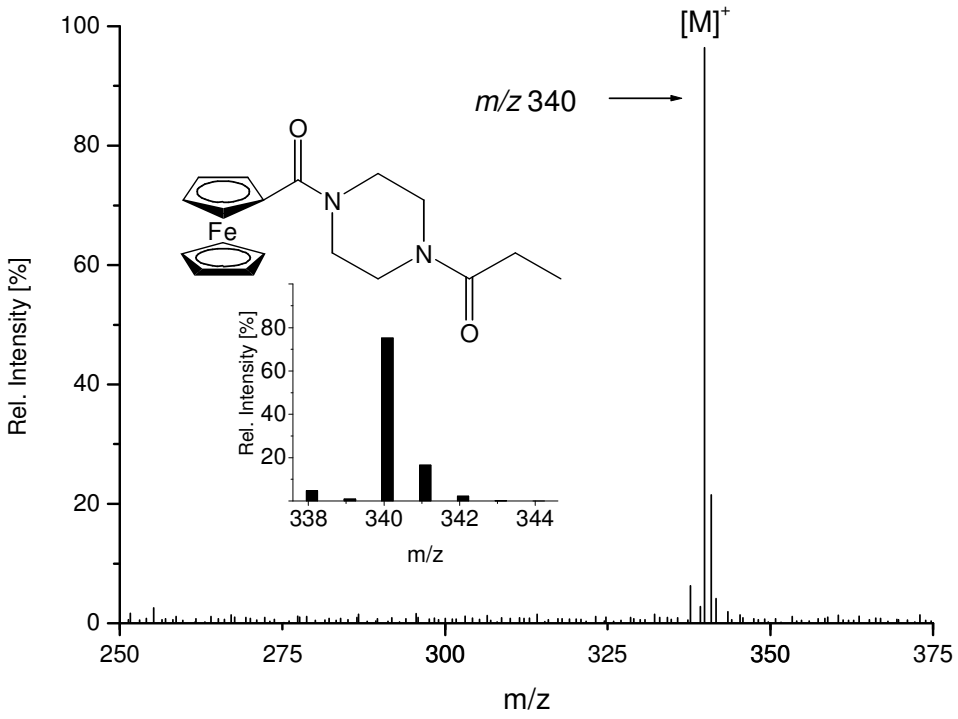

Fig. 3.10 Mass spectrum of acetyl Fc-PZ obtained with electrochemical oxidation. Inserted is the chemical structure of the analyte as well as the calculated isotopic pattern.

These findings are also important to evaluate possible interferences in the analysis of isocyanates in real samples using related reagents, which were mostly carried out with LC and UV/vis detection in previous times. In this case, misinterpretations of unknown peaks in the chromatograms as isocyanates are possible. The use of LC/MS is therefore strongly recommended to increase selectivity.

3.4 Conclusions

Fc-PZ has been introduced as promising new reagent for the analysis of isocyanates and, possibly, isothiocyanates and carboxylic acid chlorides by LC/electrochemistry/MS. Considering the used LC/MS instrument, limits of detection are very good. Future work should be directed to the development of more suitable air sampling devices, e.g., reagent-coated test tubes and passive sampling devices. Furthermore, the use of tandem mass spectrometry should allow to significantly improve the limits of detection and the selectivity of the method. Fragmentation experiments of the doubly charged diisocyanate derivatives, possibly under formation of singly charged fragments with higher *m/z* ratio than the parent compound, could lead to a very high selectivity. Applications to the analysis of isocyanates in liquid samples also appear to be possible, as it can be expected that polyurethane prepolymers and related compounds with a larger number of isocyanate functionalities will react with several Fc-PZ molecules and will then be multiply charged after electrochemical oxidation. This would allow to analyze (pre)polymers of comparably high masses, if high-resolution mass spectrometry, e.g., with a TOF instrument, would be available.

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Chapter 4

Analysis of Cysteine-Containing Proteins Using Pre-Column Derivatization with *N*-(2-Ferroceneethyl)-maleimide and LC/Electrochemistry/MS[‡]

N-(2-Ferroceneethyl)maleimide (FEM) is introduced as an electroactive derivatizing agent for thiol functionalities in proteins. Using appropriate reaction conditions, the derivatization is completed within five minutes and no unspecific labeling of free amino functions is observed. Liquid chromatography/electrochemistry/mass spectrometry was used to detect the reaction products. The reagent is a useful tool to determine the number of free thiol groups or the sum of free and disulfide-bound thiol groups in proteins. The electrochemical cell provides additional information, because the increase in mass spectrometric response upon electrochemical oxidation of the neutral ferrocene to the charged ferrocinium group is monitored. The method was applied successfully for the analysis of native proteins and their tryptic digests.

[‡] Seiwert B., Karst U., accepted for publication in *Anal. Bioanal. Chem.* **2007**, DOI:10.1007/s00216-007-1260-9.

4.1 Introduction

The on-line combination of electrochemistry (EC) and mass spectrometry (MS) has been used increasingly in recent years. The possibilities and limitations of using EC/MS to mimic phase I oxidative reactions in drug metabolism were studied by Jurva et al. [1] and compared to cytochrome P450-catalyzed oxidation reactions [2]. Furthermore, as an alternative to enzymatic protein digestion methods, the oxidation of tryptophan and tyrosin in proteins may give rise to peptide bond cleavage, as shown by Permentier et al. [3]. Using on-line oxidation, electroactive analytes may be converted into radical ions or products that yield better detection limits with mass spectrometric detection, as shown for example for PAHs [4], and phenothiazine [5] and its derivatives. For analytes without electroactive functionalities, the use of ferrocene-based reagents has been reported to increase the limits of detection. Van Berkel and co-workers [6] introduced ferrocenoyl azide for the analysis of alcohols in natural products. They used the electrolysis inherent in the electrospray instead of an external electrochemical cell and obtained impressive limits of detection. Diehl et al. [7, 8] used ferrocenoyl chloride for the determination of alcohols and phenols based on the on-line combination of liquid chromatography, electrochemistry and mass spectrometry. For this work, a commercial electrochemical cell, which can provide a quantitative conversion rate under optimum conditions, was used. The different approaches employed to combine electrochemistry and mass spectrometry are described in more detail in original papers by Van Berkel et al. [9] and in reviews by Diehl and Karst [10,11]. Ferrocene-based derivatizing agents were used for other classes of compounds, too.

Semiquantitative analysis of catechol estrogens as their ferrocene boronate esters using ESI-MS with one-electron oxidation in the source using a non-aqueous solvent system (a mixture of dichloromethane and acetonitrile (ACN) with lithium triflate as the working electrolyte) was applied by Williams et al. [12]. 1α-Hydroxyvitamin D_3 in rat plasma was determined by ESI-MS after derivatization with 4-ferrocenylmethyl-1,2,4-triazoline-3,5-dione by Murao et al. [13] At a low capillary voltage, the ferrocinium cation was produced. This was advantageous for selectivity, because the ionization of most other compounds is inefficient at the conditions used. For the analysis of isocyanates in air samples by LC/EC/MS, ferrocenoyl piperazide proved to be useful as derivatizing agent [14].

Cysteine residues and disulfide bonds are important for protein structure and function. They stabilize the conformation of molecules through disulfide linkages during the process of protein folding [15]. The activities of some enzymes directly depend on the existence of free thiol functionalities, which participate in substrate binding and catalysis [16]. Therefore, the identification of thiol functionalities in proteins and peptides is necessary to understand these properties. Thiol-containing compounds are labile due to the high reactivity of the thiol group and are easily transformed into disulfides upon oxidation. Their analysis therefore requires immediate stabilization to preserve the original oxidation state [17]. In many cases, derivatization is used for stabilization purposes [15]. *N*-Substituted maleimides are often used in protein chemistry for this purpose [18]. In those cases, where a combination with a reversed-phase separation is intended, reagents that lead to low-polarity

products are advantageous. Therefore, ferroceneethylmaleimide (FEM) appears to be a promising reagent for this work. It was originally developed by Shimada et al. for the determination of thiols by HPLC with electrochemical detection [19, 20]. Later it was also used to introduce an IR probe [21], a redox active reporter group [22], and an electroactive label into a non-electroactive enzyme [23] in order to design new electrochemical biosensors. Furthermore, the attachment of maleimide-containing ferrocene derivatives onto self-assembled alkanethiol and alkanedithiol monolayers was studied [24]. However, the reagent has not yet been used in conjunction with liquid chromatography/mass spectrometry (LC/MS), although it appears to be particularly promising.

In this work, FEM is used for the first time as a reagent in the LC/MS analysis of thiol-containing proteins. Additional investigations focus on the possible use of an electrochemical cell in order to improve the limits of detection and to obtain important additional information on the products of the tryptic digest of the peptides.

4.2 Experimental part

4.2.1 Chemicals

Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP), formic acid, ammonium hydrogencarbonate, ammonium formate, glutathione, all proteins and all fine chemicals were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) at the highest purity available unless stated otherwise below.

The solvents used for LC/MS were acetonitrile and water at LC/MS grade quality from Biosolve (Valkenswaard/The Netherlands).

N-(2ferroceneethyl)maleimide (FEM) was synthesized from ferrocenylmethyltrimethylammonium iodide (obtained from Alfa Aesar, Karlsruhe, Germany) according to a method described in the literature [21, 26, 27] that was modified as indicated below.

4.2.2 Synthesis of FEM

The synthetic route to FEM is shown in Fig. 4.1. It consists of the following three major steps:

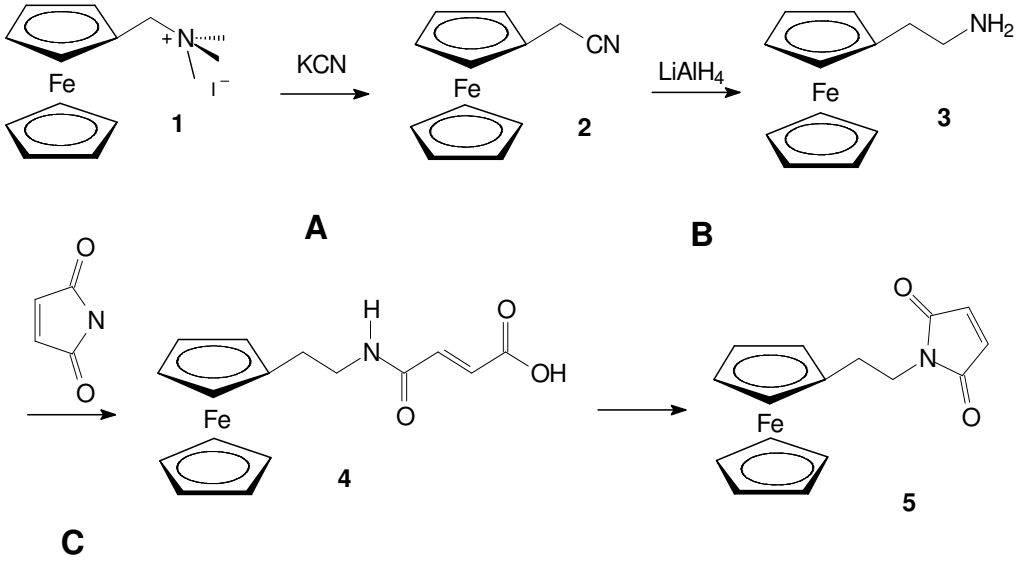

Fig. 4.1 Synthesis of FEM.

A. Ferroceneacetonitrile (**2**) was synthesized from ferrocenylmethyltrimethylammonium iodide (**1**) according to Lednicer et al. [26]. Ferrocenylmethyltrimethylammonium iodide (10 g, 26 mmol) and 10 g (153 mmol) potassium cyanide were dissolved in 100 mL water and the solution was heated under reflux. After two hours, the reaction mixture was

cooled and extracted twice with 50 mL diethyl ether, washed twice with 20 mL water and dried over magnesium sulfate. The solvent was evaporated to yield 5.12 g (85%) of a yellow substance that was used for subsequent reactions without further purification. ^{1}H-NMR (300 MHz, $CDCl_3$, TMS) δ(ppm): 3.4 (s, 2H), 4.1 (s, 4H), 4.15 (s, 5H).

B. 2-Ferrocenylethylamine (**3**) was synthesized from ferrocenylacetonitrile (**2**) by reduction with $LiAlH_4$ as described in the literature [27]. However, some modifications were performed to improve the yield and reproducibility of the reaction. According to the literature, small portions of in total 1 g (26 mmol) $LiAlH_4$ and 2.4 g (18 mmol) of $AlCl_3$ were dissolved in 40 mL dry THF while stirring on an ice bath. 2 g (9 mmol) of ferroceneacetonitrile dissolved in 20 mL THF were added, and the mixture was refluxed for two hours. After cooling, water was carefully added dropwise to decompose the excess of $LiAlH_4$. The green complex formed by $AlCl_3$ and 2-ferrocenylethylamine [28] had to be destroyed by adding 0.5 mL of concentrated NaOH. The solution turned yellow and the aqueous solution was extracted with diethyl ether three times. The combined organic extracts were dried over magnesium sulfate and evaporated to dryness. The purity of the red oily substance (1.1 g / 54 %) was investigated by ESI-MS: *m/z* 230 $[M+H]^+$; MS/MS: *m/z* 213; ^{1}H-NMR (300 MHz, $CDCl_3$, TMS) δ(ppm): 2.48 (t, 2H), 2.81(t, 2H), 4.00 (s, 4H), 4.05 (s, 5H).

C. 2-Ferrocenylethylamine (460 mg, 2 mmol), the product obtained from B, was dissolved in dry THF (20 mL) and mixed with 220 mg (2.4 mmol) maleic

anhydride, which was also dissolved in dry THF (10 mL). After stirring the reaction mixture for one hour in an ice bath, the solvent was removed by evaporation and the crude *N*-(2-ferroceneethyl)maleic acid (**4**) obtained was, in contrast to the approach described in the literature [12], purified by column liquid chromatography using silica gel with ethyl acetate/chloroform (9:1) as the mobile phase (ESI-MS: *m/z* 327 $[M+H]^+$). The product (400 mg, 1.2 mmol) was added to a solution of 4 mL acetic anhydride and 0.6 g ammonium acetate and heated for 10 min to 100 °C. Afterwards, 30 mL water and 20 mL ethyl acetate were added and the aqueous phase was extracted with ethyl acetate three times. The combined organic phases were washed with $NaHCO_3$ and brine, dried over $MgSO_4$ and evaporated to dryness. Column liquid chromatography was performed on silica gel using chloroform/ethyl acetate (2:1) as the mobile phase and 209 mg (56%) *N*-(2-ferroceneethyl)maleimide (**5**) was obtained. ESI-MS: *m/z* 309 $[M+H]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$, TMS) δ(ppm): 2.56 (t, 2H), 3.65 (t, 2H), 4.10 (s, 4H), 4.15 (s, 5H), 6.68 (s, 2H).

4.2.3 Derivatization of proteins

Stock solutions were prepared of the following compounds: FEM (20 mM) in ACN, urea (8 M) in NH_4ac buffer (100 mM, pH 6.8) or NH_4HCO_3 buffer (100 mM, pH 7.8) and TCEP (100 mM) in 0.3 M aqueous ammonia solution. The proteins of interest were dissolved in buffer and denaturated in urea to form a 0.125 mM solution. The derivatization was carried out using a tenfold molar excess of FEM with respect to the thiol groups in the proteins. Reduction of disulfide bonds was being performed with a ninefold molar

excess of TCEP with respect to the thiol groups for 30 min before derivatization. Afterwards, formic acid was added to decrease the pH value to acidic conditions. As an example, the derivatization of β-lactoglobulin A is now described: For the derivatization of free cysteine groups, 65 µL of 20 mM FEM solution was added to 1 mL of β-lactoglobulin A solution (dissolved in NH_4 ac-urea, see above). To derivatize the disulfide-bound cysteines as well, first 60 µL of TCEP were added to the protein solution (preferably in urea-NH_4HCO_3) and then 320 µL of the FEM-solution where added. Finally, the protein solutions exceeding 3 kDa were passed through a PD-10 desalting column (Amersham Bioscience, Freiburg/ Germany) to remove the excess reagent and reduction agent. For this, 0.01% formic acid was used as mobile phase.

4.2.4 Digestion with trypsin

A stock solution of trypsin (1 µg/mL in 0.01% formic acid, diluted 1:10 immediately before use in 100 mM NH_4HCO_3) was prepared first. For the in-solution digest, 150 µL trypsin solution were added to 200 µL of the desalted protein (β-lactoglobulin A) solution containing approximately 0.03 mM protein in 0.01% formic acid (trypsin to protein ratio of 1:20 (w/w)), and the mixture was incubated over night at 37°C.

4.2.5 Analysis of the derivatized proteins

A LC/MS system from Agilent Technologies (Amstelveen, The Netherlands), which consisted of a G1322 vacuum degasser, a G1316 thermostated column compartment, a G1367 well plate-sampler, a G1311 quarternary pump, and a

G1956B LC/MSD SL single quadrupole mass spectrometer was used. The column employed for the MS measurements was a Discovery® BioWidePore C5 column (Supelco, Taufkirchen, Germany) with a particle size of 5 µm, length of 150 mm and an inner diameter of 2.1 mm. The injection volume was 10 µL. A flow rate of 0.3 mL/min was selected. The following three gradient profiles were selected for the measurements:

a)

t [min]	0.03	1	20	22	23	30
c (CH_3CN) [%]	10	10	100	100	10	stop

b)

t [min]	0.03	1	25	27	29	32
c (CH_3CN) [%]	10	10	100	100	10	stop

c)

t [min]	0.03	1	5	20	22	24	25	30
c (CH_3CN) [%]	5	5	60	75	100	100	5	Stop

A binary gradient of a) 0.1% formic acid in water/0.1% formic acid in acetonitrile and b) 10 mM NH_4HCO_3 buffer and acetonitrile was employed. Scan data were recorded from *m/z* 130 to *m/z* 2000 with the following parameters: fragmentor 70, gain of 1, threshold of 150 and a step size of 0.1. For data analysis and deconvolution, Chemstation Rev. A.10.02 software (Agilent) was used.

4.2.6 Conditions for cyclic voltammograms

The FEM derivative of glutathione was prepared by mixing equimolar amounts of glutathione in 10 mL NH_4HCO_3 buffer (100 mM, pH 7.8) and FEM was dissolved in 10 mL acetonitrile to form a 0.1 mM solution of glutathione derivative. After 5 min of stirring under a nitrogen atmosphere, the stirrer was turned off and cyclic voltammograms were recorded over the potential range from -500 mV to 1000 mV at a scan rate of 50 mV/s.

4.2.7 LC/EC/MS analysis

To hyphenate the LC/MS system with on-line electrochemistry, a conditioning cell model 5021 and a Coulochem II potentiostat from ESA Bioscience Inc. (Chelmsford, MA, USA) was inserted between the outlet of the UV/vis detector and the inlet of the ionization interface of the mass spectrometer. To protect the working electrode, a PEEK in-line filter (ESA) was mounted between column and electrode. It should be noted that all potentials provided for the ESA cell were measured vs. a Pd/H_2 reference electrode, while the CV measurements were measured against Ag/AgCl, as indicated below.

4.3 Results and discussion

In this chapter, we present a general strategy for identifying free and disulfide-bound cysteine (Cys) residues in proteins. *N*-(2-Ferroceneethyl)maleimide is used as a derivatizing agent. *N*-Substituted maleimides contain an activated double bond that can undergo a Michael-type electrophilic addition reaction by forming a stable thioether bond with sulfhydryl groups, as shown in Fig. 4.2 for

cysteine (Cys). Thiols preferentially react with maleimide groups in the form of the thiolate anion.

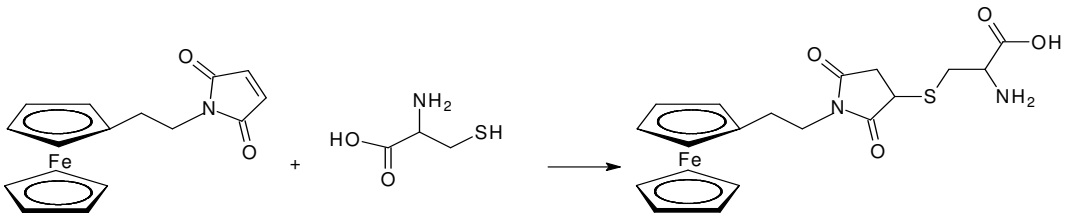

Fig. 4.2 Derivatization of cysteine with FEM.

The reaction is fast and highly selective at pH 7. The maleimide group is 1000 times more reactive towards a sulfhydryl group than towards an amine [29]. The reaction progress was investigated for glutathione, a tripeptide containing one Cys residue, using flow injection analysis (FIA) with MS detection of the derivative formed. At the conditions used (10 mM NH_4HCO_3 pH 7.8, room temperature, and tenfold molar reagent excess with respect to glutathione), the reaction was completed in less than 3 min.

