Development of an adductomic approach to identify electrophiles in vivo through their hemoglobin adducts

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List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals. Reprints were made with permission from the publishers.

I **LC–MS/MS Screening Strategy for Unknown Adducts to N-terminal Valine in Hemoglobin Applied to Smokers and Nonsmokers**
H. Carlsson, H. von Stedingk, U. Nilsson and M. Törnqvist
Chemical Research in Toxicology 27 (2014) 2062 – 2070.

II **Characterization of a Hemoglobin Adduct from Ethyl Vinyl Ketone Detected in Human Blood Samples**
H. Carlsson, H. V. Motwani, S. Osterman Golkar and M. Törnqvist
Chemical Research in Toxicology 28 (2015) 2120 – 2129.

III **Strategy for Identifying Unknown Hemoglobin Adducts Using Adductome LC-MS/MS Data: Identification of Adducts Corresponding to Acrylic Acid, Glyoxal, Methylglyoxal, and 1-Octen-3-one**
H. Carlsson and M. Törnqvist
Food and Chemical Toxicology 92 (2016) 94 – 103.

IV **Adductomic Screening of N-terminal Hemoglobin Adducts and Measurement of Micronuclei in Blood Samples from School-Age Children**
H. Carlsson, J. Aasa, D. Vare, N. Kotova,
L. Abramsson-Zetterberg and M. Törnqvist
Manuscript

The author’s contribution to the papers

I The author was responsible for most of the planning, experimental work, data evaluation and major parts of the writing.

II The author was responsible for most of the planning, experimental work, data evaluation and major parts of the writing.

III The author was responsible for all of the planning, experimental work, data evaluation and major parts of the writing.

IV The author was responsible for significant parts of the experimental work, data evaluation and of the writing.

Contents

Abbreviations

AA, acrylamide
AGE, advanced glycation endproduct
AN, acrylonitrile
AUC, area under the concentration-time-curve
CMV, carboxymethylvaline
DIA, data independent acquisition
DTC, differentiated thyroid carcinoma
EO, ethylene oxide
EVK, ethyl vinyl ketone
FITC, fluorescein isothiocyanate
fMN, frequency of micronuclei
FTH, fluorescein thiohydantoin
GA, glycidamide
GC, gas chromatography
GC/MS, gas chromatography/mass spectrometry
Hb, hemoglobin
HPLC, high-performance liquid chromatography
HRMS, high resolution mass spectrometry
HSA, human serum albumin
IS, internal standard
LC, liquid chromatography
LC/MS, liquid chromatography/mass spectrometry
LOD, limit of detection
LOQ, limit of quantification
MN, micronucleus
MS, mass spectrometry
MRM, multiple reaction monitoring
MVK, methyl vinyl ketone
PFPTH, pentafluorophenyl thiohydantoin
PRM, parallel reaction monitoring
RBC, red blood cells
RSD, relative standard deviation
Rt, retention time
SA, serum albumin

SIM, selected-ion monitoring
SPE, solid-phase extraction
Val, valine
Val-*p*NA, *L*-valine *p*-nitroanilide

1. Introduction to the thesis

During the last decades much research has concerned the genetic (and hereditary) factors contributing to cancer and other chronic diseases. Human studies of twins and genome-wide associations have however indicated that non-genetic factors, such as environmental exposures, are more important for the development of these diseases (AICR, 2007; Lichtenstein et al., 2000; Rappaport, 2016). Currently a large fraction of the total exposure of humans is unknown, and methods to detect different chemical sources are highly needed. The concept of the exposome has been introduced to describe the totality of exposures received by a person throughout life, from both endogenous and exogenous sources (Wild, 2005).

Humans are exposed to reactive compounds, such as electrophiles, from a wide range of sources. Such compounds have the potential to react with biomacromolecules, like proteins and DNA, thereby constituting risks for toxic effects. Because of the inherent reactivity of these compounds they typically have a short half-life in vivo due to detoxification. Such processes involve chemical and enzyme-mediated reactions, e.g. hydrolysis or conjugation with glutathione. This makes it practically impossible to measure electrophiles as free compounds in vivo. However, the corresponding more long-lived reaction products formed with biomolecules, defined as adducts, can be quantitatively assessed as a measure of exposure.

This thesis describes the adaption and application of a method to measure adducts, to search for exposures to unknown electrophilic compounds. This concerns adducts formed with the protein hemoglobin (Hb) in human blood. The method is based on the previously reported FI*R*E procedure for the analysis of adducts to N-terminal valine (Val) in Hb by liquid chromatography/mass spectrometry (LC/MS) (von Stedingk et al., 2010b). The term adductomics refers to the unbiased screening of adducts to biomolecules. The blood samples screened during this project represent the general population and the detected adducts represent the background load of reactive compounds in the everyday exposure. Within the context of this thesis, such adducts are defined as background adducts.