Three small proteins were selected for the investigation. Two of these are acidic (insulin with a pI of 5.3 and β-lactoglobulin A with a pI of 5.2) and one is basic (lysozyme with a pI of 11.35) [30, 31, 32]. Lysozyme contains eight Cys residues within four disulfide bridges [33]. Insulin consists of two chains (A and B) and possesses two intermolecular disulfide bridges and one intramolecular disulfide bridge [34]. β-Lactoglobulin A contains two disulfide bridges and one free cysteine [35]. TCEP was used as reducing agent. TCEP is more stable than other reducing agents such as dithiothreitol (DTT) and β-mercaptoethanol, and it has the advantages of being water-soluble and

non-volatile. Furthermore, it exhibits a faster reaction [36]. Therefore, only half of the concentration of TCEP is typically applied compared with DTT under the same conditions [37]. Furthermore, TCEP possesses no thiol function, which could compete with the analyte for the derivatizing agent. However, it is known from the literature that using high concentrations of TCEP inhibits the labeling of proteins. There is an optimum ratio of 9:1 of TCEP over thiol and 10:1 of derivatizing agent over thiol [38]. In preliminary investigations, these ratios were proved to be applicable to thiol analysis with FEM as well. To investigate the progress of the reaction, a solution containing a protein denaturized with 8 M urea was first treated with TCEP as reducing agent and then with FEM. Only one peak could be detected in the TIC chromatogram for derivatized lysozyme (Fig. 4.3 a). Deconvolution of the mass spectrum shows that only the species with a mass of 16787 Da, the corresponding sodium adduct (16807 Da) and the potassium adduct (16826 Da) are present. For comparison, a spectrum of unreduced derivatized protein and deconvolution resulted in the masses of 14306 Da and 14328 Da (sodium adduct) after deconvolution, as shown in Fig. 4.3 b. The increase of 2481 Da is due to the addition of eight FEM (8 x 309 Da = 2472 Da) molecules, which implies complete alkylation of the eight Cys residues and no additional involvement of the six Lys residues. The mass difference of 9 Da between the measured and calculated data is due to the low mass accuracy of the single quadrupole mass spectrometer.

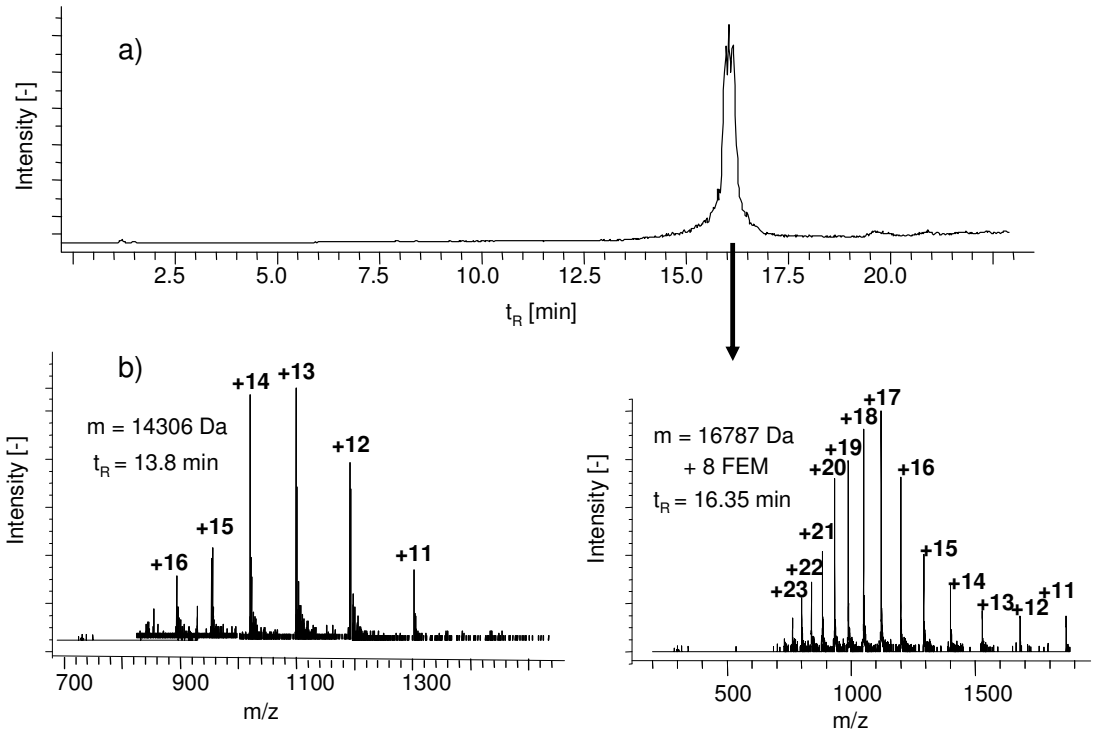

Fig. 4.3 TIC chromatogram of lysozyme, which was denaturated, reduced and derivatized with FEM after cleaning on a PD column and mass spectrum (a), solvent a and gradient A. For comparison, the mass spectrum of lysozyme that was denaturated and derivatized with FEM is shown as well (b).

The TIC chromatogram of unreduced and derivatized insulin is shown in Fig. 4.4. A peak is present due to the excess of the derivatizing agent FEM, because the solution was not cleaned by a PD column in this case. A small peak appears at t_r = 12.66 min with a deconvoluted mass of 6041 Da as well as the main peak at t_R = 11.32 min with a mass of 5732 Da. The difference of 309 Da can be traced back to the addition of one FEM molecule and it is a non-specific adduct (probably with one Lys residue in the B-chain) because no free Cys residue was present in insulin. This happened only when NH_4HCO_3-buffer (pH 7.8) is used. The peak of the non-specific adduct does not appear when NH_4ac (pH 6.8) was used to dissolve the protein (data not shown). For this reason, NH_4ac was used as the buffer in all subsequent labeling experiments. No further experiments were performed to study the

labeling of lysine in proteins with FEM at a higher pH. Therefore, other derivatizing agents will be better suited.

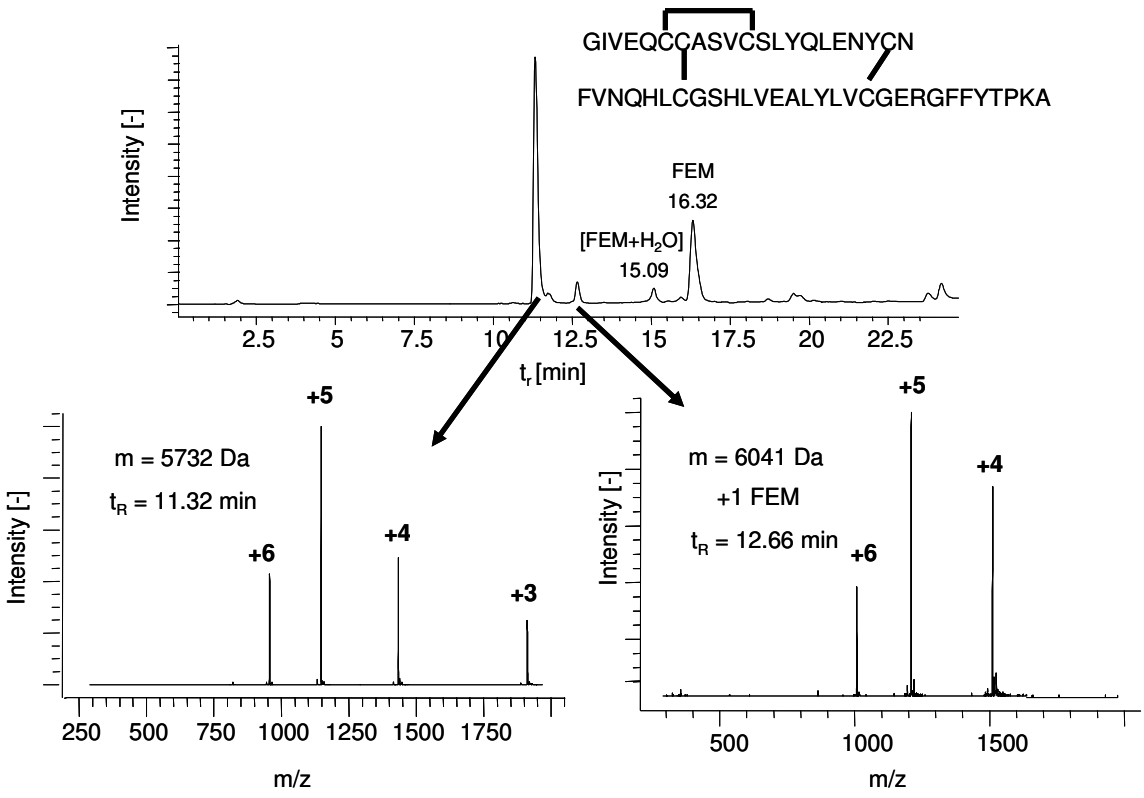

Fig. 4.4 TIC chromatogram of insulin, which was denatured and treated with FEM with the corresponding mass spectra. Conditions: solvent a and gradient A.

When insulin was reduced, two peaks appeared in the chromatogram because the two chains of insulin are not connected now (Fig. 4.5 a). The increase in mass is equivalent to two FEM in the B-chain and four FEM in the A-chain. In the A-chain, two of the cysteins are directly adjacent, but they are labeled, too. Therefore, it can be concluded that no spatial discrimination of the derivatization reaction with the ferrocene label takes place under these conditions.

With FEM, an electroactive label is introduced into the proteins. To study the electrochemical behaviour and to obtain information about the half-wave

potential of FEM derivatives, cyclic voltammetry was performed for derivatized glutathione, which serves as a small and therefore easy to study model compound. The redox system shows a reversible one-electron oxidation of the ferrocene derivative to the ferrocinium cation, and the half-wave potential is found to be 300 mV vs. Ag/AgCl.

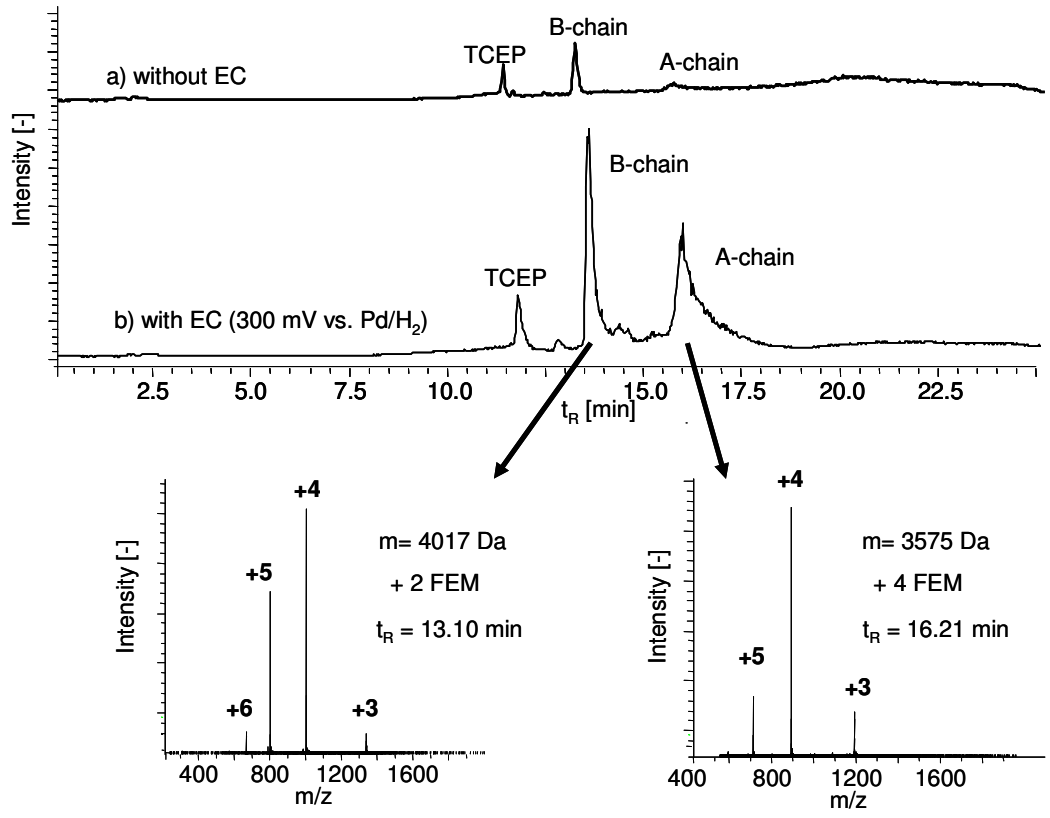

Fig. 4.5 LC/MS (a) and LC/EC/MS (b) chromatograms of insulin, reduced and derivatized with the corresponding mass spectra of the A-chain and the B-chain. Conditions: solvent b and gradient A.

For LC/EC/MS measurements, an electrochemical flow cell, containing a porous glassy carbon working electrode with a very large surface area, was used to obtain a high conversion rate for the ferrocene derivatives. It was possible to increase the intensity and the average charge state of the protein by EC/ESI-MS detection. However, it is not possible to draw conclusions from

the increase in charge to the number of Cys residues. For the A chain and the B chain, the increase is equivalent to only one charge (Fig. 4.6). This is not surprising, because the proteins are already highly charged.

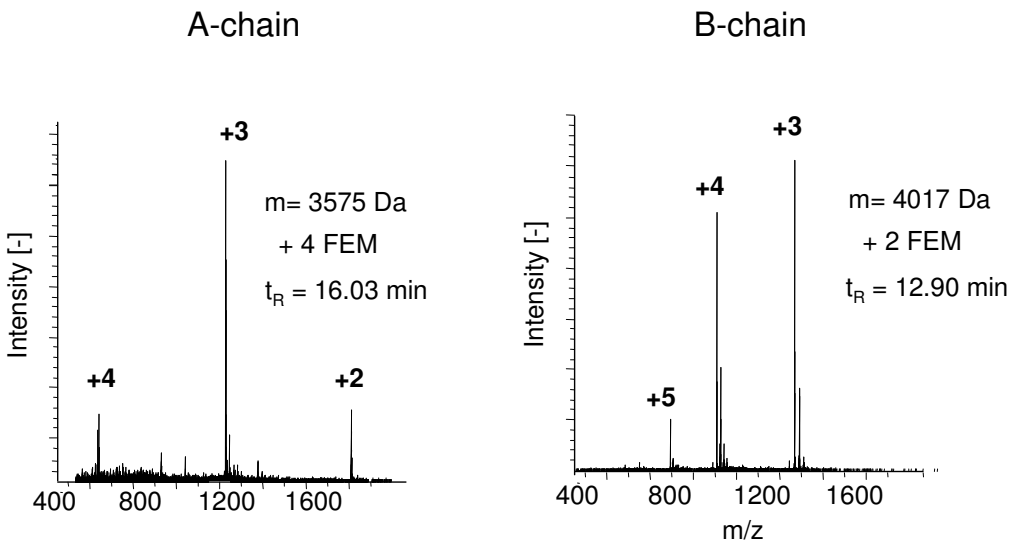

Fig. 4.6 Mass spectra of the A- and B-chain of insulin, reduced and derivatized measured with LC/MS without EC.

Peak tailing was observed for the peaks corresponding to the A- and B-chain, probably due to adsorption to the glassy carbon working electrode of the electrochemical cell (Fig. 4.5 b). However, this did not affect the reproducibility of the LC separation and the electrode has not to be cleaned after each run. The cleaning is achieved within the chromatographic run.

β-Lactoglobin A possesses one free cysteine, as confirmed by the increase of 309 Da to 18669 Da (Fig. 4.7). There are only two peaks in the chromatogram (solvent system b and gradient B were used). One of these corresponds to the excess of the derivatizing reagent FEM (t_R = 17.02 min) and the other one to the derivatized protein (t_R = 15.03 min). No unlabeled protein (18360 Da), which should elute at t_R = 13.8 min, could be detected.

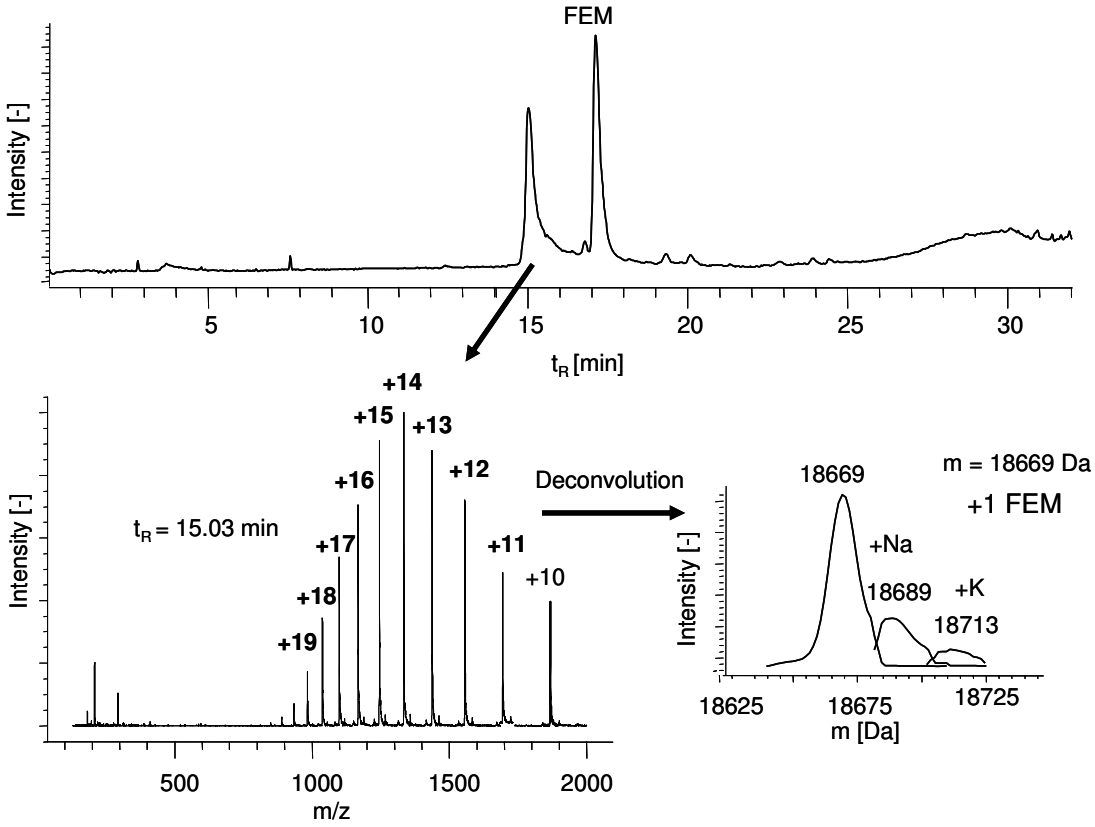

Fig. 4.7 LC/MS chromatogram of β-lactoglobulin A, treated with FEM with the corresponding mass spectra and deconvolution data. Solvent system b and gradient B were used.

After reduction with TCEP, the TIC chromatogram became more complex (

Fig. 4.8) due to the added reducing agent (recorded by using solvent system b and gradient C). The signals at t_R = 8.1 min (dominant peak *m/z* 578) and t_R = 8.5 min (dominant peak *m/z* 560) are caused by TCEP-FEM and the ferroceneethylsuccinamic acid derivative of TCEP, which is formed by the hydrolytic cleavage of the N-C bond of the succinimide ring of FEM. The peak at t_R = 9.5 min could be attributed to the excess of derivatizing agent. Deconvolution of the mass spectrum of the peak at t_R= 8.7 min leads to a mass of 19910 Da. This corresponds to an increase of 1550 Da, which is

equivalent to the addition of five FEM (calculated: 1545 Da). Again, the differences can be attributed to the low mass accuracy of the mass analyzer used.