1.1. Scope of this thesis

The aims of this thesis were:

- To develop an adductomic approach for the screening of unknown Hb adducts in human blood using liquid chromatography/mass spectrometry (LC/MS).
- To identify the detected unknown Hb adducts and propose their precursor electrophiles and probable sources.
- To characterize adduct patterns in human blood from a larger number of individuals to assess whether individual differences are observable.
- To evaluate whether this approach for adductomics can contribute with new information, broadening the insight in human exposure to electrophilic agents.

Paper I describes the development and application of an adductomic approach for the screening of Hb adducts in human blood samples.
Paper II describes the identification and quantitative evaluation of a previously unknown Hb adduct formed from ethyl vinyl ketone in human blood.
Paper III presents a general strategy for the identification of unknown Hb adducts based on collected adductome data. The strategy is applied for the identification of four unknown adducts in human blood.
Paper IV describes the semi-quantitative assessment of identified and unidentified adducts in blood samples from Swedish school children, as well as the adaption of the previously used screening procedure for high resolution mass spectrometry (HRMS).

2. Background

2.1. Formation of adducts from electrophiles and their potential toxic effects

Throughout life we are constantly, and to a large extent unavoidably, exposed to a broad range of reactive electrophilic compounds. The exposure sources are both of endogenous (e.g. lipid peroxidation and oxidative stress) and exogenous (e.g. food and air pollution) origin as illustrated in **Figure 1**. Many compounds are initially, upon exposure, not electrophilic but are metabolically activated to electrophilic species (e.g. epoxidation of alkenes by cytochrome P450). Such reactive compounds constitute risks for toxic effects. Electrophilic compounds might react at nucleophilic sites in DNA forming covalent reaction products, adducts, which if not repaired by DNA repair enzymes, can lead to mutations during cell division. If such mutations occur in critical regions of genes important for regular cell function, the effects may be crucial, such as disrupted normal cellular growth and ultimately cancer. Electrophiles also react and form adducts with other biomolecules, like proteins. The observation of protein adducts indicate that the same reactions are plausible with DNA.

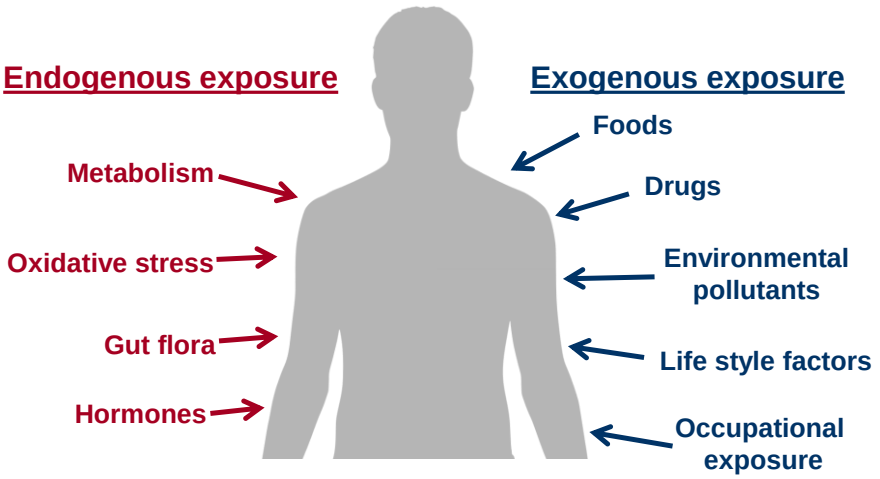

Figure 1. Illustration of the broad range of exposure to chemicals, of both endogenous and exogenous origin, that humans are exposed to.

Although protein adducts could not cause mutagenic effects, they may be associated with other diseases. In patients with diabetes (Rahbar, 2005) or renal failure (Wynckel, 2000), certain protein adducts could be monitored to follow the disease status. Oxidative modifications of proteins are used as a measures of oxidative stress (Ho et al., 2013). Contact allergens exert their activity through covalent binding, resulting in hapten-protein complexes (Karlberg et al., 2008; Smith Pease, 2003). Some electrophiles and/or their metabolites cause neurotoxic effects, for instance acrylamide (Calleman, 1994; Hagmar et al., 2001) and hexane (Huang, 2008), and have been monitored in occupational exposures by measurements of the corresponding protein adducts. Recently, the role of electrophilic metabolites as causative factors in idiosyncratic drug toxicity has been reviewed (Stepan et al., 2011; Thompson et al., 2011). For instance, quinone-like metabolites from common drugs like acetaminophen, tamoxifen and diclofenac have been observed to cause hepatotoxicity (Björnsson, 2016; James et al., 2006; Licata, 2016). The exposure to several human carcinogens and their metabolites, have been monitored by measurements of protein adducts, such as the adducts from ethylene oxide and several metabolites of 1,3-butadiene (Törnqvist et al., 2002).

Of certain importance for the development of methods for measurement of protein adducts was the discovery of glycated Hb (HbA1c) by Samuel Rahbar in the 1960s (Rahbar, 2005, 1968). Following the characterization of HbA1c, elevated levels of the adduct were found in patients with diabetes. Methods for monitoring of HbA1c levels were then developed to provide indirect long-term measurements of blood glucose levels. Today, measurement of HbA1c in diabetic patients is an established procedure of great importance since HbA1c levels reflect the risk of developing diabetes-related complications (Diabetes.co.uk, 2016). HbA1c was the first observed product of non-enzymatic glycation of proteins, and its discovery motivated studies of Maillard reactions in vivo, eventually leading to the concept of advanced glycation/lipoxidation endproducts (Rahbar, 2005).

Reactive compounds involved in adduct formation can be categorized in groups based on their reactive functional groups and reaction patterns (Enoch et al., 2011; Törnqvist et al., 2002). Of special importance for this thesis are: epoxides that form adducts through nucleophilic substitution (S_N2-type mechanism) (**Figure 2**); α,β-unsaturated carbonyl compounds that form adducts through Michael addition (**Figure 3**); and aldehydes that form Schiff base-type adducts via carbinolamine intermediates (**Figure 4**). Other reactive species may react according to other patterns, such as nitrosamines through S_N1-type mechanisms (**Figure 5**) or free radicals by radical-mediated reactions.

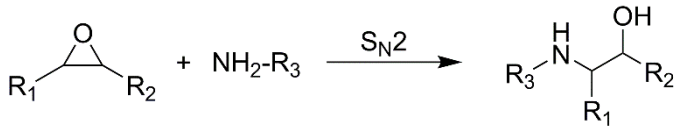

Figure 2. Epoxides react with nucleophiles, exemplified with an amine, to form adducts through nucleophilic substitution (S_N2-type mechanism). The figure shows the reaction with the carbon next to R_1, but the same reaction is possible with the carbon next to R_2.

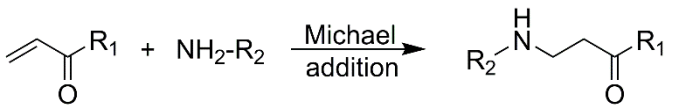

Figure 3. α,β-Unsaturated carbonyl compounds form adducts via Michael addition, as exemplified with an amine.

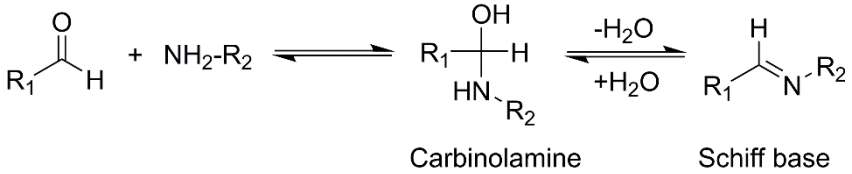

Figure 4. Aldehydes form Schiff base-type adducts through carbinolamine intermediates, as exemplified with an amine.

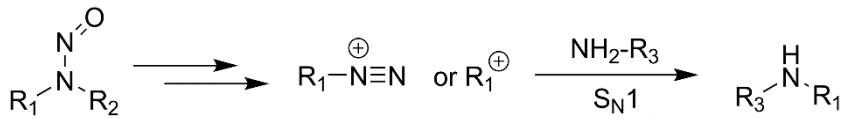

Figure 5. Nitrosamines are activated by forming alkyl diazonium ions or carbocations, which then form adducts through nucleophilic substitution (S_N1-type mechanism), as exemplified with an amine. The figure shows the reaction with the R_1 ion, but the same reaction is possible with the R_2 ion.

The extent of adduct formation depends on the nucleophilicity and pK_a of the nucleophilic atom as well as of steric hindrance and neighbouring group participation at the site of reaction (Törnqvist et al., 2002). Functional groups that are deprotonated at the physiological pH (pH 7.4) are more favorable for adduct formation, compared to protonated functional groups.

Some sites for adduct formation in proteins and DNA are shown in **Figure 6** and **Figure 7**, respectively. For many electrophiles the major sites for adduct formation in proteins are cysteine-S, the ring-nitrogens of histidine, and the NH_2 group of N-terminal amino acids (Törnqvist et al., 2002). In DNA, sites for adduct formation are e.g. N7-guanine, O^6-guanine, and N3-adenine (Koc and Swenberg, 2002).

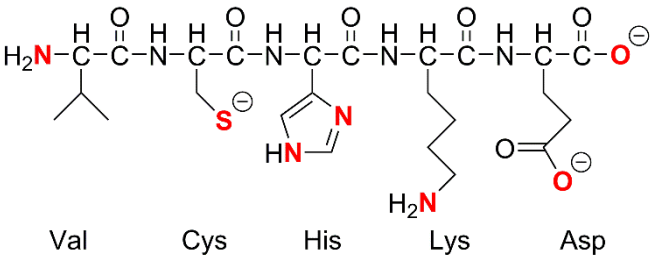

Figure 6. An example of a peptide, with Val as the N-terminal amino acid, showing the amino acids in their most favorable form for adduct formation (occurring at different pH), with the nucleophilic targets in red.