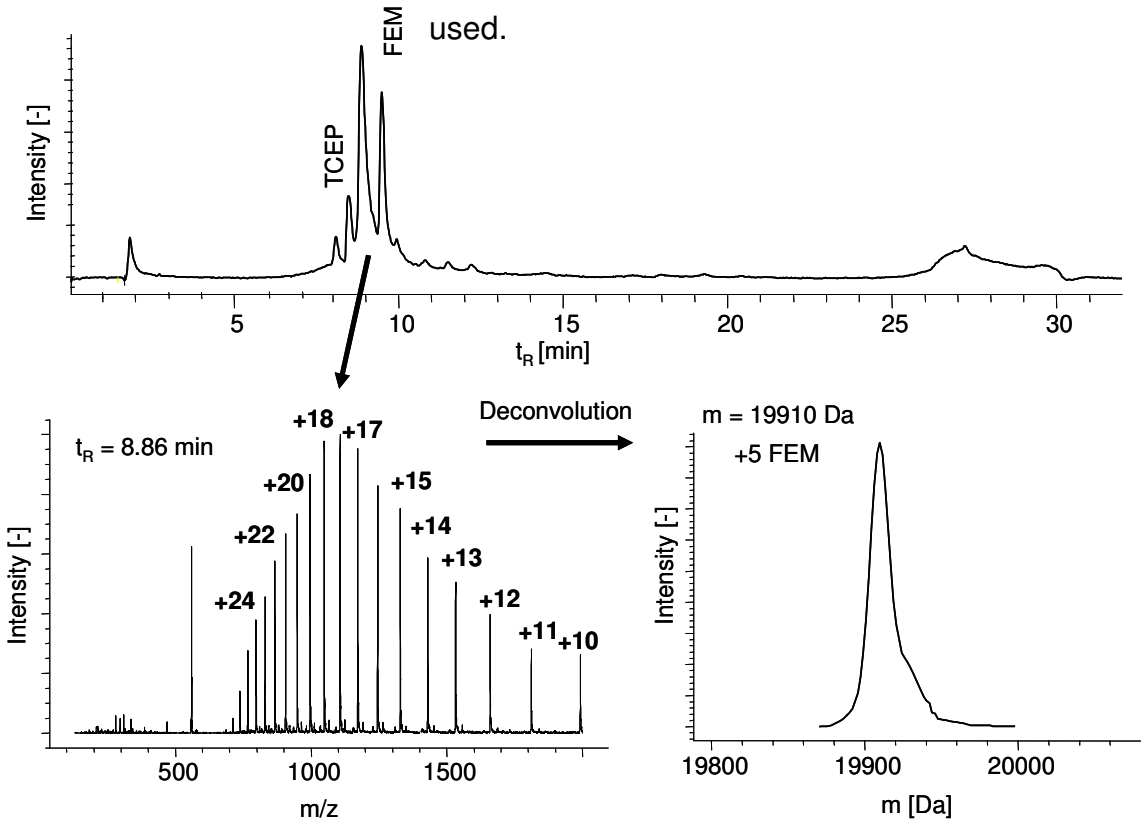

Fig. 4.8 LC/MS chromatogram of β-lactoglobulin A. Disulfide bonds reduced with TCEP and treated with FEM with the corresponding mass spectra and deconvolution data. Solvent system b and gradient C were used.

To obtain information about the position of the Cys residues in the proteins, a tryptic digest was performed. Trypsin cleaves proteins at the C-terminal side of lysine and arginine amino acid residues. The tryptic digest of the reduced and derivatized β-lactoglobulin A yields the TIC chromatogram shown in Fig. 4.9. By using electrochemistry/MS, an increase in the intensities of the peaks corresponding to peptides that contain one or more ferrocene units compared with those from the non-derivatized peptides could be detected. The increase

was greatest at an optimum potential of 300 mV vs. Pd/H_2, which confirms the earlier CV measurements.

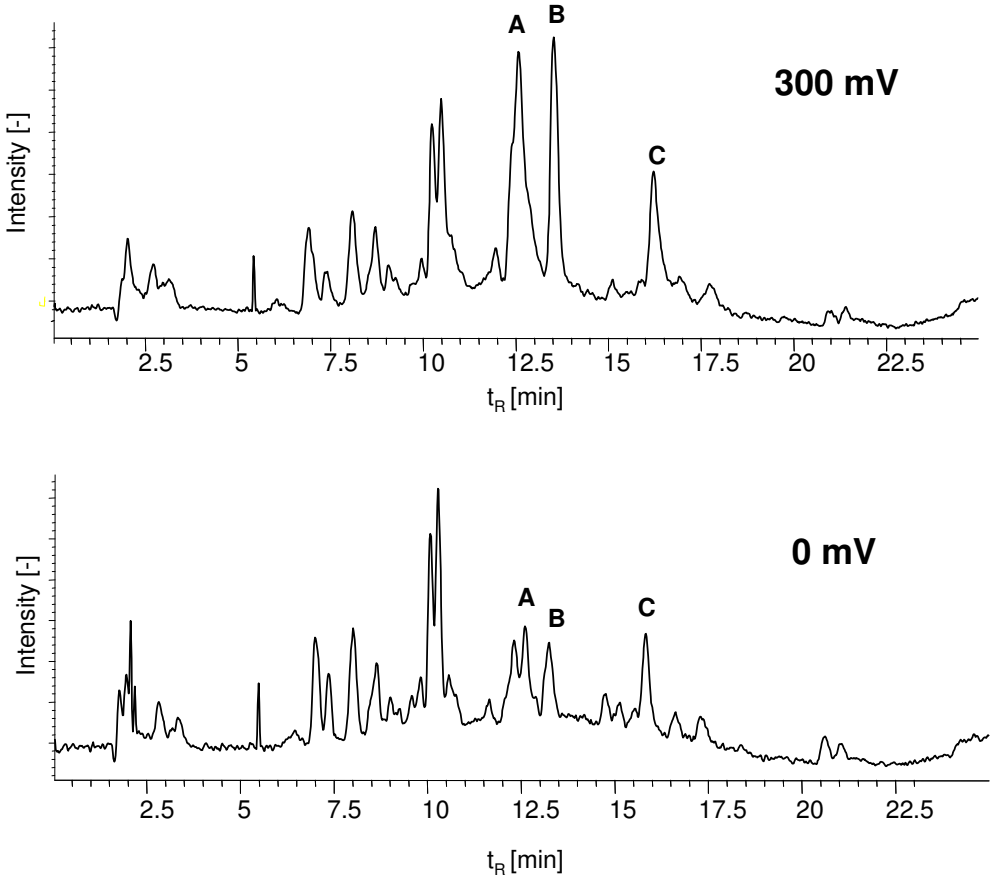

Fig. 4.9 TIC Chromatogram of the trypsin digest of β-lactoglobulin A with LC/EC/MS at a potential of 300 mV (a) and LC/MS (b) detection, LC conditions: gradient A, solvents a. The peak intensity of the peaks A, B and C are enhanced at 300 mV compared to 0 mV.

Further, the increase of the potential results in the same or less intensity. This may be explained by the behaviour of the model peptide glutathione. When glutathione is analysed by ESI-MS, the main peak corresponds to the protonated molecular ion $[M+H]^+$. Furthermore, there is an enhanced molecular ion peak $[M]^+$. By switching on the electrochemical cell, which is inserted between the outlet of the LC column and the inlet of the mass spectrometer (EC/ESI-MS) at 300 mV, the $[M]^+$ becomes the base peak and

the $[M+H]^+$ disappears. Because the intensity is not distributed between two peaks any more, the intensity of the $[M]^+$ is increased. The ferrocene unit is oxidized to the ferrocinium cation, and protonation of the already charged molecule in the ESI-interface is unlikely. Since the oxidation occurs in the ESI source, the $[M]^+$ peak is already present in the mass spectra, which were recorded under regular ESI-MS conditions. That explains why the average charge states and the intensities of the derivatized peptides are increased by LC/EC/MS compared to LC/MS measurements. As already observed for proteins, this increase does not correlate with the number of thiol functionalities and ferrocene labels, respectively. This can be explained by the principle of electrospray as an ionization technique. It is likely that additional charges were transferred either by protonation or by electrochemistry in the electrospray needle and not just by electrochemistry in the interface. An increase in signal intensity was observed for the three signals marked A, B and C in the TIC-chromatogram of Fig. 4.9, due to the electrochemical oxidation. Peak B is the excess of derivatizing agent. Peak A corresponds to the tryptic peptide LSFNPTQLEEQCHI, and peak C to YLLFCMENSAEPEQSLVCQCLVR (Fig. 4.10). Only one Cys residue (C 66) was not found in the tryptic digest, either derivatized or underivatized. However, a basic lysine residue is located directly adjacent to the peptide WENDECAQK. It is known from the literature [39] that the likelihood of tryptic cleavage is reduced in this case.

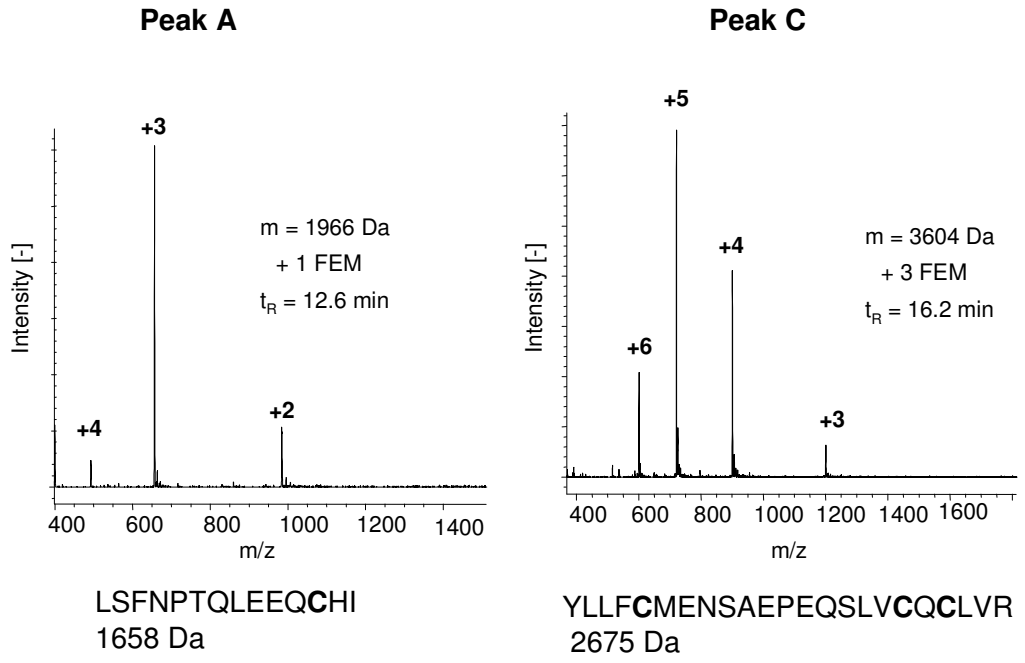

Fig. 4.10 Mass spectra, composition and mass of tryptic digest peptides containing derivatized thiol functionalities (taken from the chromatogram shown in Fig. 4.9).

4.4 Conclusions

FEM has proven to be a useful new derivatizing agent for the determination of thiol functionalities by LC/MS, whether or not it is used in conjunction with thiol reduction. Under the optimized conditions employed in this work, the highly selective derivatization of cysteine residues can be achieved, even in the presence of lysine groups. Additional information is obtained by using an electrochemical cell, where the ferrocene units are converted to ferrocinium cation. An increase in intensity after electrochemical treatment indicates the presence of a derivatized cysteine residue in the complete or the tryptically digested peptide. Future work is presented in chapter 5. It focuses on the simultaneous labeling of free and disulfide-bound thiols using two different ferrocene-based reagents, which can then be distinguished by tandem mass spectrometric measurements.

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Chapter 5

Simultaneous LC/MS/MS Determination of Thiols and Disulfides in Urine Samples Based on Differential Labeling with Ferrocene-Based Maleimides‡

A method for the simultaneous determination of a series of thiols and disulfides in urine samples has been developed based on the sequential labeling of free and bound thiol functionalities with two ferrocene-based maleimide reagents. The sample is first exposed to N-(2-ferroceneethyl)maleimide (FEM), thus leading to the derivatization of free thiol groups in the sample. After quantitative reaction and subsequent reduction of the disulfide-bound thiols by tris(2-carboxyethyl)phosphine (TCEP), the newly formed thiol functionalities are reacted with ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide (FMEA). The reaction products are determined by LC/MS/MS in the multiple reaction mode (MRM), and precursor ion scan as well as neutral loss scan are applied to detect unknown further thiols. The method was successfully applied to the analysis of free and disulfide-bound thiols in urine samples. Limits of detection (LODs) are 30 to 110 nM, limits of quantification (LOQs) 100 to 300 nM and the linear range comprises two decades starting at the limit of quantification, thus covering the relevant concentration range of thiols in urine samples. The thiol and disulfide concentrations were referred to the creatinine content to compensate for different sample volumes. As some calibration standards for the disulfides are not commercially available, they were synthesized in an electrochemical flow-through cell. This allowed the synthesis of disulfides based on two identical or different thiols.

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5.1 Introduction

Most thiols occurring in body fluids are related to metabolic processes. Cysteine is an endogenous precursor of glutathione and a metabolite of *N*-acetylcysteine [1]. Cysteinylglycine is a catabolite of glutathione and may be cleaved into cysteine and glycine [2]. Homocysteine, a product of methionine demethylation, may either be catabolized to cysteine or remethylated to methionine [3]. All these aminothiols circulate as protein-bound, low molecular weight disulfides and in a free form [4]. Besides symmetrical disulfides, mixed disulfides may be formed as well by oxidative coupling of two different thiols [5]. In recent studies it has been found that changes in the amount of thiols and disulfides in body fluid occur when cellular processes do not work properly [6]. Thus, the analysis of all of these aminothiols is important for the diagnosis of inherited (homocystinuria, cystinuria, etc.) and of acquired (cobalamine and folic acid deficiencies) metabolic disturbances and several other diseases. Furthermore, the concentrations and the ratio of thiols and disulfides are influenced by various factors as drug administration, oxidative stress and infections and may provide information on the stage of disease progression. Therefore, it is required to determine the concentration of the individual disulfides and thiols [1]. Thiols are labile due to the high reactivity of the thiol group and are easily transformed into mixed and symmetrical disulfides. In biological samples, it is important to stabilize thiol groups by immediately derivatizing them with appropriate reagents, as described in a large number of publications as summarized in various reviews [1, 7]. The functional groups with a selective reaction towards the thiol function include, but are not limited to *N*-substituted maleimides, active halogens and

aziridines. Among those, maleimides were found to be most selective and reactive [8]. The determination of disulfides and thiols is performed sequentially in most cases [9]. For the simultaneous determination of disulfides and thiols, only a few reports exist. The detection methods mostly used for that purpose are electrochemical [10] and mass spectrometric detection [11-15]. However, electrochemical methods suffer from severe sensitivity problems due to interferences caused by electroactive impurities [16]. It is not easy to separate polar thiols and disulfides on commercial reversed-phase (RP) columns. At this moment, only one mass spectrometric method for the simultaneous quantitative determination of derivatized thiols and disulfides in biological samples is described in literature. The method uses a specialized RP18 column [12], but the derivatives of cysteine and the underivatized cystine, as well as homocystine and the derivative of homocysteine were not separated within the LC run, thus increasing the likelihood of ion suppression even in the MRM mode. A drawback of the direct analysis of disulfides is that other polar compounds, depending on the sample used, may coelute with the analytes. Even more important, it is known that homocystine and cystine quickly aggregate to proteins and therefore spontaneously disappear from plasma or serum samples when stored [17].

We propose that the derivatization of both thiols and disulfides will lead to more reliable results. No additional extraction from the sample matrix has to be performed and it is ensured that both disulfides and thiols are stabilized. Furthermore, the real total amount of bound thiol is determined because the thiol that is bound in mixed disulfides or proteins is determined as well. Direct

determination of all possible mixed disulfides would, on the other hand, be very difficult due to the very large number of possible permutations. Until now, only one differential labeling method for thiols and disulfides is currently known from literature [18]. The thiols are detected as ABD-F (4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole) derivatives and the disulfides were detected after reduction as SBD-F (7-fluoro-2,1,3-benzoxadiazole-4-sulfonate) derivatives by fluorescence. The main drawbacks of this method are that the cysteine-ABD-F derivative is unstable at the conditions used for the labeling of the disulfides and that an extraction with ethyl acetate is used to remove the excess of ABD-F [18].

In this chapter, a new differential labeling method for disulfides and thiols is presented. This method was applied for the determination of cysteine, glutathione, cysteinylglycine, *N*-acetylcysteine, homocysteine and their disulfides in urine. For thiol derivatization, *N*-(2-ferroceneethyl)maleimide (FEM) is used. FEM, originally developed by Shimada et al. [19] for the determination of thiols by HPLC with electrochemical detection was used recently as a mass tag for the derivatization of cysteine functions in proteins [20]. The thiols that occur as disulfides were derivatized by the newly developed derivatizing agent ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide (FMEA).

5.2 Experimental part

5.2.1 Chemicals

All reagents and chemicals were obtained from Sigma Aldrich (Steinheim, Germany) or Fluka Chemie GmbH (Buchs, Switzerland) in the highest quality available and were used without further purification. The water used for HPLC was purified using a Milli-Q Gradient A 10 system and filtered through a 0.22 µM Millipak 40 filter unit (Millipore, Billerica, MA, USA). The acetonitrile (ACN) for HPLC was obtained in gradient grade quality from Merck KGaA (Darmstadt, Germany). *N*-(2-Ferroceneethyl)maleimide (FEM) was synthesized from ferrocenylmethyltrimethylammonium iodide (obtained from Alfa Aesar (Karlsruhe, Germany)) according to a method described in literature [19, 20]. Ferrocenecarboxylic acid chloride was synthesized from ferrocenecarboxylic acid (obtained from Alfa Aesar (Karlsruhe, Germany)) according to [21].

5.2.2 HPLC/MS/MS

The LC/MS setup comprised a Shimadzu (Duisburg, Germany) LC system and a QTrap mass spectrometer (Applied Biosystems, Darmstadt, Germany), equipped with an electrospray ionization (ESI) source. The LC system consisted of two LC-10ADVP pumps, a DGC-14A degasser, a SIL-HTVP autosampler, a CTO-10AVP column oven, and a SPD-10AVVP UV detector. The software used for controlling LC and MS was Analyst 1.4.1 (Applied Biosystems, Darmstadt, Germany). The analytes were ionized in the ESI interface with an ionspray voltage of 5000 V, using 50 psi nebulizer gas and 70 psi dry gas with a temperature of 500°C. The analytes were detected in the

positive ion mode using tandem mass spectrometry in the multiple reaction monitoring (MRM) mode with the collision activated dissociation (CAD) gas set to high. Declustering potential (DP), entrance potential (EP) and cell exit potential (CEP) as well as the collision energy were optimized under HPLC conditions for each analyte and were used for all LC/MS measurements. Two MRM transitions were monitored for each of the derivatives: GSH-FMEA 660→213, 185; Cys-FMEA 474→213, 185; HCys-FMEA 488→213, 185; Nac-FMEA 516→213, 185; 2-MPG-FMEA 516→213, 185; GSH-FEM 617→422, 144; CysGly-FEM 488→422, 144; Cys-FEM 431→365, 212; Hcys-FEM 445→379, 246; Nac-FEM 473→407, 278; 2-MPG-FEM 473→407, 278. The first transition was used for quantification because of its higher signal intensity.

A binary gradient consisting of ACN and formic acid/ammonium formate buffer (10 mM, pH 4) was applied for the liquid chromatographic separation. The column employed for the HPLC separation was an Agilent Zorbax Eclipse XDB-C8 column (Agilent Technologies, Palo Alto, CA, USA) with a particle size of 5 µm, a pore size of 120 Å, a length of 150 mm and an inner diameter of 4.6 mm. The injection volume was 10 µL. As flow rate 0.9 mL/min was selected. The derivatives were eluted with the following gradient profile:

t [min]	0.03	3	6	16	17	18	19	25
c (CH_3CN) [%]	19	19	26	55	90	90	19	Stop

5.2.3 Synthesis of ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide (FMEA)

Ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide was synthesized from ferrocenecarboxylic acid chloride. 180 mg (0.7 mmol) of *N*-(2-aminoethyl)-maleimide trifluoroacetate and 300 µL triethylamine (TEA) were mixed and dissolved in dichloromethane. In another flask, 150 mg (0.6 mmol) ferrocenecarboxylic acid chloride was dissolved in 10 mL dry dichloromethane assisted by an ultrasonic bath. This solution was added to the first solution and stirred for 10 min. The color of the solution changed immediately from red to orange and thin-layer chromatography (TLC) on silica gel 60 with ethyl acetate as mobile phase proved quantitative conversion. The solution was extracted with diluted hydrochloric acid, washed with water and dried over magnesium sulfate. The solvent was evaporated under reduced pressure to yield 150 mg (67%) of a yellow to orange powder. Purity was checked by TLC. If necessary, ferrocenecarboxylic acid-(2-maleimidoyl)-ethylamide was cleaned on a silica column (mobile phase: ethyl acetate, silica gel 60, R_f 0.5). ^{1}H-NMR (300 MHz, $CDCl_3$, TMS) δ(ppm) 3.60 ppm (2H, m); 3.80 ppm (2H, m); 4.09 ppm (5H, s); 4.12 ppm (2H, s); 4.32 ppm (2H, s); 6.75 ppm (2H, s); ESI-MS: *m/z* 352.3 $[M]^+$, MS/MS: m/z 213.

5.2.4 Determination of creatinine

Creatinine was determined using an HPLC-UV method reported elsewhere [22] with some modifications. 20 µL of a urine sample diluted 1:100 with mobile phase was injected on the HPLC column (Nucleodur RP, Macherey-Nagel (Düren, Germany), 4.6 mm x 150 mm, 5 µm particle size). The mobile

phase consisted of water and ACN (95/5, v/v) containing 10 mM of sodium 1-octanesulfonate with the pH adjusted to 3.2 with orthophosphoric acid. The flow rate was 1 mL/min and creatinine was detected at 236 nm.

5.2.5 Preparation of symmetric disulfides

Some symmetric disulfides like *N*-acetylcystine $(Nac)_2$ and 2-mercaptopropionylglycine disulfide $(2\text{-MPG})_2$ are not commercially available and have to be prepared before quantification. This was achieved by pumping a 5 mM thiol solution containing 10 mM ammonium bicarbonate (pH 7.8) through an electrochemical flow cell (conditioning cell model 5021) connected to a Coulochem II potentiostat (both from ESA Bioscience Inc., Chelmsford, MA, USA) and operated at 800 mV vs. Pd/H_2 with a flow rate of 100 µL/h. The working electrode material was glassy carbon with a Pd counter electrode and a Pd/H_2 reference electrode. For protection of the working electrode, a PEEK inline filter (ESA Bioscience Inc.) was mounted between the syringe and the electrochemical cell. The electrochemical conversion was controlled by mass spectrometry and was found to be quantitative in all investigated cases when using the conditions stated above. No further oxidation of the disulfides occurred. Standard disulfide solutions could therefore be prepared by this procedure from thiol solutions of known concentration.

5.2.6 Thiol and disulfide solutions

Thiol and disulfide solutions (5 to 10 mM and 0.5 to 1 mM) were prepared in 1 mM EDTA solution containing 0.05 % formic acid. An ultrasonic bath was used to dissolve the disulfides. However, no heating of the bath was carried

out, because otherwise thiol formation was observed from the disulfides. The prepared disulfides were diluted 1:10 with the EDTA solution stated above.

5.2.7 Sample collection and pretreatment

Urine was collected from eight healthy volunteers (2 female, 6 male). Directly after sampling, 97 µL of a 1 mM EDTA solution and 3 µL formic acid were added to 900 µL of urine to prevent the autoxidation of thiols by complexing potentially catalytic metal cations and by acidifying the solution. The samples were stored at 4°C and derivatized within 2 hours. The urine was diluted directly before derivatization by a factor of 2.5 with 1 mM EDTA solution containing 0.05 % formic acid.

5.2.8 Differential derivatization of thiols and disulfides

A volume of 60 µL internal standard (containing 5 µM 2-mercaptopropionyl-glycine (2-MPG) and 2.5 µM disulfide of 2-MPG $(2\text{-MPG})_2$) was added to 125 µL of the standard solution or diluted urine. After mixing, 40 µL of FEM solution (5 mM, in ACN) and 30 µL of ammonium bicarbonate solution (1 mM) were added. 4-Acetamidothiophenol (AATP) solution (25 µL, 20 mM in ACN) was added, vortex-mixed and treated with 20 µL of tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 25 mM in 10 mM ammonium bicarbonate solution). After 30 min, first 75 µL of FMEA solution (20 mM in ACN) and after mixing, 75 µL of AATP solution were added. The sample was acidified by adding 20 µL of formic acid (25%) and finally 30 µL of ascorbic acid solution (100 mM, 10% (v/v) formic acid) to prevent oxidation reactions in solution.

For the precursor ion scan measurement, 250 µL of urine were used and treated as stated above.

5.2.9 Derivatization of the sum of thiols and disulfides

As for the differential labeling, a volume of 60 µL internal standard solution was added to 125 µL of the standard solution or diluted urine sample. The pH was adjusted to 7 by adding 30 µL of the 1 mM ammonium bicarbonate solution and the mixture was treated with 20 µL of TCEP solution (25 mM in 10 mM ammonium bicarbonate solution). After 30 min, 65 µL ACN and 75 µL FMEA solution (20 mM in ACN) were added, vortex-mixed and treated as stated above for the differential labeling.

5.3 Results and discussion

5.3.1 Derivatizing agents

For differential derivatization, two different derivatizing agents were needed. Ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide (FMEA) was synthesized as a slightly more polar derivatizing agent than *N*-(2-ferroceneethyl)maleimide (FEM). Both derivatizing agents convert polar analytes to less polar products with the goal to enable reversed-phase separation of the thiol derivatives. Thus, the separation from other polar compounds in the sample is achieved without intensive sample preparation. FMEA was synthesized as a new derivatizing agent for the analysis of thiols following a two-step route, which is presented in Fig. 5.1. The first step consists of the reaction of the commercially available ferrocenecarboxylic acid to the respective

ferrocenecarboxylic acid chloride (FCC) by using oxalyl chloride and catalytic amounts of *N,N*-dimethylaminopyridine as reported in literature [21].

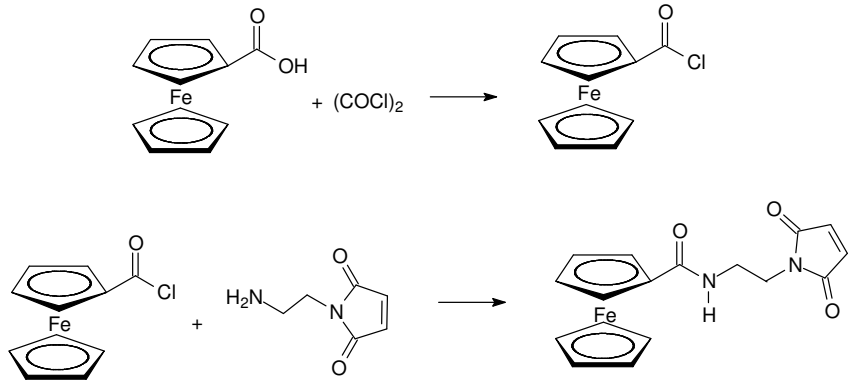

Fig. 5.1 Reaction scheme for the synthesis of FMEA.

FCC then reacts with *N*-(2-aminoethyl)maleimide to yield FMEA. FMEA and FEM have, under the derivatization conditions mentioned above, comparable reaction velocities of less than 3 min for quantitative conversion with all thiols investigated (as controlled by FIA-ESI-MS experiments, data not shown). One advantage of FMEA is the easy accessibility with only a two-step synthesis without preparative chromatographic clean up, whereas for FEM four steps of synthesis are needed.

5.3.2 Differential labeling of thiols and disulfides

The general strategy of this methodology involves the following four steps: (1) derivatization of the free thiols in the mixture with FEM at pH 7; (2) binding the excess of FEM with 4-acetamidothiophenol (AATP); (3) reduction of the disulfides with TCEP; (4) derivatization of the generated thiols (originally present as disulfides) with FMEA (Fig. 5.2).

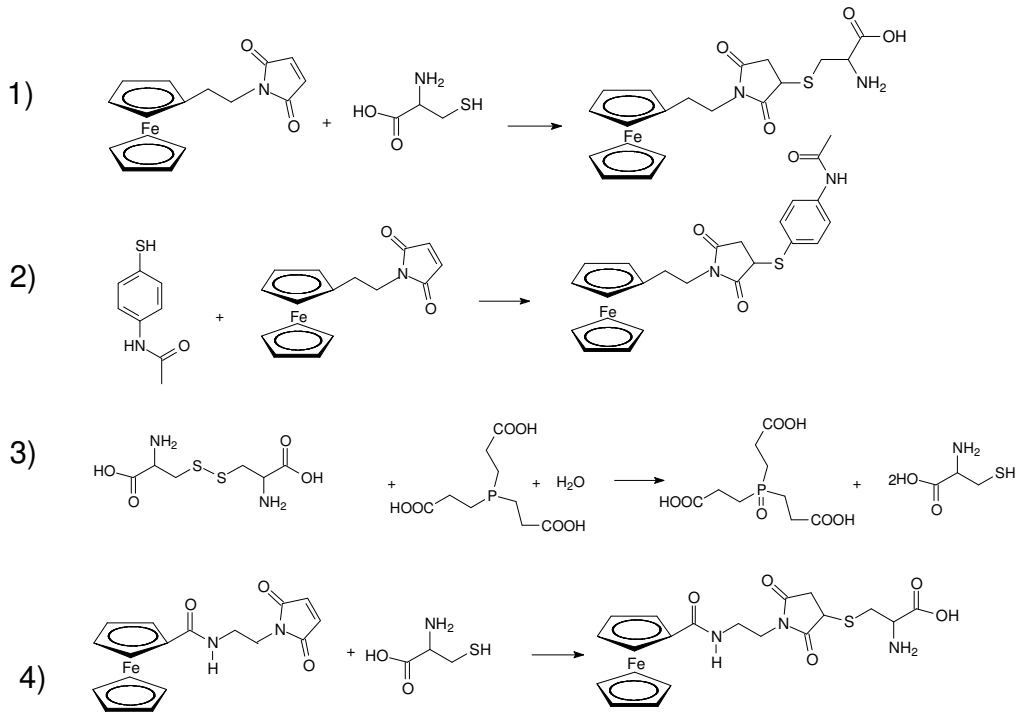

Fig. 5.2 Derivatization of cysteine with FEM (1), reaction of the FEM excess with the blocking reagent (2) and derivatization of cystine with FMEA (4) after reduction with TCEP (3).

2-Mercaptopropionylglycine (MPG), a drug that has been used in the treatment of cystinuria [23], was selected as internal standard. In urine of persons without respective treatment, MPG is not present. The reaction between thiol and maleimide is a Michael type addition reaction that involves the thiolate anion. Therefore, the reaction velocity depends on the pH. Typically, a pH of 7 is selected for the derivatization of thiols with maleimides. Under these conditions, other functional groups (amino, hydroxyl) do not react according to literature data [24]. The derivatizing agent FEM was added before the pH was adjusted to 7 to ensure that no oxidation of thiols to disulfides occurs. FEM was selected as first derivatizing agent because of its

lower water solubility compared with FMEA and because of the requirement of a larger reagent excess in the second reaction step.

Compared to other thiols (pK_a around 8) the pK_a value of the thiol functionality in 4-acetamidothiophenol (AATP) (pK_a 6.1 [25]) is low. To a large extent, the thiolate anion is formed even at neutral pH, thus leading to a very efficient derivatization. Because disulfide formation may occur rapidly as well in aqueous solutions, AATP is dissolved in ACN. Another advantage of using AATP as blocking reagent is that it forms low polarity products with the derivatizing agents, which elute very late in the chromatographic run (t_R=14.3 min (AATP-FMEA), t_R=18.6 min (AATP-FEM). Even the AATP-FMEA derivative elutes after the other aminothiol-FEM derivatives.

TCEP was selected as a reducing agent due to its good water solubility and stability in aqueous systems. Furthermore, it is known from literature that TCEP is the most efficient reductant for disulfides and cannot form a covalent adduct with cysteine [26]. The reduction was finished within 30 min. TCEP was found to react with the derivatizing agent as well. No TCEP-FEM (*m/z* 531) derivative was detected, which proves that no FEM was present during and after the reduction. Further experiments with only thiols and no disulfides present in the sample showed that none of them was detected in the disulfide elution window of the chromatogram. FMEA was added as second derivatizing agent in a large excess over TCEP and AATP, which are still present in the solution. Because of the large excess of FMEA added, AATP was added afterwards to ensure that FMEA is quantitatively converted to its AATP derivative and does not disturb the chromatographic separation of

the FEM derivatives. An acidic solution of ascorbic acid is added at the end to ensure the stability of the formed derivatives. The FEM derivatives have a low oxidation potential (300 mV vs. Pd/H_2). In some cases, oxidation was found to take place in the solution mixture, resulting in ferrocinium cations. These lead to an elution of derivatives with the injection peak in case that no ascorbic acid was added to reduce the ionic species prior to chromatography. The acidification is necessary to stabilize the derivatives. Under alkaline conditions, hydrolytic cleavage of the N_1-C_2 or the N_1-C_1 bond may lead to the formation of an open chain adduct (a succinamic acid derivative) The α-amino group of cysteine has a position, which allows the attack at the N_1-C_1-bond of the succinimde ring, thus forming a thiazine type adduct. Both side reactions take place for all maleimide-based derivatizing agents and have been intensively investigated in literature [27]. The formation of these expected side products was investigated for the ferrocene-based maleimides. No hydrolytic cleavage of the derivatives (formation of $[M+H_2O]^+$) was detected when the sample was acidified immediately after sampling. However, the formation of the thiazine adduct occurred only to a low extent. Additional peaks with *m/z* 431 and *m/z* 474 were detected in this case. The intensity was low for the Cys-FMEA derivative (approximately 1.8% of the area of the unchanged derivative) and higher for the Cys-FEM derivative (approximately 9.5% of the area of the unchanged peak). However, the ratio of the thiazine to the main product did not change when the same time intervals were used for the derivatization steps. This was confirmed by comparing the proportion at different concentrations with and without matrix. To avoid problems for the

quantitative measurements, the peaks of the thiazine adducts were integrated as well and the peak area ratio was checked.

The organic content added to the sample causes precipitation of the present proteins. Therefore, no addition of sulfosalicylic acid or other dedicated additives for protein precipitation is needed.

5.3.3 Tandem MS experiments

The derivatives of FEM and FMEA were detected as molecular ions $[M+H]^+$ with ESI-MS. The product ion spectra of the FMEA derivatives show three common fragment ions at *m/z* 129, *m/z* 185 and *m/z* 213 (Fig. 5.3). The fragment at *m/z* 213 corresponds to the cleavage of the ester function next to the carbon atom of the carbonyl group. Further loss of carbon monoxide leads to *m/z* 185. The same product ions were observed for phenol derivatives of ferrocene carboxylic acid chloride [28].

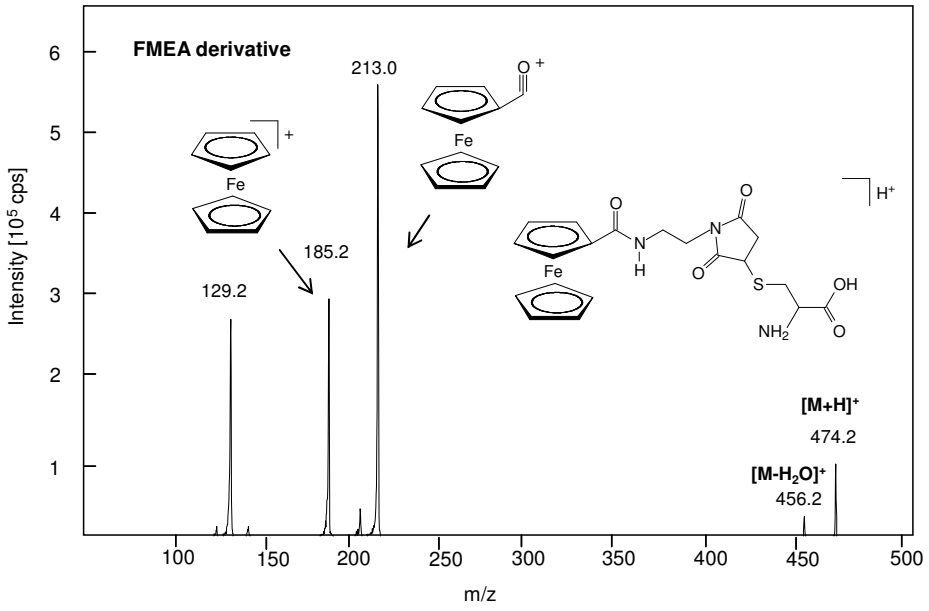

Fig. 5.3 Product ion spectra of the cysteine derivative of FMEA (*m/z* 474).

The fragment ion spectra of FEM derivatives are completely different and no common fragment ions were observed. However, a neutral loss of *m/z* 66 was detected for all derivatives (Fig. 5.4). The neutral loss of 66 Da corresponds to the symmetrically coordinated ligand cyclopentadienyl (leaving as a neutral). For glutathione-FEM, the most abundant fragment is *m/z* 422 and not *m/z* 551. The additional difference of 129 Da is due to the loss of the glutamyl residue, which is the main fragment in tandem MS experiments with glutathione.

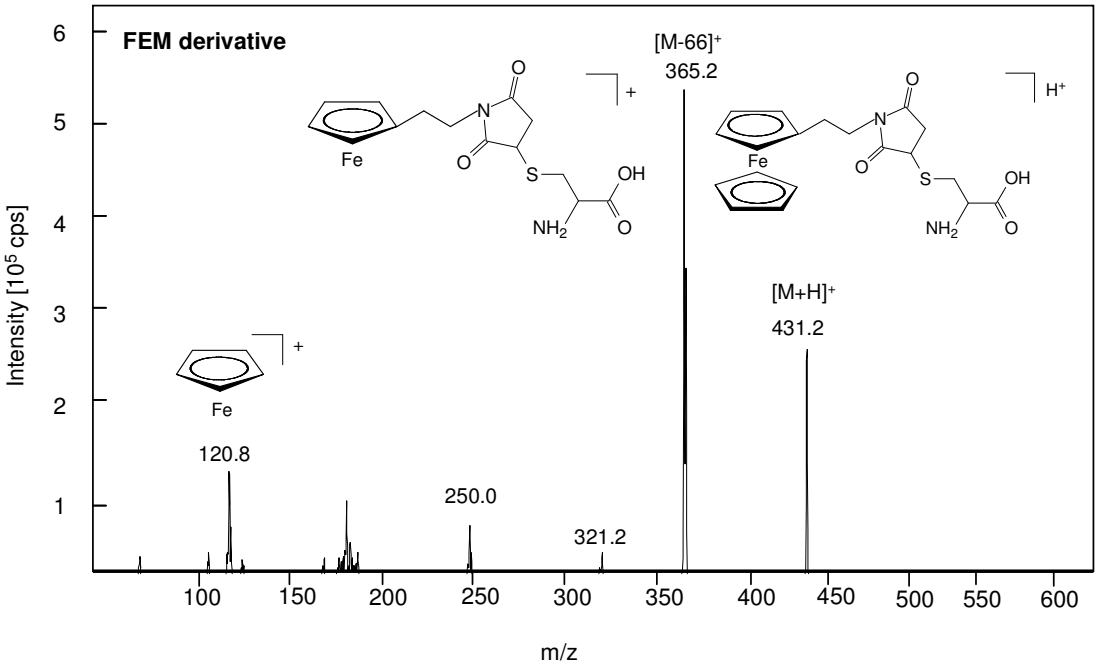

Fig. 5.4 Product ion spectra of the cysteine derivative of FEM (*m/z* 431).

5.3.4 HPLC separation of FMEA derivatized thiols and FEM derivatized disulfides

A reversed-phase liquid chromatographic separation for the investigated compounds was achieved using an Agilent Zorbax C8 column with a binary gradient consisting of ACN and aqueous formic acid/ammonium formate

buffer (10 mM, pH 4). The respective chromatogram is presented in Fig. 5.5. The FMEA derivatives elute in the first part of the chromatogram, and the FEM derivatives elute in the second part. The UV trace (254 nm) shows the formed by-products (TCEP-FMEA, AATP-FMEA, AATP-FEM and AATP disulfide) that do not coelute with the derivatives of the physiologically relevant thiols and disulfides.

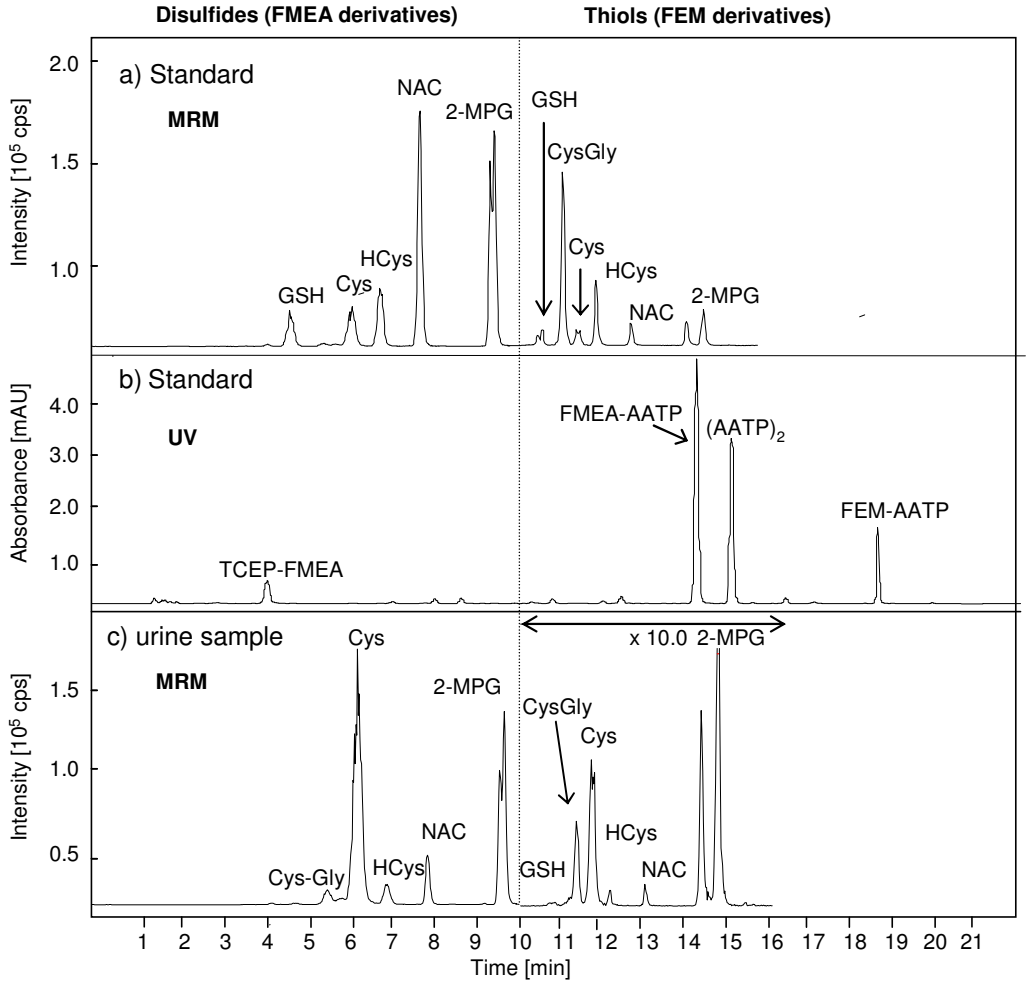

Fig. 5.5 a) Chromatogram for the separation of a mixture of FMEA and FEM derivatives, mass spectrometric determination in the multiple reaction monitoring (MRM) mode; FMEA derivatives: GSH-FMEA 660→213, Cys-FMEA 474→213, Hcys-FMEA 488→213, Nac-FMEA 516→213, 2-MPG-FMEA 516→213; FEM derivatives: GSH-FEM 617→422 , CysGly-FEM 488→422, Cys-FEM 431→365, HCys-FEM 445→379, Nac-FEM 473→407, 2-MPG-FEM 473→407; b) UV chromatogram recorded at 254 nm; c) Chromatogram of a typical urine sample (mass spectrometric detection, MRM mode).