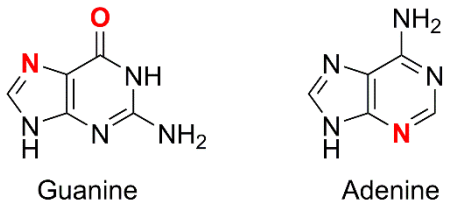

Figure 7. N7-guanine, O^6-guanine, and N3-adenine, in red, are examples of sites for the formation of DNA adducts.

2.2. Adduct determination: Methods and applications

2.2.1. Early work

Among the first to realize the potential of adduct measurements of genotoxic and carcinogenic compounds, were Groth and Neumann whom in 1971 proposed that Hb adducts could be used to measure the bioavailability of reactive metabolites of aromatic amines (Groth and Neumann, 1972). At Stockholm University the development of methods to measure in vivo doses of reactive cancer-risk increasing compounds as protein adducts were initiated

by Ehrenberg et al. in the early 1970s (Ehrenberg et al., 1974). By 1976 Hb had been chosen as a suitable protein for dose monitoring (Osterman-Golkar et al., 1976), based on its high abundance in blood samples and relatively long life-span (approximately 4 months in humans (Furne et al., 2003)). The first methods for adduct measurements were developed to monitor occupational exposures of carcinogenic compounds in workers. An early application of the methodology was the measurement of histidine adducts from ethylene oxide (EO) to Hb in occupationally exposed workers (Calleman et al., 1978).

About the same time as the developments of methods for dosimetry of reactive genotoxic compounds in vivo by the Ehrenberg group and others, analogous developments were done in the field of medicine for clinical applications. The most famous example is HbA1c, as described above (Ch. 2.1.). Another early example is the measurement of acetylated Hb. Bridges et al. found that aspirin (acetylsalicylic acid) acetylates Hb at multiple sites, and that the levels of the adducts were elevated in patients receiving long-term high-dose aspirin therapy (Bridges et al., 1975).

While the basic concepts remain, many advancements and discoveries have been made since those early experiments. An important observation was the background level of several adducts in non-exposed control subjects. This has demonstrated that many methods for adduct measurements are sufficiently sensitive to detect internal exposures to electrophiles within the general population. This has stimulated the interest in characterizing the background load of reactive chemicals, both of endogenous and exogenous origin, using adduct measurements. This concerns for instance ethylene oxide (Törnqvist et al., 1986b), aromatic amines (Bryant et al., 1988), and tobacco-specific nitrosamines (Hecht et al., 1993).

2.2.2. Choice of target molecule for adduct measurements

Most methods for adduct measurements use either DNA or the abundant blood proteins, human serum albumin (HSA) and Hb, as the monitor molecules. DNA adducts are mechanistically interesting since DNA is the primary target for carcinogenic and mutagenic action, whereas protein adducts are better indicators of exposure/internal dose. The high abundance of HSA and Hb in blood, compared to DNA, allows analysis of samples of small volumes. Typically, one mL of human blood sample contains approximately 150 mg Hb, 30 mg HSA, and 0.005 – 0.008 mg DNA. The lack of repair and relatively long half-lives make protein adducts more suitable for measurements of internal doses. Adducts to blood proteins accumulate over long periods of time and are suited for monitoring of continuous exposures, so called background exposure. DNA adducts usually have shorter half-lives due to DNA repair. The measurement of DNA adducts is not limited to blood, and has been

performed also in human urine (Bransfield et al., 2008; Chen and Chang, 2004), saliva (Bessette et al., 2010), and oral cells (Balbo et al., 2012). DNA and protein adducts should be considered complementary, but the different approaches are only rarely used in conjugation.

2.2.3. Analytical techniques for adduct measurements

The method of choice for detection of adducts is mass spectrometry, since it provides the necessary selectivity as well as some structural information. MS also provides the possibility to perform reliable quantitative measurements, using stable isotope-substituted internal standards (ISs). Historically, a range of different methodologies have been used for adduct detection, some of which will be briefly mentioned here.

For the detection of protein adducts, MS has been the primary method from an early phase, initially based on GC/MS (see e.g. (Calleman et al., 1978)). A limitation of GC/MS analysis is the requirement for volatile analytes, meaning that molecules of higher molecular weights, e.g. adduct-modified peptides, in general are not possible to determine. Prior to GC/MS analysis, adducts or modified amino acids have to be detached from the proteins. In many cases, derivatization is a further requirement, either to increase sensitivity or improve the retention properties of polar analytes. Many reagents used for derivatization are fluorinated, to increase the signal when performing analysis in the negative ion mode. During the last decade LC/MS-based methodologies have to a large extent replaced GC/MS methods.

LC/MS is a more versatile method for protein adducts compared to GC/MS, since thermolabile, hydrophilic, and non-volatile compounds may be determined. Electrospray ionization in the positive mode is normally used for adduct measurements. Tandem MS is typically employed in the multiple reaction monitoring (MRM) mode when performing targeted analysis. High resolution LC/MS instruments provide other useful modes of analysis, which are discussed in following chapters (Ch. 7.1. and 8.1.). Other methods for protein adduct detection include laser-induced fluorescence (Özbal et al., 2000) and immunochemical approaches, such as radioimmunoassays (Wraith et al., 1988).

For the measurements of DNA adducts, LC/MS-based methods have had a strong development and have been increasingly used during the last decade. Most instrumental setups employ tandem mass spectrometry and electrospray ionization in the positive mode. Methods for GC/MS are seldom used due to the thermal instability and low volatility of many DNA adducts. Other approaches for the determination of DNA adducts include HPLC with fluorescence or electrochemical detection, immunoassay-based techniques,

and the ^{32}P-postlabeling method, which for a long time was the most frequently used method (Phillips, 2013). These techniques have been reviewed, in comparison with mass spectrometric approaches, by Farmer and Singh (Farmer and Singh, 2008).

2.2.4. Enrichment and work-up of adducts

In vivo adduct levels only correspond to modifications of a very small fraction of the total amount of the studied biomolecule. Using Hb adducts as an example, an adduct level of 30 pmol/g Hb[1] corresponds to a fraction of about 5 modified Hb chains per 10^7 Hb chains in a human blood sample. DNA adducts are typically measured at levels corresponding to 1 adduct per 10^6 – 10^9 nucleotides. Enrichment of adducts is therefore normally needed to separate the modified proteins/DNA from the much larger bulk of unmodified biomolecules.

To enrich adducts, adducted biomacromolecules are typically isolated and the modified moieties detached prior to measurement. The detachment is often accomplished by hydrolysis of the protein, for instance by enzymatic digestion. For enzymatic hydrolysis of proteins, trypsin is frequently used, and has for instance been applied prior to measurement of N-terminal adducts to Hb from diepoxybutane and isoprene diepoxide (Boysen et al., 2004; Fred et al., 2005, 2004b). Another enzyme, endoproteinase Glu-C, was used for hydrolysis of glycated Hb (HbA1c) (Jeppsson et al., 2002). The highly specific cleavage of proteins provided by those enzymes results in a limited number of peptides and provides site-specific information useful for adduct measurements. In some cases complete hydrolysis, digesting proteins into individual amino acids, may be an alternative. This may be accomplished by the use of various proteases. Westberg et al. used pronase (a commercially available mixture of proteases) to digest serum albumin prior to the determination of adducts from diolepoxides of polycyclic aromatic hydrocarbons (Westberg et al., 2014).

Further cleanup is most often needed prior to analysis, to enrich the adducts and remove interfering species from the complex matrices. Contaminants, such as inorganic salts and other polar compounds (Hess, 2013), may suppress the electrospray ionization process at the LC/MS analysis and reduce the signal. Other contaminants may increase the noise and thereby affect detection limits. The removal of contaminants is often done by solid-phase extraction (SPE). Other extraction procedures, like liquid-liquid extraction, are also

[1] 30 pmol/g Hb correspond to the average background level of acrylamide adducts originating from intake of acrylamide from food.

commonly used as well as more sophisticated methods, such as immuno-affinity chromatography (Boysen et al., 2004).

In general, the sample preparation methods applied prior to LC/MS are more straightforward and involve fewer steps than those intended for GC/MS analysis, which often involve derivatization of polar groups. The work-up procedure should also be considered with regard to the stability of the studied adducts.

2.2.5. Quantification

To achieve accurate and precise quantification the choice of standards is of outmost importance. For MS-based procedures, standards of the measured adduct analytes, substituted with stable isotopes, are the optimal choice. Isotope-substituted internal standards are almost identical to the native compounds and will exhibit nearly the same behaviors in the entire analytical chain, but they differ in mass, which is utilized in the MS analysis. When performing the analysis, tandem mass spectrometers are most often used in the multiple reaction monitoring (MRM) mode to increase selectivity and thereby achieve as good detectability as possible of the adducts.

An aspect to consider at measurement of adduct levels is the possibility of artefactual adduct formation, particularly of low molecular weight adducts. Artefacts may form both during sample treatment and storage of samples. One example of an artefact is 2-hydroxyethyl (corresponding to EO) adducts to N-terminal Hb, formed during the storage of blood samples, probably by oxidation processes (Törnqvist, 1990). To check for any artefactual formation various control experiments may be performed (cf. *Paper I*), and if possible, the conditions causing risk for artefactual formation should be avoided.