With the maleimide, diastereomers were formed for optically active thiol-containing compounds. Under the chromatographic conditions described above, the diasteromers elute in a single peak, thus enabling an easier quantification than in case of separated diastereomers. Because of the high selectivity and excellent limits of detection, MRM transitions were used for quantification purposes. Limits of detection (LODs) were determined as a signal-to-noise ratio of 3 and range from 30 nM to 110 nM. The limit of quantification (LOQ) ranges from 100 to 300 nM and was determined as a signal to noise ratio of 10. The linear ranges comprise two decades, starting at the limits of quantification for all compounds investigated. The relative standard deviations (RSDs) for multiple subsequent analyses of three solutions (separately derivatized) calculated for cysteine in the urine samples average 6.4% and range from 1.5 to 9.5 %.

5.3.5 Precursor ion scan and neutral loss experiments

The fragmentation behaviour of the FEM and FMEA derivatives enables the detection of unknown disulfides by a precursor ion scan of FMEA derivatives and the detection of unknown thiols by a neutral loss experiment of FEM derivatives. An example is shown in **Fig. 5.6**. A mixture of penicillamine (PenSH) (20 µM in urine) and penicillamine disulfide (10 µM in urine) was added to a urine sample and detected. As these MS experiments lead to a reduction in sensitivity compared with the MRM mode, a larger sample volume (250 µL instead of 50 µL) is used.

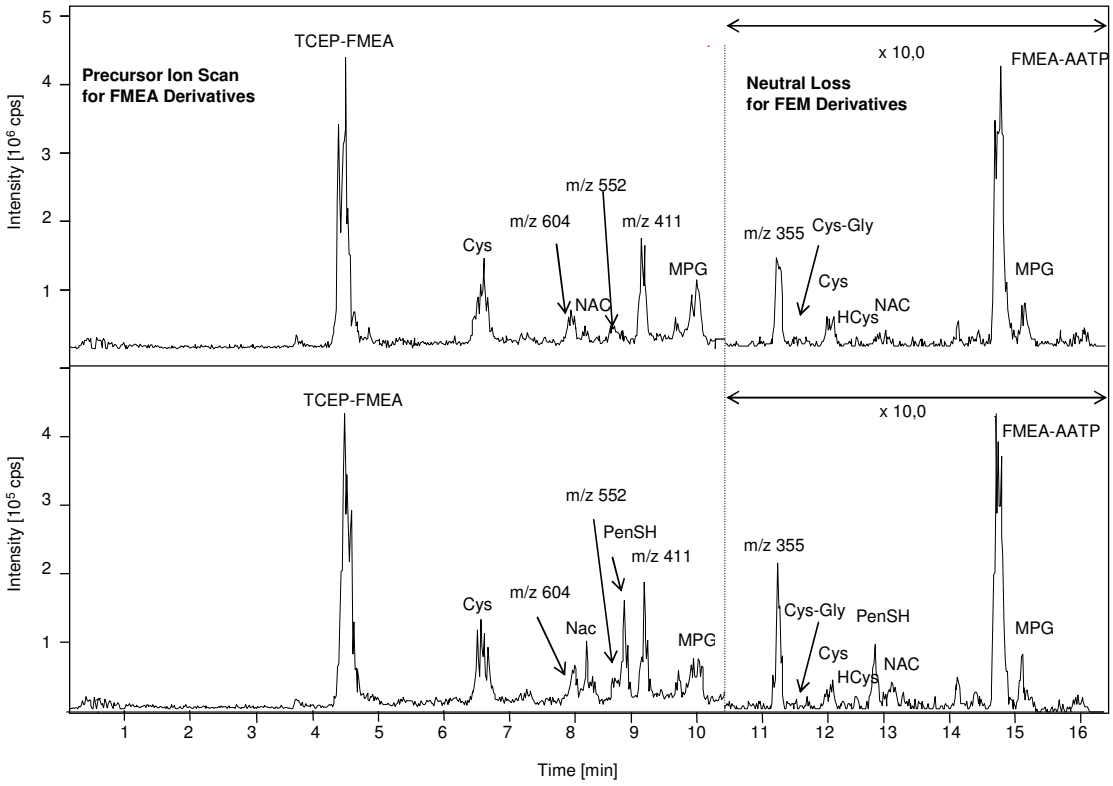

Fig. 5.6 Combination of precursor ion scan for FMEA derivatives and neutral loss scan for FEM derivatives for the identification of thiols; a) urine sample; b) urine sample with a mixture of penicillamine (PenSH) and penicillamine disulfide added.

Thus, two other disulfide-bound thiols were detected in urine (Fig. 5.7). These are mercaptolactic acid (t_R = 8.5 min, FMEA derivative: *m/z* 476) and mercaptoacetic acid (t_R = 9.6 min, FMEA derivative: *m/z* 446). No FEM derivatives of these two compounds were detected, which corresponds to literature data. It is known that these thiols exist in urine of healthy people as mixed disulfides with cysteine [29]. Because only mercaptoacetic acid was commercially available, the retention time of this thiol was additionally confirmed by the respective standard compound.

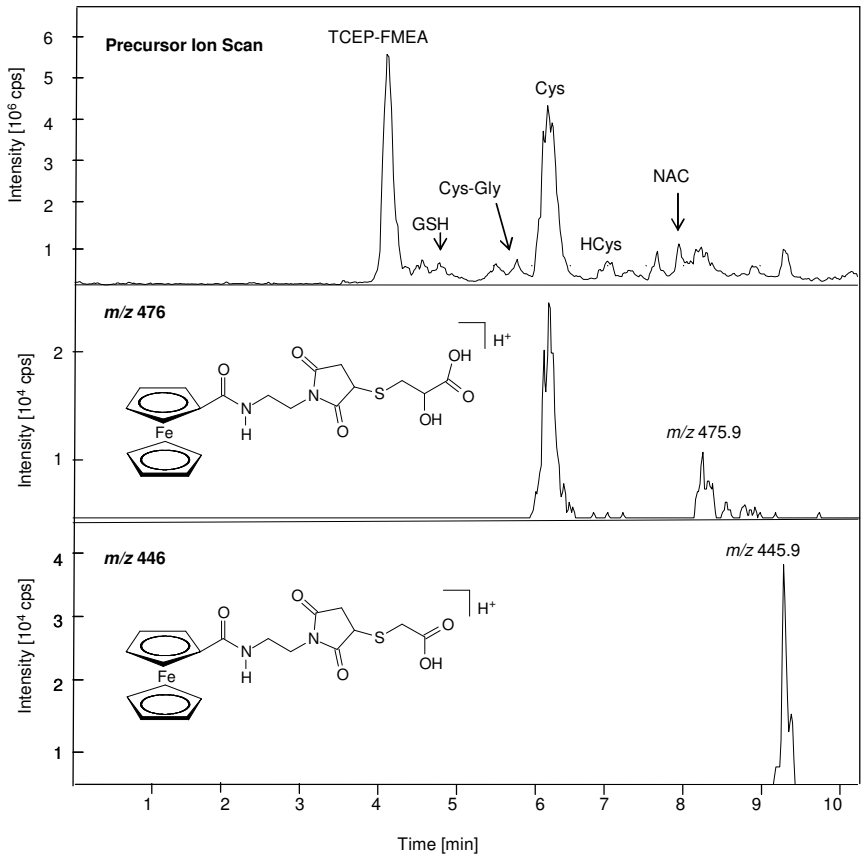

Fig. 5.7 Precursor ion scan of *m/z* 213 of a urine sample with extracted mass traces *m/z* 476 and *m/z* 446.

5.3.6 Simultaneous determination of thiols and disulfides in urine samples

In the eight urine samples, cysteine, *N*-acetylcysteine, homocysteine, cysteinylglycine and glutathione were detected. The concentrations of the thiols and disulfides in solution cover a large range from 50 µM for cysteine to below 0.5 µM for glutathione. Due to the relatively high concentration of cysteine, the urine sample was diluted by a factor of 2.5. As a consequence, in some cases the concentrations of other analytes were below
the limits of quantification, but the physiologically relevant concentration range was covered for the other thiols and disulfides as well.

The concentration of the analytes in urine depends on the constitution of the different individuals. To facilitate the comparison between the data, the analytical results for urinary thiols were normalized to creatinine. Creatinine is an endogenous metabolite and is extracted by the kidney predominantly by filtration and in proportion to the total extracted filtrate [29]. Concentrations between 1.8 mmol/L to 9.8 mmol/L creatinine were found in the urine samples. This is within the expected concentration range according to literature [30].

The free thiol, the bound thiol and the total content of each analyte was determined in order to prove the quality of the new method (Tab. 5.1). The concentration of the reduced and disulfide-bound thiols correlates well with the sum parameter, which was determined independently (Tab. 5.1). As an additional proof of principle, standard addition experiments were performed, which confirmed the applicability of the method (data not shown).

Tab. 5.1 Thiol concentrations and disulfide bound thiol concentrations determined in eight urine samples as well as the total amount (measured and calculated) including the corresponding RSD and ratio of thiol to total thiol.

sample	c $(Nac)_2$ [µmol/L]	RSD [%] n=3	c (Nac) [µmol/L]	RSD [%] n=3	total $(Nac)_2$ [µmol/L] measured	RSD [%] n=3	total $(Nac)_2$ [µmol/mol] calculated	[%]	ratio c (Nac) to total c (Nac) [%]	c (Nac) [mmol/mol creatinine]
1	6.5	5.2	2.2	1.3	7.7	1.3	7.6	98.7	14.3	3.4
2	5.0	11.8	2.2	7.1	5.7	1.7	6.1	107.0	19.3	1.7
3	7.4	3.9	1.7	7.7	7.9	4.1	8.3	105.0	10.3	1.6
4	4.8	3.4	1.3	13.1	5.2	5.4	5.5	105.8	11.8	1.5
5	0.6	14.0	n.d.	n.d.	1.3	7.5	n.d.	n.d.	n.d.	1.4
6	1.6	8.0	n.d.	n.d.	2.0	2.8	n.d.	n.d.	n.d.	1.3
7	3.1	5.5	0.9	8.1	3.8	8.6	3.6	94.7	11.8	1.5
8	1.3	12.7	n.d.	n.d.	1.8	4.3	n.d.	n.d.	n.d.	1.3

sample	c (GSH) [µmol/L]	RSD [%] n=3	c (GSSG) [µmol/L]	RSD [%] n=3	total c (GSSG) [µmol/L] measured	RSD [%] n=3	total c (GSSG) [µmol/L] calculated	[%]	ratio c (GSH) to total c (GSH) [%]	c (GSH) [mmol/mol creatinine]
1	n.d.	n.d.	n.d.	n.d.	0.7	9.9	n.d.	n.d.	n.d.	0.3
2	0.6	13.7	0.9	6.2	1.0	7.3	1.2	120.0	30.0	0.3
3	0.5	4.4	1.0	1.5	1.2	3.4	1.3	108.3	20.8	0.2
4	n.d.	n.d.	n.d.	n.d.	1.1	1.3	n.d.	n.d.	n.d.	0.2
5	n.d.	n.d.	n.d.	n.d.	0.2	8.5	n.d.	n.d.	n.d.	0.2
6	n.d.	n.d.	0.4	3.3	0.5	8.8	n.d.	n.d.	n.d.	0.3
7	n.d.	n.d.	0.9	10.8	1.2	3.1	n.d.	n.d.	n.d.	0.5
8	n.d.	n.d	n.d.	n.d	1.0	4.7	n.d	n.d	n.d	0.4

sample	c $(Hcys)_2$ [µmol/L]	RSD [%] n=3	c (Hcys) [µmol/L]	RSD [%] n=3	total c $(Hcys)_2$ [µmol/L] measured	RSD [%] n=3	total c $(Hcys)_2$ [µmol/L] calculated	[%]	ratio c (Hcys) to total c (Hcys) [%]	c (HCys) [mmol/mol creatinine]
1	2.4	3.6	0.6	9.1	2.8	4.9	2.7	96.4	10.7	1.2
2	2.8	7.2	1.0	3.3	3.6	8.7	3.3	91.6	13.8	1.1
3	2.6	12.8	0.9	3.4	2.8	5.8	3.1	110.7	16.0	0.6
4	3.3	6.2	0.7	0.4	4.0	4.1	3.7	92.5	8.8	1.2
5	1.5	8.9	0.3	1.1	1.8	7.3	1.7	94.4	26.7	2.0
6	1.4	5.0	0.7	5.1	1.5	2.8	1.8	83.3	23.3	0.9
7	1.5	7.2	0.4	6.3	1.5	0.8	1.7	113.2	13.3	0.6
8	1.2	3.4	0.6	2.0	1.7	7.8	1.5	88.2	17.6	1.2

sample	c $(Cys)_2$ [µmol/L]	RSD [%] n=3	c (Cys) [µmol/L]	RSD [%] n=3	total c $(Cys)_2$ [µmol/L] measured	RSD [%] n=3	total c $(Cys)_2$ [µmol/mol] calculated	[%]	ratio c (Cys) to total c(Cys) [%]	c (Cys) [mmol/mol creatinine]
1	56.9	6.6	24.2	7.1	75.5	4.7	69.0	91.4	16.0	33.3
2	90.9	6.9	34.5	1.5	116.8	5.5	108.2	92.6	14.7	34.6
3	77.7	10.4	45.8	9.9	108.4	1.6	100.6	92.8	21.1	22.1
4	79.3	9.4	27.5	16.1	103.8	6.5	93.1	89.7	13.2	30.5
5	21.8	1.4	7.64	4.2	27.5	6.8	25.6	93.1	13.8	29.9
6	38.8	8.8	19.4	7.6	49.2	7.5	48.5	91.0	19.7	30.8
7	54.4	6.3	19.4	3.0	70.8	7.8	64.1	90.5	13.7	28.4
8	41.0	2.0	14.0	2.3	49.4	9.9	48.0	97.2	14.2	35.9

sample			c (Cys-Gly) [µmol/l]	RSD [%] n=3	total c $(Cys\text{-}Gly)_2$ [µmol/L] measured	RSD [%] n=3			ratio c (Cys-Gly) to total c (CysGly) [%]	c (Cys-Gly) [mmol/ mol creatinine]
1			n.d.	n.d.	4.2	8.4			n.d.	1.8
2			5.5	8.8	5.6	4.3			49.1	1.7
3			6.9	5.7	6.5	3.7			53.4	1.3
4			n.d.	n.d.	6.9	2.8			n.d.	2.0
5			n.d.	n.d.	1.3	n.d.			n.d.	1.4
6			n.d.	n.d.	2.6	1.1			n.d.	1.6
7			n.d.	n.d.	4.5	4.5			n.d.	1.8
8			n.d.	n.d.	2.6	9.3			n.d.	1.9

It was stated by other authors [31] that glutathione is not present in urine. In another publication, 0.5 µM glutathione was found in urine [4]. This is within the same concentration range as found in this work. We determined 0.2 to 0.5 mmol glutathione/mol creatinine. Cysteinylglycine (Cys-Gly) was found to be present at 1.6±0.3 mmol/mol creatinine in agreement with literature (1.70±0.85 mmol/mol creatinine [30]). Because there are no reports in literature about the corresponding disulfide in urine, we only determined the thiol of Cys-Gly. Our measurements of 'total Cys-Gly' show, however, that the respective disulfide is present in urine as well. The ratio of Cys-Gly to 'total Cys-Gly' for two samples was (51.3±2.5)%, whereas for the other analytes, the values were significantly smaller. The reason and the relevance of this difference should be investigated in the future. The homocysteine content determined in this work ((1.1±0.4) mmol/mol creatinine) is less than found by Bald et al. (1.9 mmol/mol creatinine) [30]. A reason for the higher concentrations found by Bald et al. may be due to the homocysteine thiolactone, which is hydrolyzed to homocysteine at strongly alkaline pH as used in their reduction protocol [30]. In our experiments, the pH was carefully

adjusted not to exceed 7.5, as a result of which no hydrolysis of homocysteine thiolactone should occur. An average of (1.7±0.7) mmol/mol creatinine was determined for *N*-acetylcysteine which is in line with the concentrations found by others of (2.9±1.7) mmol/mol creatinine [32]. Cysteine was determined at a concentration of (30.7±4.3) mmol/mol creatinine, thus matching well the literature data of (31±14.5) mmol/mol creatinine. In urine, the ratio of cysteine to 'total amount of cystine' is between 11 to 33 % [33], which coincides well with the measurements carried out in this work (average: (15.8±2.9)%). However, in more recent publications, the ratio is stated to be 36% [3] and 37% [34]. The ratio of 'thiol' to 'total thiol' concentrations found in urine is rarely reported in literature for the other thiols. The following data was found: *N*-acetylcysteine (13.5±3.2%, (lit.: 21.7% [3]), homocysteine (16.3±6.1%), and glutathione (51.3±2.5%).

5.4 Conclusions

A powerful method for the simultaneous determination of free and disulfide-bound thiols in urine was developed based on sequential derivatization with ferrocene-based maleimide reagents. First, the free thiols glutathione, cysteine, *N*-acetylcysteine, homocysteine and cysteinylglycine in urine samples were derivatized with *N*-(2-ferroceneethyl)maleimide (FEM). After reaction of the reagent excess with a quencher, disulfide-bound thiols were reduced and subsequently derivatized with ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide (FMEA). The reaction products were separated by reversed-phase liquid chromatography and determined by tandem mass spectrometry in the multiple reaction monitoring mode. Unknown thiols or

disulfides in the urine samples were identified by additional tandem mass spectrometric experiments in the precursor ion scan and neutral loss mode, respectively. The simple sample preparation and reliable analytical data could lead to the application of this method as a valuable routine tool in clinical chemistry.

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Chapter 6

Differential Labeling of Free and Disulfide-Bound Thiol Functions in Proteins‡

A method for the simultaneous determination of the number of free cysteine groups and disulfide-bound cysteine groups in proteins has been developed based on the sequential labeling of free and bound thiol functionalities with two ferrocene-based maleimide reagents. Liquid chromatography/electrochemistry/mass spectrometry was used to assign the N-(2-ferroceneethyl)-maleimide (FEM) labeled free cysteine functionalities in a tryptic digest mixture, whereas a precursor ion scan enables the detection of peptides with ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide (FMEA) labeled disulfide-bound cysteine groups after reduction. Fragment spectra of the labeled peptides yield an excellent coverage of b-type and y-type ions. The ferrocene labeled cysteines were fragmented as 412 Da (FEM) and 455 Da (FMEA). These fragment masses are significantly higher than unlabeled amino acids or dipeptides and are easily detected. The position of free and disulfide-bound cysteine may therefore be assigned in an amino acid sequence.

‡ Seiwert, B.; Hayen, H.; Karst, U., manuscript in preparation.

6.1 Introduction

The presence of cysteine residues in peptides and proteins is the base for building disulfide bridges. This common post-translational modification has a strong influence on the three-dimensional structure of proteins, and it is essential for their function. Free cysteines may participate in substrate binding and catalysis [1]. Therefore, the determination of the number and position of both bound and free cysteine residues may provide important information allowing to understand protein structure and function.