3. Adductomics

3.1. The exposome

During the last decades, much research in the field of biology has focused on various omics studies, such as genomics, proteomics, and metabolomics (Horgan and Kenny, 2011). The suffix -ome refers to some sort of totality and omics refers to comprehensive studies of these "omes". Before the "omics revolution" research within these fields generally focused on a determined set of constituents, whereas omics aim to study the "whole". By performing omics, unknown constituents, as well as unknown interactions and relationships between constituents can be observed. The previous praxis of studying the effects of single or several predetermined constituents only allow narrow hypotheses and may skirt important observations. Metabolomics and proteomics, have both gained enormously from the advancements of LC/MS instruments during the last decades.

The concept of the exposome was first introduced by C. Wild in 2005, "to draw attention to the critical need for more complete environmental exposure assessment in epidemiological studies" (Wild, 2012, 2005). The exposome was suggested as a complement to the genome; whereas the genome has been studied with high precision the environmental exposure to individuals is largely unknown. Non-genetic factors are though considered to contribute to a larger portion of chronic diseases than genetic factors (Lichtenstein et al., 2000; Manolio, 2010; Manolio et al., 2009). There is a large imbalance in what is known about genetics contra the environment, which is not improved by the fact that most studies of environmental exposures focus on the effects of individual compounds, one at a time (Wild, 2005).

The exposome encompasses all the exposures to an individual through life, from conception and onwards, including both endogenous and exogenous sources. The assessment of the exposome is thus highly challenging, and several combined techniques and methodologies will be needed for comprehensive measurements. A broad range of compounds of exposure remains unknown and to truly assess the exposome, untargeted methods are needed.

3.2. The adductome

Reactive compounds, such as electrophiles, may be considered among the most important constituents of the exposome, because of their ability to react and form modifications with DNA and proteins. The totality of such adducts, is defined as the adductome and methods aiming at studying this ome are called adductomic approaches. For practical reasons methods for adductomics focus on adducts to specific biomacromolecules, and often to specific nucleophilic sites. Such sites may be defined as sub-adductomes (Rappaport et al., 2012). The ultimate aim of adductomic approaches is to characterize a priori unknown adducts in the general population to discover hitherto unknown sources, of importance for chronic diseases or other widespread disorders. Adductomic methods may be used to study adduct patterns of different populations, representing different exposure situations or diseases. This type of studies could provide valuable observations normally not obtained with targeted approaches.

The ambition to screen for a priori unknown adducts in the general population has been expressed for several decades among researchers (Törnqvist, 1989, 1988). In the 1990s such efforts were made by applying the N-alkyl Edman procedure (cf. Ch. 4.1.) for GC/MS screening of adducts to N-terminal Val in Hb (Rydberg, 2000). The potential to use the method for the detection of hitherto unknowns was early realized and a strategy similar to the one used in this thesis was formulated, but was not published.

The possibility to perform adductomic experiments has finally been realized during the last 10-15 years, along with the improvements of LC/MS technologies and the increased access to suitable instruments. The term "adductome" was first used by Kanaly et al. in 2006 (Kanaly et al., 2006), in their pioneering work concerning screening of DNA adducts. About the same time similar approaches were applied for the screening of mercapturic acids (Wagner et al., 2007, 2006) and glutathione conjugates (Castro-Perez et al., 2005). The first work on protein adductomics, published in 2011, concerned the screening of cysteine adducts (Cys34) in HSA (Li et al., 2011). Tryptic peptides containing the Cys34 adducts were enriched by HPLC prior to adductomic screening.

Even though DNA adducts might be more interesting with regard to genotoxic damage, it can be argued that the abundant blood proteins, HSA and Hb, are more useful and relevant for adductomic studies. The advantages of protein adducts compared to DNA adducts are described in Ch. 2.2.2.

The field of adductomics is still in an early phase and most published work to date concerns method development. Most studies on human samples

concern small sample series and further developments are needed for adductomic approaches to reach their full potential. A summary of adductomic studies of human samples published to date is given in **Table 1**. This summary does not offer a complete list of published studies, for instance in vitro studies have been excluded, but it gives an overview of the various methodologies available for adductomic analysis of human samples. This summary illustrates the small scale of the published adductomic studies to date.