Reactions of sulfhydryl groups with other compounds in solution are a common problem in detecting peptides, which contain free cysteine residues. A general approach to overcome this problem is to derivatize the thiol groups with iodoacetamide [2], iodoacetic acid [3], or 4-vinylpyridine [4]. However, labeling strategies based on alkylhalides and haloacetamides may exhibit a cross reactivity towards histidine, tyrosine and methionine [5]. Maleimide-based derivatizing agents are known to be, depending on the pH, more selective for thiol groups. There are two common strategies for the determination of disulfide bonds and free cysteine residues: One is based on the derivatization of the free cysteines and enzymatic cleavage of the protein, followed by the analysis of the resulting fragments containing the intact native disulfide bonds. The second uses the comparison of peptide maps of the reduced and the native protein [6]. Suitable enzymes and digestion conditions are critical issues in this case. Another promising approach is to combine derivatization and chemical cleavage at cysteine residues by 2-nitro-5-thiobenzoic acid (NTCB) [7]. However, it is more difficult to obtain structural

fragments from the process of collision induced dissociation (CID) for peptides containing disulfide bonds than for reduced and alkylated ones [8]. Therefore, cysteine alkylation is required to ensure maximum coverage [9]. To overcome these problems, a differential labeling may be employed, using two different derivatizing agents before and after the reduction of the disulfide bonds [10]. Together with LC/MS/MS data of the unreduced protein, bio-informatics enables the calculation of the number and position of disulfide bonds and free cysteines. In combination with partial reduction by tris-(2-carboxyethyl)-phosphine (TCEP), complex disulfide bond patterns can be analyzed [11]. However, complex data analysis has to be employed to determine the complete amino acid sequence. Selective analysis of cysteine residues enables the reduction of the amount of data. For the selective analysis, there are two strategies: Selective enrichment or selective detection. The analysis after selective enrichment can be achieved by quaternary amine tags [12], a method based on avidin and biotin [13] or by the covalent capturing of cysteine containing peptides with pyridyl disulfide reactive groups on agarose beads [14]. Fluorescence may be used as a selective method to detect the derivatized peptides [15]. Although the analysis of cysteine residues and disulfide bonds is simplified, the selective analysis of cysteines was, to the best of our knowledge, never applied in combination with the differential labeling approach. We describe a new approach of differential protein labeling using two ferrocene-based derivatizing agents. In a recent chapter, we reported the development of a LC/MS method based on the derivatization of thiol groups with the derivatizing agent *N*-(2-ferroceneethyl)maleimide (FEM) [16]. The method presented in this chapter uses a two-reagent differential

labeling of thiol and disulfide functions in proteins. The combination of electrochemistry and precursor ion scan mass spectrometry allows the selective analysis of the cysteine containing peptides and simplifies the analysis of complex tryptic digest mixtures without applying dedicated bioinformatic tools.

6.2 Experimental part

6.2.1 Chemicals

All reagents and chemicals were obtained from Sigma Aldrich (Steinheim, Germany) or Fluka Chemie GmbH (Buchs, Switzerland) in the highest quality available and were used without further purification. The water used for HPLC was purified using a Milli-Q Gradient A 10 system and filtered through a 0.22 µM millipak 40 (Millipore, Billerica, MA, USA). The acetonitrile (ACN) for HPLC was obtained from Merck KGaA (Darmstadt, Germany). *N*-(2-Ferroceneethyl)maleimide (FEM) was synthesized from ferrocenyl-methyltrimethylammonium iodide (obtained from Alfa Aesar, Karlsruhe, Germany) according to a method described in literature [16,17]. Ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide (FMEA) was synthesized in a two-step reaction from ferrocenecarboxylic acid (obtained from Alfa Aesar Karlsruhe, Germany) according to [18,19]. TPCK treated trypsin from bovine pancreas (12.4 units/mg) from Sigma Aldrich (Steinheim, Germany) was used.

6.2.2 Differential derivatization

Stock solutions were prepared of the following compounds: FEM (20 mM in ACN), ammonium acetate (NH_4ac) buffer (100 mM, pH 4), ammonium bicarbonate (NH_4HCO_3) buffer (1 M, pH 7.8) and tris-(2-carboxyethyl)-phosphine (TCEP) (100 mM) in 0.3 M aqueous ammonia solution. α-Lactalbumin, β-lactoglobulin B and β-lactoglobulin A were dissolved in NH_4ac to form a 0.3 mM solution. The differential derivatization was carried out by adding 100 µL FEM to 1.5 mL protein solution. Directly after adding the derivatizing agent, 0.72 g of urea were dissolved and 50 µL NH_4HCO_3 buffer (1 M) were added. After mixing, 10 µL formic acid were added to decrease the pH value to acidic conditions. The protein solution was passed through a PD-10 desalting column (Amersham Bioscience, Freiburg/Germany) to remove the excess of the reagent. Formic acid (0.01% in water) was used as mobile phase. Urea (0.48 g) was dissolved in 1 mL protein solution and 50 µL NH_4HCO_3 solution (1 M) were added. Reduction of the disulfide bonds was performed by adding 50 µL TCEP and allowing it to react for 30 min. Afterwards, 200 µL FMEA were added and the solution was acidified again and passed through a PD column as described above.

6.2.3 Tryptic digest

First, a stock solution of trypsin (1 µg/mL in 0.01% formic acid, diluted 1:10 with 100 mM NH_4HCO_3 immediately prior to use) was prepared. For the in-solution digest, 150 µL of the trypsin solution were added to 200 µL of the desalted protein (β-lactoglobulin A) solution containing approximately

0.03 mM protein in 0.01% formic acid (trypsin:protein ratio of 1:20 (w/w)), and the mixture was incubated overnight at 37°C.

6.2.4 HPLC/MS/MS

The LC/MS setup comprised a Shimadzu (Duisburg, Germany) LC system and a QTrap mass spectrometer (Applied Biosystems, Darmstadt, Germany), equipped with an electrospray ionization (ESI) source. The LC system consisted of two LC-10ADVP pumps, a DGC-14A degasser, a SIL-HTVP autosampler, a CTO-10AVP column oven, and a SPD-10AVVP UV detector. The software used for controlling LC and MS was Analyst 1.4.1 (Applied Biosystems). The analytes were ionized in the ESI interface with an ionspray voltage of 5000 V, using 50 psi nebulizer gas and 70 psi dry gas with a temperature of 500°C. The proteins and tryptic peptides were detected in the positive ion mode using a Q1 scan (*m/z* 550 to *m/z* 1400). The collision energy was set to 30 V, entrance potential to 10 V and exit potential to 15 V for the precursor ion scan. The collision activated dissociation (CAD) gas was set to medium.

A binary gradient of ACN and ammonium formate (10 mM, pH 4) was used for the liquid chromatographic separation. The column employed for the HPLC separation was a Discovery BioWidePore C5 column (Supelco, Taufkirchen, Germany)) with a particle size of 5 µm, a length of 150 mm and a inner diameter of 2.1 mm. The injection volume was 10 µL. A flow rate of

0.25 mL/min was selected. The following gradients were applied for the separation of the untreated (a) and the differentially labeled (b) whey proteins and for the tryptic peptides, gradient (c) was used:

a)

t [min]	0.03	5	10	30	33	35	36	40
c (CH_3CN) [%]	5	5	33	40	90	90	5	stop

b)

t [min]	0.03	5	10	30	33	35	38	40
c (CH_3CN) [%]	30	30	40	50	90	90	30	stop

c)

t [min]	0.03	1	21	22	23	30
c (CH_3CN) [%]	5	5	90	90	5	stop

6.2.5 Fragment ion spectra

All fragment ion spectra of the tryptic peptides were carried out using a Finnigan LTQ^{TM} linear quadrupole ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an offline nano-ESI source. The instrument was operated in the positive ion mode. The parameters were: source voltage, 1.0-1.3 kV; capillary voltage, 35 V; capillary temperature, 150 °C; and tube-lens voltage, 110 V. Besides collision induced dissociation (CID), a new activation technique was used. The pulsed Q collision induced dissociation (PQD) eliminates the low mass cut-off of quadrupole ion traps.

The normalized collision energies were 22% for CID and 30% for PQD, respectively.

6.2.6 LC/EC/MS analysis

To hyphenate the LC/MS system with on-line electrochemistry, a conditioning cell model 5021 controlled by a Coulochem II potentiostat from ESA Bioscience Inc. (Chelmsford, MA, USA) was inserted between the outlet of the UV/vis detector and the inlet of the ionization interface of the mass spectrometer. To protect the working electrode of the electrochemical cell, a PEEK in-line filter (ESA) was mounted between column and cell.

6.2.7 Cyclic voltammetry

FEM and FMEA were dissolved in a mixture of NH_4HCO_3 buffer (100 mM, pH 7.8) and ACN (1:1, v/v) to form a 0.1 mM solution. After 5 min of stirring in a nitrogen atmosphere, the stirrer was turned off and cyclic voltammograms were recorded over the potential range from -500 to 1000 mV at a scan rate of 0.35 V/s. Ferrocene was added to the solution as reference.

6.3 Results and discussion

6.3.1 Differential derivatization of cysteine residues in proteins

In this chapter, an approach for differential derivatization with two different derivatizing agents based on ferrocene to characterize free and bound cysteine residues is presented. Two maleimide based derivatizing agents are used for this purpose. *N*-(2-Ferroceneethyl)maleimide (FEM) was already

used for the analysis of proteins and peptides by HPLC/MS [16]. The second reagent, ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide (FMEA) was recently introduced [19] for the detection of low molecular weight aminothiols and was synthesized for the differential labeling approach together with FEM.

As previously demonstrated, the ferrocene-based derivatizing agents FEM and FMEA react selectively with sulfhydryl groups of cysteine residues in proteins at a pH around 7 [19]. The first step of the differential derivatization is the labeling of the free thiol functionalities. This is achieved by simultaneous protein denaturation and derivatization of by FEM. The reaction time needed for derivatization is less than five minutes under the conditions stated above. The excess of FEM has to be removed prior to the second step (the reaction of the disulfide bound cysteines with FMEA). The difference in size between protein and low molecular weight reagents was utilized for a respective separation on a PD 10 column. Afterwards, TCEP is added to reduce the disulfide bonds in the presence of urea, which denatures the protein in order to yield accessible thiol functionalities. TCEP is known as an easily soluble and very stable compound, which reduces disulfide bonds more efficiently than DTT. It does, according to literature, not react with other functional groups in proteins [20]. The second step of the differential derivatization is the labeling of the cysteine groups of the former disulfide bonds. Therefore, FMEA is added as second derivatizing agent. The sequence of the reagents is selected due to their water solubility. FEM is poorly water soluble whereas FMEA, due to the additional carboxyl group, is well soluble in water and may be added in a large excess even under aqueous conditions. The excess is needed because TCEP reacts with maleimides as well. However, there is no

reduction agent available, that does not react with the derivatizing agent. Subsequently, the number of cysteine residues involved in disulfide bonds and the number of the free cysteines can be determined quasi-simultaneously by MS. Therefore, the mass of the untreated and the differentially labeled protein have to be exactly determined by mass spectrometry. The mass tags (352 Da, FMEA and 309 Da, FEM) increase the mass of the protein significantly. Furthermore, the mass tag difference (43 Da) is large enough to distinguish between the derivatizing agents even after a reaction with larger proteins.

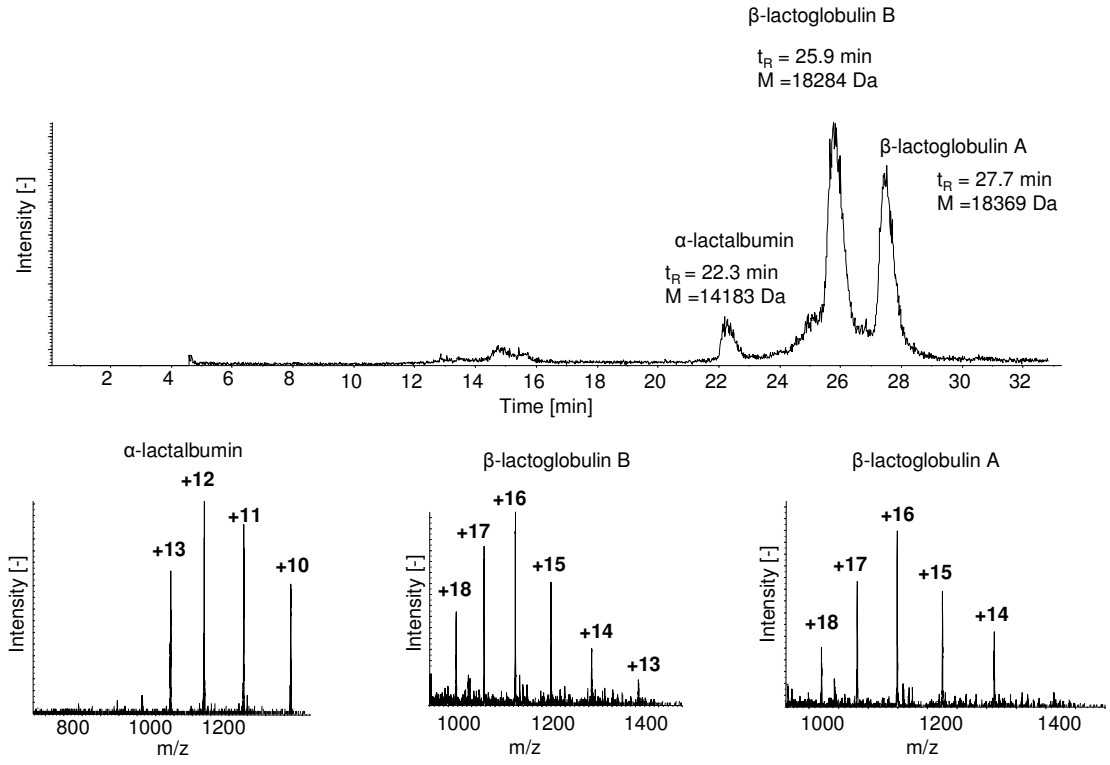

Fig. 6.1 Total ion current (TIC) chromatogram of the separation of the three whey proteins α-lactalbumin, β-lactoglobulin B and β-lactoglobulin A. Below: Mass spectra of the protein peaks and annotated charge states.

A mixture of three whey proteins was selected as model compounds to demonstrate the applicability of the method to protein analysis.

β-Lactoglobulin B (18284 Da) and A (18369 Da), which differ in two amino acid (AA) residues (var. A/var. B, AA 64: Asp/Gly and AA 118: Val/Ala) are clearly, although not baseline separated. β-Lactoglobulin B and A both contain two disulfide bridges (AA66-AA119 and AA106-AA119) and one free cysteine (AA121) [21]. α-Lactalbumin contains four disulfide bridges (AA6-AA120, AA28-AA111, AA61-AA77 and AA73-AA91) [22]. The untreated proteins elute from a BiowidePore C5 column in the order α-lactalbumin, β-lactoglobulin B and β-lactoglobulin A (Fig. 6.1). α-Lactalbumin (14183 Da) is well separated from the other peaks.

After derivatization of β-lactoglobulin B (18593 Da) and A (18679 Da) a mass gain of 309 Da and 310 Da, respectively was observed. Considering the mass accuracy of the instrument used, one FEM label can unambiguously be assigned to any of the proteins. The mass of α-lactalbumin remains unchanged. This is consistent with one free cysteine in the lactoglobulins and no free cysteine in lactalbumin. After the second labeling step with FMEA, the proteins elute in reversed order: First β-lactoglobulin B, then β-lactoglobulin A and finally α-lactalbumin (Fig. 6.2). Deconvolution of the mass spectrum of α-lactalbumin provides a mass of 17009 Da. The mass difference of 2826 Da shows that the protein is derivatized by 8 FMEA (2824 Da). Consequently, all labeled cysteines were parts of previous disulfide bonds. The mass spectrum of β-lactoglobulin B provides a mass of 20006 Da and the spectrum of β-lactoglobulin A a mass of 20093 Da. The mass difference compared with the unlabeled proteins is 1722 Da. This correlates well with four FMEA and one FEM. Therefore, both β-lactoglobolin A and B contain two disulfide bonds

and one free cysteine. As shown for the mixture of whey proteins, it is possible to determine the number of free and bound cysteines of a protein by a single mass spectrometric measurement after the differential derivatization.

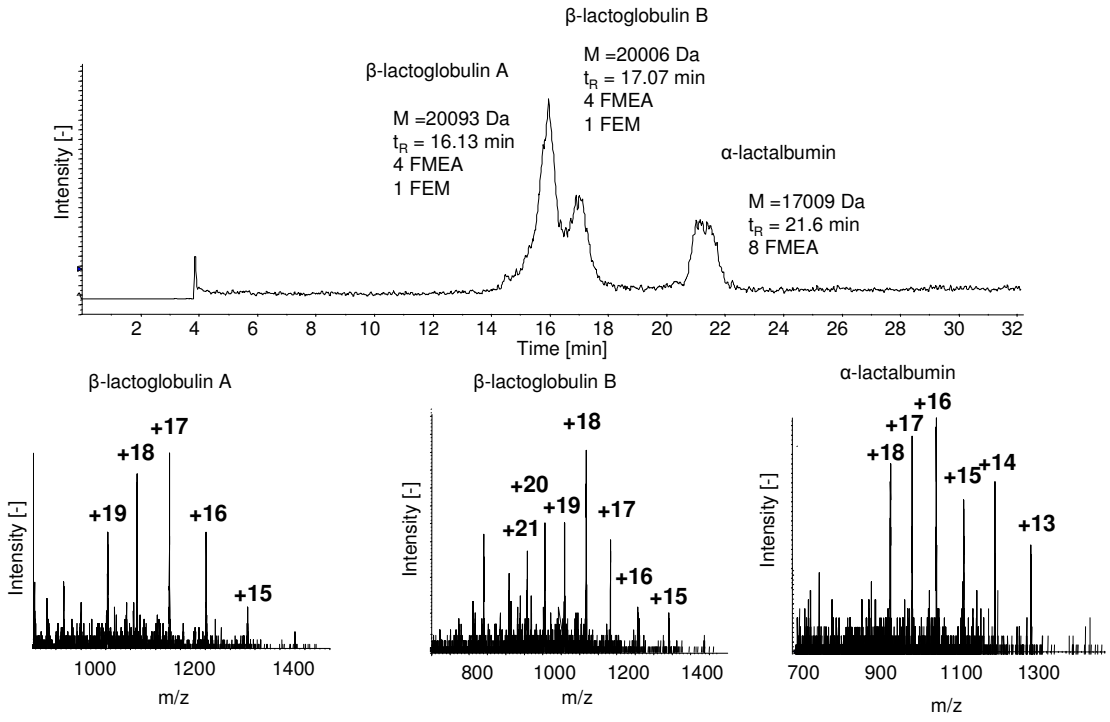

Fig. 6.2 TIC chromatogram of the separation of the differentially labeled proteins β-lactoglobulin A, β-lactoglobulin B and α-lactalbumin. Below: Mass spectra of the protein peaks and annotated charge states.

6.3.2 Identification of ferrocene-labeled peptides by MS/MS

To obtain more information about the location of the free cysteines and the disulfide bonds in the protein, a tryptic digest is performed. For the following peptide separation, the ferrocene units have the attractive feature to reduce the polarity of the peptide significantly, thus leading to the separation of the ferrocene-containing peptides at comparably high organic content (long retention times). Furthermore, a predetermined fragmentation site, an

electrochemical active group and a metal ion is introduced. Thus, three different analytical methods may be applied to determine the ferrocene-derivatized peptides: (1) a precursor ion scan in LC/MS, (2) the combination of LC with electrochemical conversion and MS and (3) atomic spectroscopy. The latter option is not used within this work, while the other two options are applied as indicated below. The identification of the ferrocene-labeled tryptic peptides is shown for the differentially labeled β-lactoglobulin A.

The fragment ion spectra of the FMEA derivatives show three common fragment ions at *m/z* 129, *m/z* 185 and *m/z* 213. The fragment at *m/z* 213 corresponds to the cleavage within the ester function next to the carbonyl carbon atom. The FMEA-containing peptides may therefore be detected by a precursor ion scan with the fragment ion mass of *m/z* 213 (Fig. 6.3).

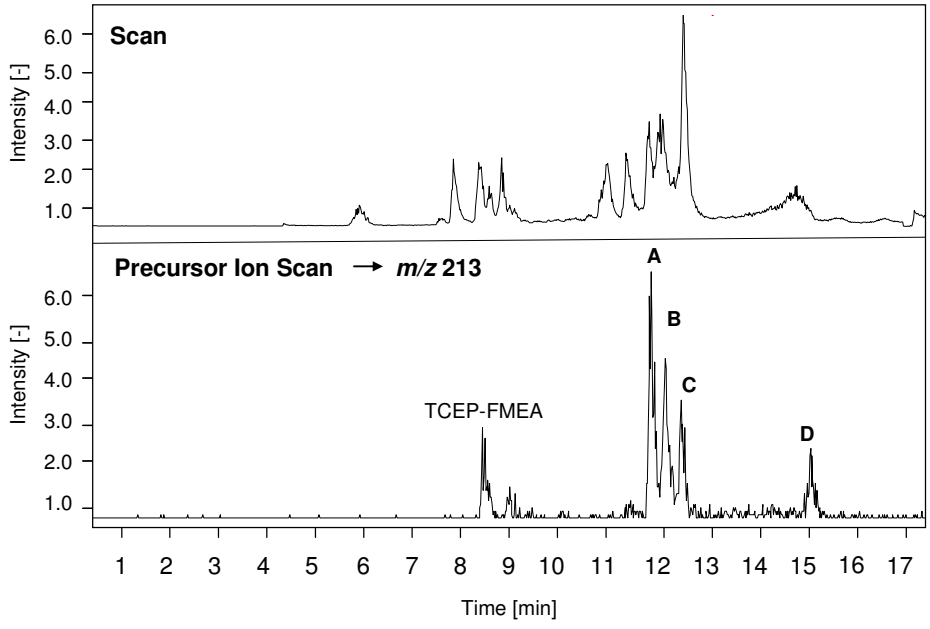

Fig. 6.3 TIC chromatogram of a tryptic digest of β-lactoglobulin A (upper chromatogram) and the corresponding precursor ion scan (below).

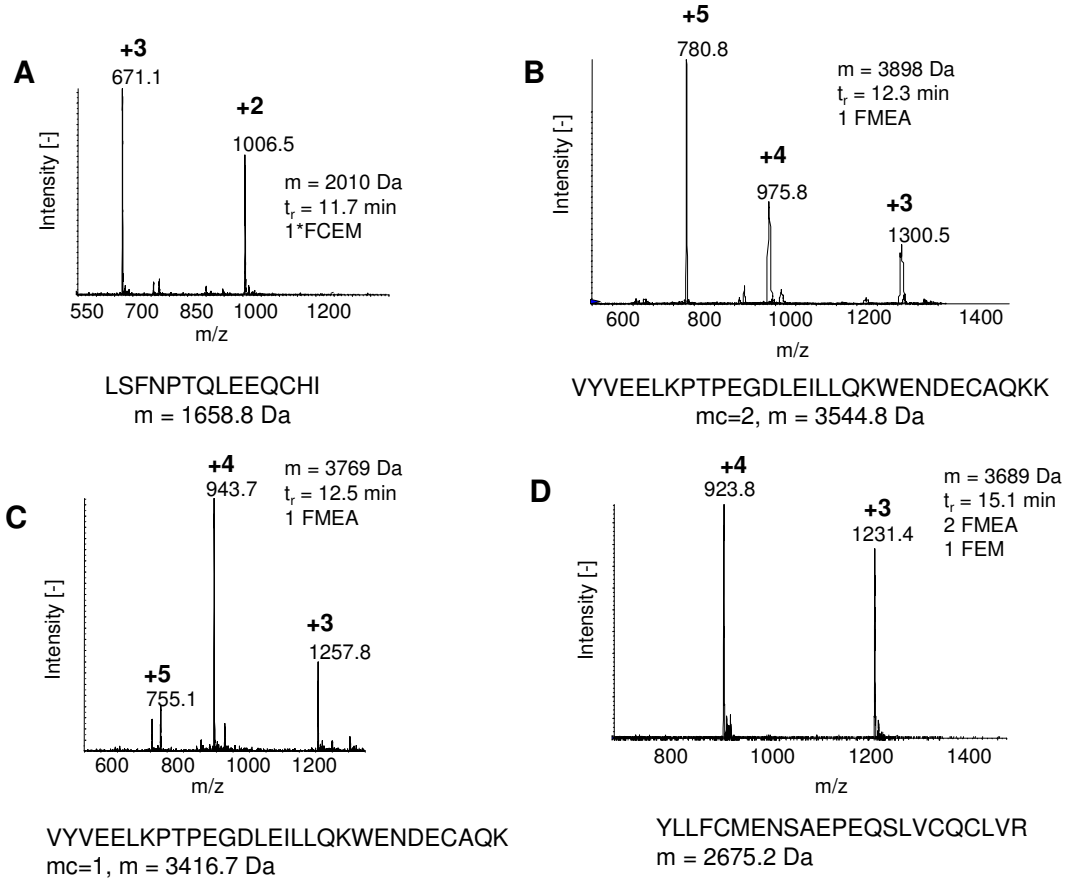

Fig. 6.4 Mass spectra of selected tryptic peptides of β-lactoglobulin A as identified by precursor ion scan measurements.

Beside the TCEP derivative, there are four additional peaks present in the precursor ion scan spectrum. These peptides are labeled by at least one FMEA. The four peptides A, B, C, D selected by the precursor ion scan are analyzed in more detail. Amino acid sequence analysis (*N*-terminal b-type ions and *C*-terminal y-type ions) of the tryptic fragments is possible by obtaining fragment spectra. The ferrocene labels attached to the cysteines were fragmented to yield b-type and y-type ions with a mass of 412 Da (FEM) and 455 Da (FMEA). These fragment masses are significantly higher than unlabeled amino acids or dipeptides and can be easily detected. Furthermore, especially singly labeled peptides and fragments exhibit a typical isotopic

pattern resulting from the iron atom. The nature and the sequence of the amino acid residues is obtained by comparing the fragment masses obtained by theoretical cleavage of the protein sequence to the measured one. At t_R=11.71 min a peptide with the mass of 2010 Da elutes with the charge state +2 and +3 (Fig. 6.4a). The tandem mass spectrum of the precursor ion (AA149-162) with *m/z* 1006.42 (doubly charged) is shown in Fig. 6.5. The mass difference (455.12 Da) between fragment y_2 (*m/z* 269.08) and y_3 (*m/z* 724.20) is equal to the mass of one cysteine derivatized by FMEA. Consequently, the cysteine C160 is part of a disulfide bond.

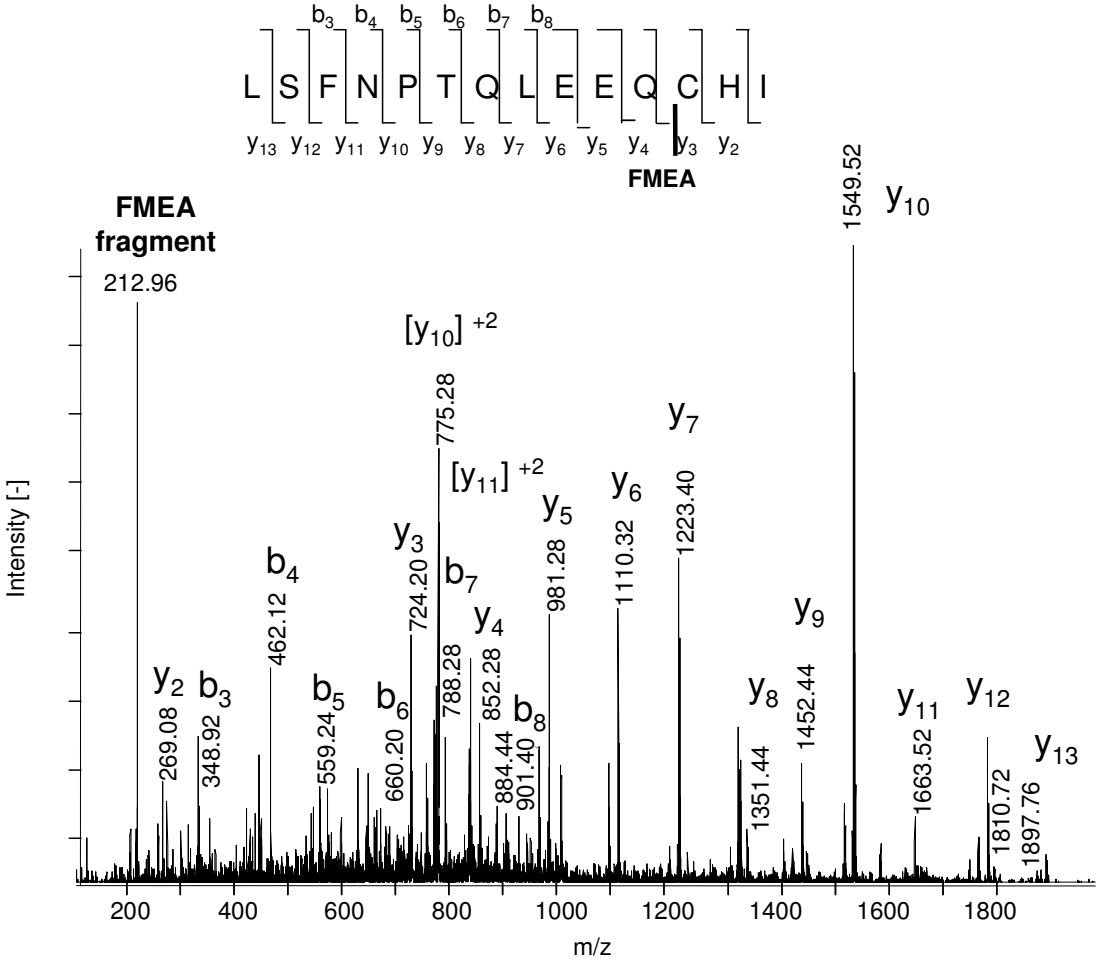

Fig. 6.5 Tandem MS spectrum of the peptide with *m/z* 1006 (peptide AA 149-162).

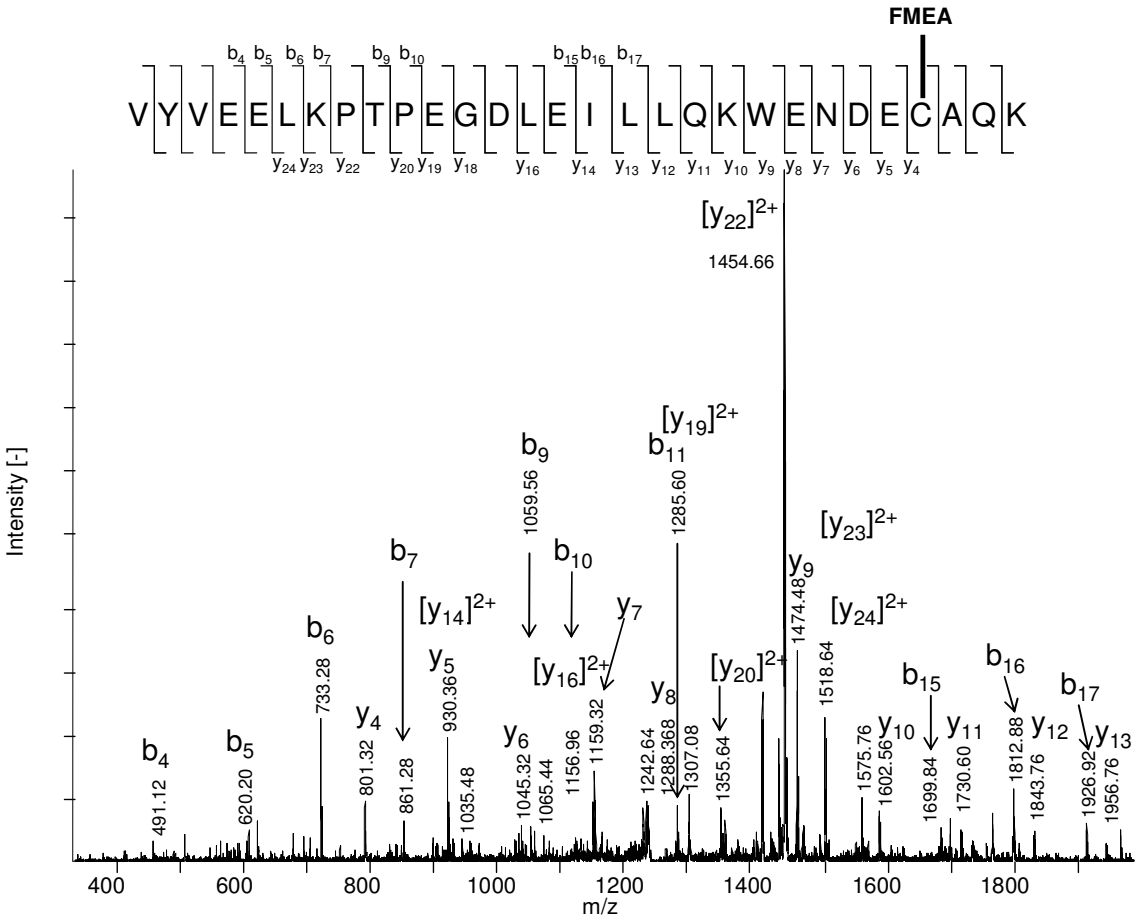

Fig. 6.6 Tandem MS spectrum of the peptide with *m/z* 1257 (peptide AA 101-124).

The peptide with the mass of 3898 Da elutes at t_R = 12.34 min (Fig. 6.4b, AA41-69). Deconvolution of the peak with t_R = 12.45 min leads to a mass of 3769 Da present in the charge states +3 to +5 (Fig. 6.4c, AA41-70). Due to the mass difference of 129 Da (Lys 129 Da), peak B corresponds to the miscleaved peptide of peak C. The mass difference between fragment y_4 (*m/z* 801.32) and the triply charged *m/z* 1257.59 of the peptide C (Fig. 6.6) is equal to the mass of FMEA-labeled Cys (455.12 Da) connected to Ala (71.03 Da), Gln (128.06 Da) and Lys (128.09 Da). Therefore, Cys 66 is a FMEA-labeled cysteine, which indicates that it is bound in a disulfide bond.

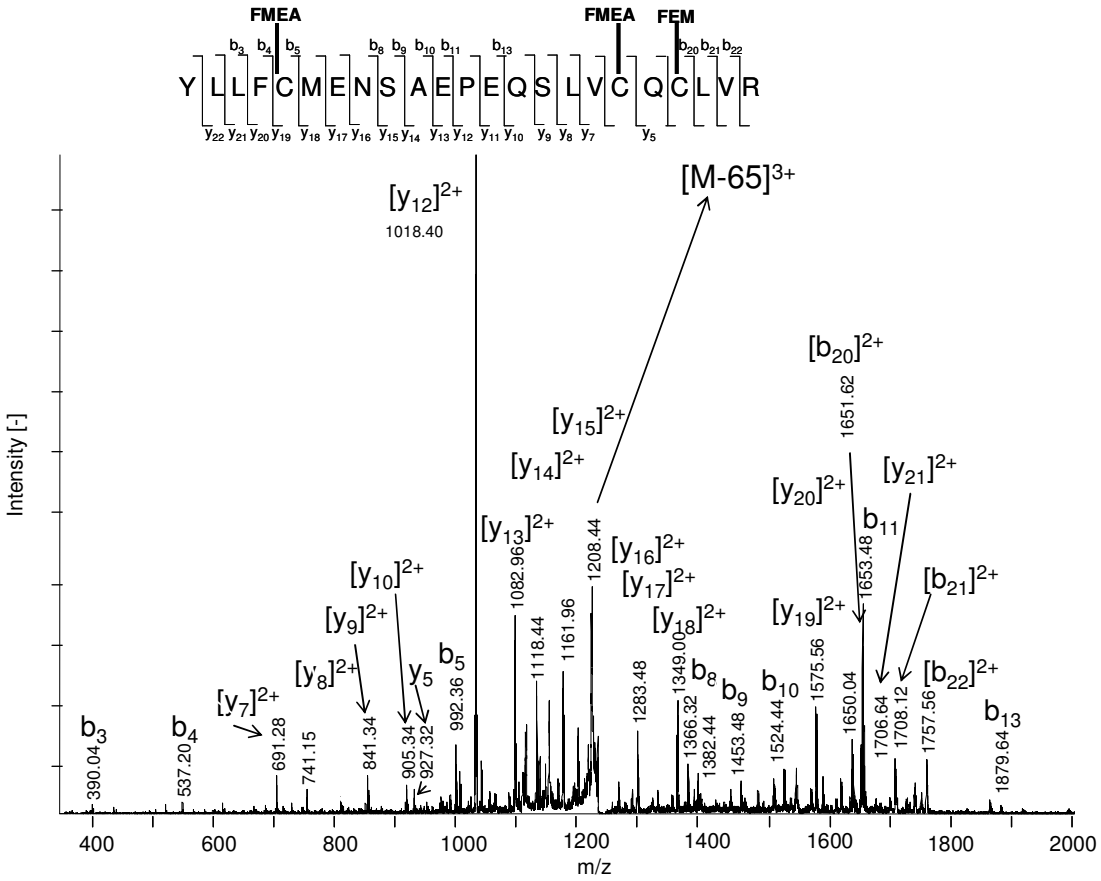

Fig. 6.7 Tandem MS spectrum of the peptide with m/z 1230 (peptide AA 102-124).

Peak D elutes at t_R = 15.07 min (AA102-124). MS/MS analysis of the triply charged tryptic peptide with m/z 1230.46 is shown in Fig. 6.7. From the mass loss of 65.1 Da yielding *m/z* 1208.76 (triply charged) it is obvious that at least one FEM label is present. A loss of 65 Da is the most abundant fragment of FEM derivatives in tandem MS spectra. It corresponds to the symmetrically coordinated ligand cyclopentadienyl (leaving as a neutral radical). However, neutral loss scans are not sensitive enough to get additional information. The mass difference of 445.16 Da between fragment b_4 (*m/z* 537.20) and b_5 (*m/z* 992.36) is consistent with the mass of a cysteine labeled with one FMEA. This demonstrates that Cys106 is a FMEA labeled Cys. Consequently Cys106 is bound in a disulfide bond.

The difference between the y_7 (*m/z* 691.75, doubly charged) and y_5 (*m/z* 927.32) can be assigned to Cys 119 (455.12 Da) derivatized by FMEA and an additional Val (99.07 Da). Fragment b_{20} (*m/z* 1651.62, doubly charged) has a mass difference of 387.36 Da compared to the peptide mass (3691.38 Da). The difference of fragment y_5 (m/z 927.32) and fragment b_{20} is 539.9 Da, referring to Gln (128.06 Da) bound to one FEM-labeled Cys residue (411.81 Da). FMEA labeling is not possible because there is no amino acid with a mass 85.01 Da. Consequently, Cys121 is labeled by FEM, which is consistent with the literature [21]. The free cysteine in the peptide is Cys121. Therefore, it was assigned correctly by our approach.

6.3.3 Identification of ferrocene-labeled peptides by LC/EC/MS

Fig. 6.8 shows a cyclic voltammogram for a mixture of FMEA and FEM.

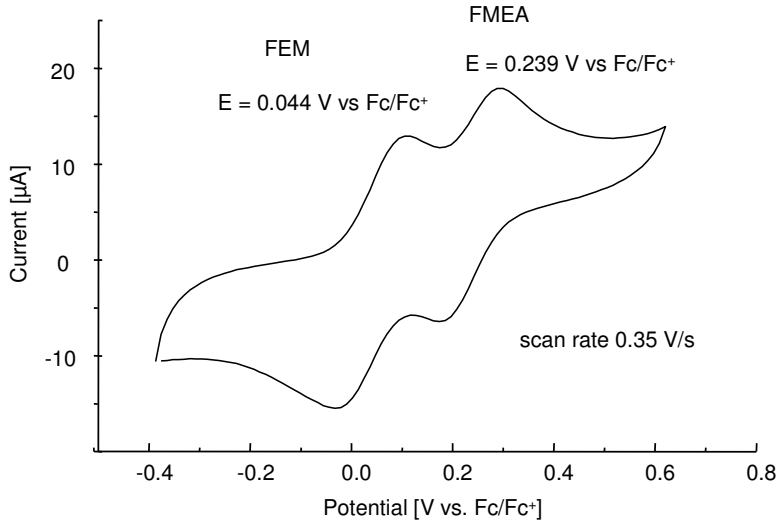

Fig. 6.8 Cyclic voltammogram of a 0.1 mM solution of FMEA and FEM in 100 mM ammonium formate pH 7.8/acetonitrile (1:1, v/v).

Compared to FEM, the oxidation potential of FMEA is increased by 200 mV. If the carbonyl group is located next to the ferrocene, its electron-withdrawing effect explains the increase. The two methylene groups of FEM directly bound to one cyclopentadienyl ring facilitate the oxidation of the ferrocene group by stabilizing the ferrocinium cation with their +I-effect.

For FEM-labeled peptides, it was already shown [17] that it is possible to enhance the intensity of the peak by an on-line electrochemical oxidation. A comparison of LC/MS and LC/EC/MS measurements of a tryptic digest of β-lactoglobulin A is shown in Fig. 6.9. The intensity of peptide A is enhanced significantly.