Table 1. Summary of published adductomics studies using human samples

Biomacro molecule	Type of sample	MS instrument	Purpose of study	Ref.
DNA	Lung tissue (n=2)	Triple quad.	Introduction of the adductome concept, method development, screening of unknowns	Kanaly et al., 2006
DNA	Lung (n=1) and esophagus (n=1)	Triple quad.	Screening of unknowns	Kanaly et al., 2007
DNA	Buccal cells (n=unknown)	Quadrupole ion trap	Screening of tobacco related adducts in samples from smokers	Bessette et al., 2009
DNA	Various tissues (colon, liver, lung, pancreas, spleen, kidney, heart, small intestine; total n=68)	Triple quad.	Screening of unknowns	Chou et al., 2010
DNA	Gastric mucosa (n=2, pooled samples)	Triple quad.	Screening of unknowns, lipid peroxidation-induced adducts, screening followed by targeted analyses of individual samples	Matsuda et al., 2013
DNA	Colon tumors (human) (n=10)	Orbitrap	Method development, database construction, screening	Hemeryck et al., 2015
HSA	Blood (n=6, pooled samples)	Triple quad.	Method development, screening of unknowns	Li et al., 2011
HSA	Blood (n=3)	Orbitrap	Method development, screening of unknowns	Chung et al., 2014
Hb	Blood (n=12)	Triple quad.	Method development, screening of unknowns	Carlsson et al., 2014 (*Paper I*)

A successful MS screening of a priori unknowns within a certain class of compounds (such as detached Val adducts from Hb) requires that the analytes of interest exhibit similar behaviors, such as a common fragmentation

pathway in MS/MS. Such common properties are often first observed in the development of general analytical procedures and could then be applied for screening procedures. All published methodologies for adductomics use LC/MS/MS to monitor some type of fragmentation common for all analytes. For all DNA adducts the deoxyribose group, is a neutral loss (116 Da) and is screened during adductomic experiments. Similarly, common fragmentation pathways have been used in the screening of mercapturic acids, by monitoring the common loss of glutamate (Wagner et al., 2007, 2006), as well as for glutathione conjugates, by monitoring the characteristic loss of pyroglutamic acid (Castro-Perez et al., 2005).

Use of a constant (common) neutral loss (CNL) in the LC/MS/MS screening of adducts is often a suitable choice. In CNL mode both mass analyzers are scanning in full scan mode, with the second mass analyzer at a specific m/z off-set from the first. However, due to the often low adduct levels in human samples, only few adducts can be conveniently detected using full scan mode.

To increase detectability and reduce noise many reported methods for adduct screening use methods of sequential lists of MRM transitions set-up similar to CNL scans (cf. eg. (Kanaly et al., 2006; Li et al., 2011)). Instead of CNL scans discrete MRM transitions representing the analytes and separated by a fixed m/z are used. This dramatically increases the duty cycle and signal intensity for each analyte in the mass spectrometric run. Another major advantage of the MRM methods is the more straight-forward interpretation of data, compared to the complex situation with continuous data from full scan experiments. To cover wide m/z ranges multiple injections are often performed. This means that the cumulative time of analysis for each sample will be relatively long and susceptible to instrumental variations, which accentuates the use of suitable internal standards. Recent developments for adductomics are discussed in Ch. 8.

4. Method

4.1. The N-alkyl Edman procedure

Of special importance for this thesis is the N-alkyl Edman procedure, used for the detachment and subsequent determination of adducts to N-terminals in Hb by GC/MS. The method was developed at Stockholm University by the research group of Ehrenberg et al. in the 1980s (Jensen et al., 1984; Törnqvist et al., 1986a), to replace previous methods that utilized complete hydrolysis of Hb by hydrochloric acid prior to analysis (see e.g. (Calleman et al., 1978)).

The original Edman procedure was developed at Lund University by Pehr Edman in 1950 (Edman and Begg, 1967; Edman, 1950). The procedure has been of great importance for the field of molecular biology, since it was the first established method to allow protein sequencing on a routine basis. Protein sequencing was accomplished by derivatization and subsequent detachment of N-terminal amino acids, sequentially, without disruption of peptide bonds between other amino acid residues. The reagent originally used was phenyl isothiocyanate, but several different isothiocyanate reagents have later been used in applications based on the Edman procedure.

The derivatization and detachment involve the following two steps; first the isothiocyanate reagent is coupled to the N-terminal amino acid to form a cyclic thiocarbamyl adduct under mildly alkaline conditions, then the amino acid derivative is detached under acidic conditions, through the attack of the sulfur of the thiocarbamyl adduct on the carbonyl component of the first peptide bond (**Figure 8**). The detached amino acid derivative is then selectively extracted with an organic solvent and treated with acid to form a more stable isomer, a thiohydantoin, prior to analysis using one of several possible methods, like chromatography and electrophoresis. This procedure is then repeated to identify the next amino acid.