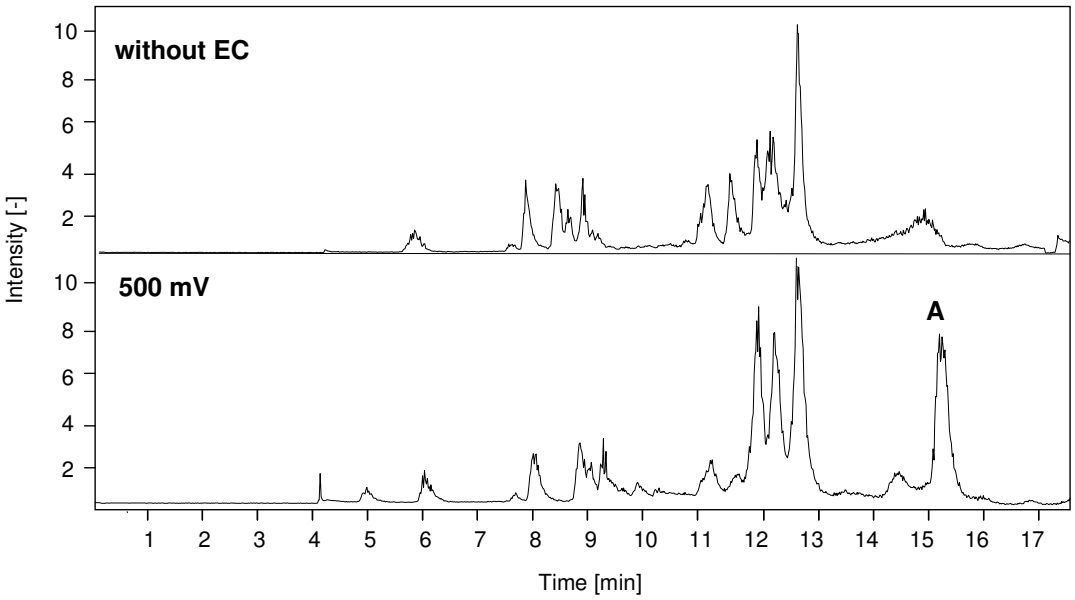

Fig. 6.9 TIC chromatogram of a tryptic digest of β-lactoglobulin A without and with electrochemical cell (500 mV vs. Pd/H_2).

As already known from the MS/MS experiments (see above), peak A corresponds to the tryptic peptide that contains both derivatizing agents (peptide D, Fig. 6.4).

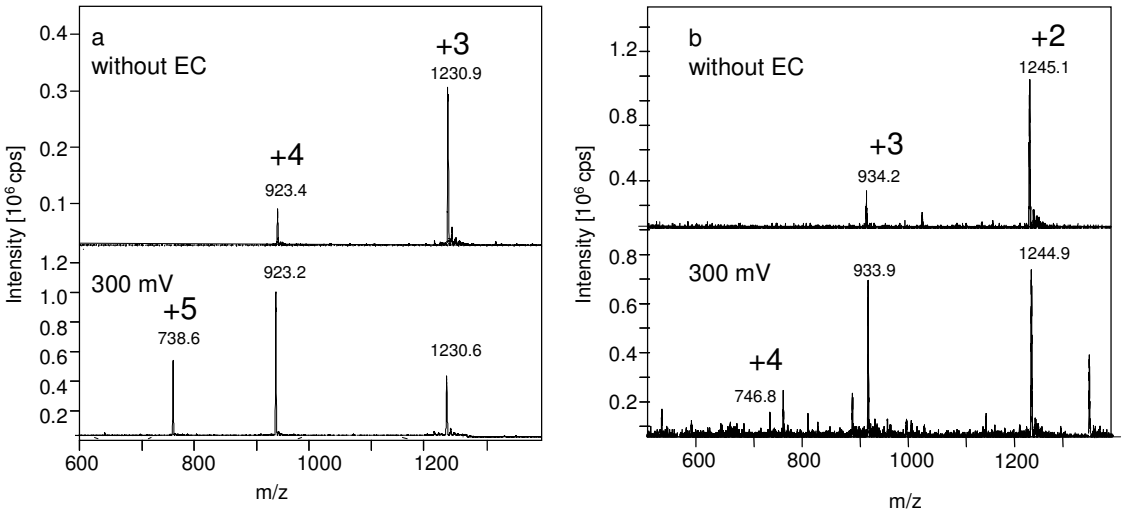

Fig. 6.10 Comparison of the influence of the electrochemical conversion on FMEA derivatives and FEM derivatives.

In Fig. 6.10, the behaviour of the tryptic peptide AA102-124, differentially labeled by one FEM and two FMEA (a), is compared with the behaviour of the same peptide exclusively labeled by FMEA (b). In both cases, the charge distribution is shifted to higher values compared to the unlabeled peptide due to electrochemical oxidation/ionization at the ferrocene functions. This behaviour is not observed for unlabled peptides and can be applied to distinguish labeled and unlabled peptides. As observed in [18], labeling with FEM (a) results in an increase in signal intensity when applying an electrochemical potential. If the same peptide is exclusively labeled by FMEA (b) the signal intensity stays constant or even decreases after electrochemical treatment. This effect is most likely caused by the distribution of the signal intensity over more peaks, which is caused by the electrochemical oxidation.

The LC/EC/MS measurements are useful for the differential labeling, because peptides labeled by both ferrocene reagents are detected by the precursor

scan (peptide D, Fig. 6.3) and by the LC/EC/MS approaches (peptide A, Fig. 6.10). Peptides labeled by only one of the derivatizing agents were detected by only one of the methods. Furthermore, the change in charge state distribution does not occur for unlabeled peptides. The identification of peptides by single quadrupole instruments is simplified by a broader charge state distribution. With these instruments, limited information about the charge state is available from the isotopic pattern. In addition, the peaks are shifted towards smaller *m/z* values, which is advantageous in case of mass spectrometers with a limited mass range.

6.4 Conclusions

A differential derivatization procedure for free and disulfide-bound thiol functionalities in proteins based on two maleimide reagents with ferrocene groups has been introduced. Tandem mass spectrometry and EC/MS may be used to assign the individual cysteine residues as free or thiol-bound. The method may be used for intact proteins or their tryptic digests.

In the future, the differential labeling strategy may be combined with partial reduction by TCEP, which will be useful for the analysis of complex disulfide bridges. TCEP may be used to reduce a particular disulfide bond selectively [11]. The sample amount required for the differential derivatization may further be reduced based on variations in the sample preparation procedure, and the use of capillary LC columns and a nanospray interface would most likely lead to significantly improved limits of detection.

6.5 References

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Chapter 7

Concluding remarks and future perspectives

In analytical chemistry, derivatization reactions are typically not the first choice, because the respective procedures are laborious and the reactions may be a potential source of additional problems. However, derivatization does not only lead to a stabilization of labile analytes, but may also improve the detection properties or the chromatographic resolution of the analytes. Therefore, derivatization is frequently carried out in the analysis of complex samples. Ferrocenes are a particularly promising group of derivatizing agents in analytical chemistry, because they are characterized by a well-established chemistry, exhibit excellent electrochemical properties and may improve the HPLC separation of highly polar analytes.

By derivatization with ferrocene, a mass tag, an electroactive group and a predetermined fragmentation site are introduced. Furthermore, polar analytes are rendered more non-polar upon labeling, what facilitates their extraction from the sample and separation on reversed-phase columns. Another important property is the possibility to ionize the non-polar compounds by oxidation to the ferrocinium cation. This takes place under relatively low potentials in an electrochemical flow-through cell. A heated nebulizer interface (APCI interface with corona voltage set to low values) can be used to simply transfer ions into the mass spectrometer, which leads to high sensitivity, as shown in this thesis for isocyanate detection. The Fc-Pz derivatization of diisocyanates leads to doubly charged analytes. Consequently, future

investigations shall be focused on the analysis of ferrocene-derivatized polyisocyanates.

Dedicated fragmentation reactions after ferrocene derivatization are useful to add selectivity to the analytical methods. Precursor ion scans and neutral loss scans enable the detection of unknown ferrocene-labeled molecules. Furthermore, the differential derivatization of different functional groups increases the amount of data obtained within one single measurement.

The differential derivatization of small thiols and disulfides in biological matrices has some advantages over direct methods or methods that imply only derivatization of the thiols. Disulfides are not as stable as expected, especially at acidic pH, at which thiol derivatives are usually stored. The presented methods stabilize both the free thiols and the thiols liberated from the disulfides. Furthermore, derivatization of the disulfides enables the determination of the amount of the symmetric (for example cystine) and unsymmetric (for example GSH-Cys) as well as the protein bound thiols, whereas most of the quantification methods in literature include only symmetric disulfides.

The oxidation potential of the ferrocenes is tuneable via the linker attached to the cyclopentadienyl ring as shown for FEM and FMEA. The difference in oxidation potential between the two is sufficiently large to enable selective oxidation. Thus, another possibility to quantify thiols and disulfides is HPLC with electrochemical detection. This method would be much cheaper than the

LC/MS approach. Unfortunately, the lack of compatibility between gradient elution and electrochemical detection has only been overcome yet with very expensive instrumentation (Coularray from ESA Bioscience Inc. (Chelmsford, MA, USA)).

Another example for differential derivatization is the labeling of free and bound cysteine residues in proteins, which is also presented in this thesis. A remarkable feature of ferrocene-labeled analytes is the specific enhancement of their MS response when applying an oxidation potential to the electrochemical cell inserted into the LC/MS system. Furthermore, the selective determination of cysteine-containing peptides in a complex mixture is possible. The cleavage of the particular ferrocene label together with the cysteine is a powerful tool to determine the position of free cysteine in tryptic peptides, which contain both free and disulfide-bound cysteines. This is a unique feature of differential labeling. As a further extension of the method, ferrocenes as mass tags for amino acid assignment are an interesting option that should be evaluated for different amino acids, e.g. lysine residues, in the future. Thus, a ternary labeling of cysteine (free and bound) and lysine would be possible.

In future research, differential labeling could also be used in combination with partial reduction by TCEP. Thereby, complex disulfide bridges (intermolecular and intramolecular disulfide bonds) can be assigned.

Currently, the on-line LC/EC/MS investigations are limited to a glassy carbon working electrode. Problems with analyte absorption were observed for some applications, as shown in this thesis for proteins. Electrodes made of other materials or the use of surface modifications would minimize absorption effects. Furthermore, electron transfer could be simplified by dedicated surfaces, possibly leading to higher conversion rates and reduced electrode potentials. The development of electrochemical cells with lower volumes is still in process. Such cells will enable the use of LC/EC/MS with capillary columns and nanospray MS. Sample consumption is then minimized and the respective methods will be even more attractive for applications in the field of proteomics.

The detection of ferrocenes by element selective detection methods is still in its infancy, but appears to be very promising. Furthermore, other stable metallocenes, like the cobaltocinium ion, can be introduced as derivatizing agents for LC/MS and CE/MS. Differential labeling approaches using those cobaltocinium salts, especially in combination with ferrocene, are particularly promising. Electrophoretic separation methods in conjunction with MS or ICP/MS are most likely to be used for that purpose, because of the ionic character of the stable cobaltocinium species. For ferrocenes, the water solubility may be improved by incorporation of a sulfonic acid group. Furthermore, the polarity is enhanced, which enables and improves their electrophoretic separation, although sacrificing the RP-LC separation. Subsequently, the determination of iron, cobalt and sulphur enables an easy analysis of free and bound thiols in proteins. Using this approach, the

determination is not limited to proteins with a defined mass, so that glycoproteins with their mass distributions may be analyzed as well.

Summary

Within this thesis, the development and application of ferrocene-based derivatizing agents for LC/MS and LC/EC/MS is presented.

The advantages of derivatization by ferrocenes are the similtaneous introduction of a mass tag and an electroactive group, which make them ideally suited for LC/MS and especially LC/EC/MS detection. Furthermore, due to the reaction of polar functionalities with less polar labels, the HPLC reversed-phase separation is strongly improved. Thiols and isocyanates have to be derivatized because of their instability. Two new ferrocene-based derivatizing agents were developed for these analytes: Ferroceneoyl piperazide (Fc-Pz) for isocyanates and ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide (FMEA) for thiols. Both derivatizing agents are highly reactive towards the respective functional groups and yield stable derivatives.

The combination of liquid chromatography, online electrochemical oxidation and mass spectrometry was employed for the quantification of the most common mono- and diisocyanates. The non-polar ferrocene derivatives were separated by means of RP-HPLC and oxidized at a low potential in an electrochemical flow-through cell, where the ferrocinium cation was formed. The latter is subsequently detected by means of MS, equipped with an APCI interface operated as heated nebulizer. Ions were detected as $[M]^+$ for monoisocyanates and $[M]^{2+}$ for diisocyanates. Limits of detection were in the low nanomolar range using a single quadrupole mass spectrometer. The

usefulness of the method is shown for the analysis of vapors formed by heat-degraded MDI-based polyurethane foam (**chapter 3**).

The derivatizing agent FEM was investigated as a mass tag for cysteine residues in proteins (**chapter 4**). By labeling before and after reduction, it is possible to find out which cysteine residues in proteins are part of disulfide bridges and which of them are free. With the ferrocene, an electrochemically active label is introduced into the protein or peptide. At a low potential, the derivatives are oxidized online in the electrochemical cell to form the ferrocinium cation, which is determinded with lower limits of detection in the mass spectrometer. The intensity enhancement may be used for the selective analysis of cysteine-containing peptides in complex mixtures, e.g. tryptic digests, by comparing chromatograms of LC/EC/MS with LC/MS measurements. The method was successfully applied for several model proteins (insulin, lysozyme, β-lactoglobulin A).

FMEA was designed to be slightly more polar than FEM in order to separate the derivatives of both reagents easily by HPLC. Furthermore, the oxidation potential is shifted by 200 mV, which allows a differential electrochemical oxidation. FEM and FMEA were used in combination to perform differential derivatization of disulfides and thiols, for small molecules (**chapter 5**) and proteins (**chapter 6**).

The differential labeling method was applied to quantify cysteine, homocysteine, glutathione, cysteinylglycine and N-acetylcysteine and

corresponding disulfides in urine (**chapter 5**). The proposed method avoids difficult extraction steps of the derivatizing reagents. It allows the determination of the total amount of bound thiols and not only the amount of symmetrical disulfides. Precursor ion scans of FMEA derivatives and neutral loss experiments of FEM derivatives allow the identification of unknown thiols or disulfides in solution.

The differential labeling method was also applied to a protein mixture of α-lactalbumin and β-lactoglobulin A and B. The free cysteine residues were labeled with FEM and the bound cysteine residues with FMEA (**chapter 6**). The mass difference of 43 Da between the two labels is sufficient to differentiate between FEM and FMEA. The assignment of FMEA labels in tryptic peptides is possible by a precursor ion scan (m/z 213) and changes in the charge state distribution in LC/EC/MS. The FEM derivatives show a change in charge state distribution together with an increase in sensitivity. Fragmentation of tryptic digest peptides yields b- and y-type fragment ions with excellent coverage. The FEM and the FMEA are cleaved off together with the cysteine as fragments of 412 Da and 455 Da, thus allowing the selective assignment of free and bound cysteine in a single peptide.

Samenvatting

Dit proefschrift beschrijft de ontwikkeling en toepassing van op ferroceen gebaseerde derivatiseringsreagentia voor LC/MS en LC/EC/MS analyses.

Het voordeel van derivatisering met ferrocenen is de simultane introductie van een massalabel en een electroactieve groep, waarmee de derivaten uitermate geschikt zijn voor LC/MS en bovenal voor LC/EC/MS analyse. Bovendien wordt de reversed phase HPLC-scheiding sterk verbetert vanwege de reactie van polare functionaliteiten die met minder polare labels. Vanwege hun instabiliteit is derivatisering voor thiolen en isocyanaten een vereiste. Voor deze analyten zijn twee nieuwe ferroceenreagentia ontwikkeld: ferroceneoyl piperazide (Fc-Pz) voor isocyanaten en ferroceencarbonzuur-(2-maleimidoyl)ethylamide (FMEA) voor thiolen. Beide derivatiseringsreagentia hebben hoge reactiviteit met de betreffende functionele groepen en leveren stabiele derivaten.

De combinatie van vloeistofchromatografie, online electrochemische oxidatie en massa spectrometrie is toegepast voor de kwantificering van de meeste algemene mono- en diisocianaten. De apolaire ferroceenderivaten zijn gescheiden middels RP-HPLC en bij lage potentialen in een electrochemische doorstroomcel geoxideerd, onder vorming van het ferrocinium cation. Laatstgenoemde is vervolgens gedectecteerd middels APCI-MS, met de APCI interface in de ‘heated nebulizer mode’ bedreven. Daarbij zijn de

monoisocyanaten als $[M]^+$ en de diisocyanaten als $[M]^{2+}$ gedetecteerd. Bij detectie met een single quadrupole massaspectrometer zijn de detectielimieten in het nanomolaire bereik. De toepasbaarheid van de methode is aangetoond voor de analyse van dampen, ontstaan bij de thermische degradatie van op MDI gebaseerd polyurethaanschuim (**hoofdstuk 3**).

Het derivatisatiereagens FEM is ingezet als massalabel voor cysteïneresiduen in proteinen (**hoofdstuk 4**). Door middel van labelling voor en na reductie, kan bepaald worden welke cysteïneresiduen in de eiwitten betrokken zijn in disulfidebruggen en welke vrij zijn. Met het ferroceenlabel bevatten de eiwitten en peptiden een electrochemisch actieve groep. Bij een lage potentiaal worden de derivaten in de electrochemische cel online geoxideerd tot het ferrocinium cation dat met lagere detectielimieten kan worden bepaald in de massaspectrometer. De verhoogde intensiteit kan worden benut voor selectieve analyse van cysteïnehoudende peptiden in complexe mengsels, zoals tryptische digesten, door de chromatogrammen van LC/EC/MS en LC/MS metingen te vergelijken. Dergelijke metingen zijn uitgevoerd voor modeleiwitten (insuline, lysozym, β-lactoglobuline A).

FMEA is ontwikkeld als reagens met een lichtelijk hogere polariteit dan FEM, teneinde de respectievelijke derivaten makkelijk te kunnen scheiden middels HPLC. Bovendien is de oxidatiepotentiaal van het FMEA reagens met 200 mV verschoven, waarmee diferentiële oxidatie mogelijk wordt gemaakt. De combinatie van FEM en FMEA is toegepast voor differentiële derivatisering

van disulfiden en thiolen, voor kleine moleculen (**hoofdstuk 5**) en eiwitten (**hoofdstuk 6**).

De differentiële labelling methode is toegepast voor kwantificering van cysteïne, homocysteïne, glutathion, cysteïnylglycine en *N*-acetylcysteïne, alsmede de bijbehorende disulfiden in urine (**hoofdstuk 5**). Met de voorgestelde methode worden moeilijke extractiestappen van het derivatiseringsreagens vermeden. Het maakt de bepaling van het totale gehalte aan gebonden thiolen mogelijk, in plaats van alleen dat van symmetrische disulfiden. Met behulp van precursor ion scans van FMEA derivaten en neutral loss scans van FEM derivaten kunnen onbekende thiolen en disulfiden in oplossing geïdentificeerd worden.

De differentiële labelling methode is tevens toegepast voor analyse van een mengsel van lactalbumin en lactoglobulin A en B, waarbij de vrije cysteïne residuen met FEM en de gebonden residuen met FMEA gelabeld worden (**hoofdstuk 6**). Het massaverschil van 43 Da tussen beide labels is groot genoeg om te kunnen onderscheiden tussen FEM en FMEA. FMEA labels in peptiden van een tryptisch digest kunnen vastgesteld worden middels een precursor ion scan (*m/z* 213) en aan de hand van een bredere verdeling in de ladingstoestand in geval van LC/EC/MS analyse. De FEM derivaten tonen een bredere verdeling van de ladingstoestand in samenhang met een verhoogde detectiegevoeligheid. Fragmentatie van peptiden van tryptische digesten resulteert in een behoorlijk spectrum aan type b en type y fragmentionen. De FEM en FMEA labels worden samen met het cysteïne

afgesplitst als fragmenten van respectievelijk 412 Da en 455 Da, daarmee selectieve bepaling van vrij en gebonden cysteïne in een enkel peptide mogelijk makend.

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Curriculum Vitae

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List of Publications

Original Papers

1. Seiwert, B.; Karst, U., *Ferrocene-based Derivatizing Agents in Analytical Chemistry*, submitted for publication.

2. Seiwert, B.; Karst, U., *Simultaneous LC/MS/MS Determination of Thiols and Disulfides in Urine Samples Based on Differential Labeling with Ferrocene-Based Maleimides*, submitted for publication.

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3. Ferrocene-based derivatization reagents for biological thiols for ionization by electrochemical oxidation, Pittcon 2006, Orlando (USA)

4. LC/Elektrochemie/MS: Eine neue Methode zur Bestimmung von Isocyanaten, Anakon 2005, Regensburg (Germany)

5. LC/Elektrochemie/MS: Eine neue Methode zur Bestimmung von Isocyanaten, CIA 2005, Berlin (Germany)

6. LC/Electrochmistry/MS-Analysis of isocyanates as ferrocenoyl piperazide derivatives, Pittcon 2005, Orlando (USA)

7. LC/Elektrochemie/MS: Eine neue Methode zur Bestimmung von Isocyanaten, Doktorandenseminar 2005, Kloster Banz (Germany)