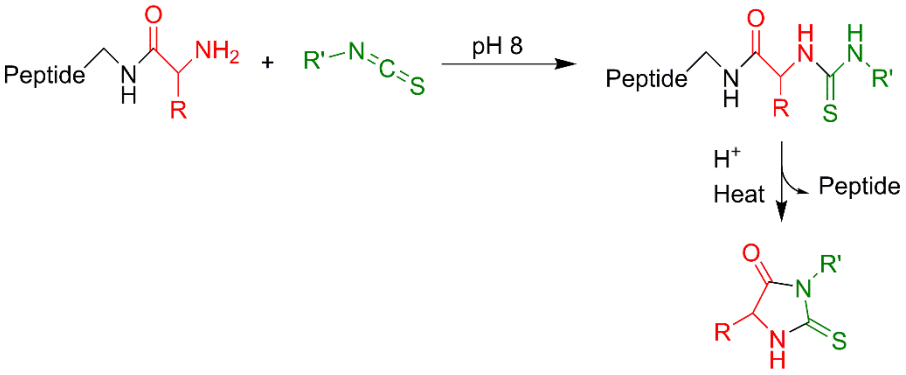

Figure 8. Illustration of the Edman procedure: an isothiocyanate reagent (in green) is used to detach an N-terminal amino acid (in red), from a peptide, as a thiohydantoin.

Ehrenberg's group explored Edman degradation as a possible method for adduct measurement. In the study of adducts from radiolabeled EO to N-terminal Hb, it was observed that when using Edman degradation the modified N-terminal Val detached spontaneously without the need for acidification (Jensen et al., 1984). That is, it seemed possible to couple the reagent and detach the thiohydantoin derivative of the Val adduct in a single step (cf. **Figure 8**). The observation of this specific detachment of adducts led to the development of the N-alkyl Edman procedure within the PhD work of M. Törnqvist (Törnqvist, 1989). Later it was concluded, from mechanistic studies, that the detachment of N-substituted N-terminal Val in Hb is favored over the detachment of non-substituted Val due to a gem-dialkyl effect, which favors ring-closure and detachment without requirement of acidification (Rydberg et al., 2002).

The different steps involved in the N-alkyl Edman procedure will be briefly described in the following since a basic understanding of the methodology is needed to understand the further developments discussed in the following chapters. First, globin is isolated from red blood cells by precipitation (Mowrer et al., 1986). The precipitated globin is then dissolved in formamide and treated with a fluorinated Edman reagent, pentafluorophenyl isothiocyanate, at mildly alkaline pH (Törnqvist et al., 1986a). The detached derivatives of N-terminal Val adducts are then isolated by liquid-liquid extraction without fractionation. A washing procedure of the extract, involving hydrolysis of by-products formed from the reagent, and subsequent evaporation then, to a large extent removes the by-products. Structures of the reagent and product are shown in **Figure 9**. If the adducts under study contain several hydrophilic functions, such as hydroxyl groups, small adjustments to the extraction procedure is needed. Such adduct analytes also need to be

further derivatized to obtain suitable lipophilicity and volatility for GC/MS separation. The GC/MS analysis is carried out with electron-capture negative ionization to obtain maximal detectability of the fluorinated adduct derivatives.

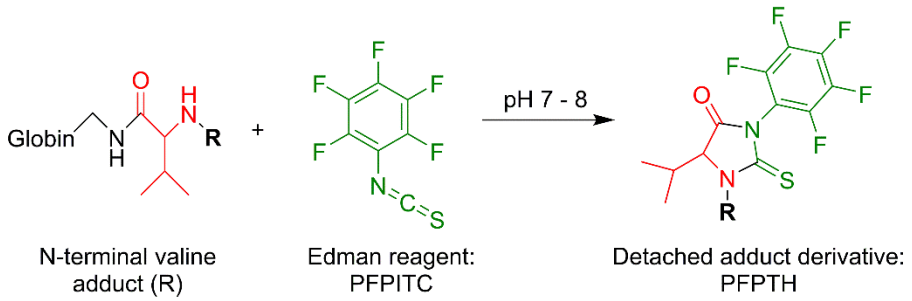

Figure 9. An illustration of the principle of the N-alkyl Edman procedure: N-terminal Val adducts (adduct denoted as R) are derivatized with the Edman reagent pentafluorophenyl isothiocyanate (PFPITC), and form detached adduct derivatives (pentafluorophenyl thiohydantoins, PFPTH).

The N-alkyl Edman procedure has been used in the determination of many different adducts, and is applicable to modifications from a broad range of electrophiles, such as ethylene oxide (Tates et al., 1991) and other epoxides, acrylamide (Bergmark, 1993) and other α,β-unsaturated carbonyl compounds, as well as aldehydes like malondialdehyde (Kautiainen et al., 1993). The method has shown high detectability and high reproducibility. One important limitation is however that when the N-terminal is blocked for reaction with the Edman reagent, with no free electron pair at the N-terminal nitrogen, it cannot be detached. This means that only mono-substituted N-terminal amino acids can be derivatized and detached. Examples of adducts which cannot be measured with this methodology are the ring-closed adducts from diepoxybutane (Kautiainen et al., 2000) and isoprene diepoxide (Fred et al., 2004a).

Compared to other nucleophilic targets, an advantage with measuring N-terminal Val is that there is no risk of misincorporation of NH_2-substituted amino acids during protein synthesis in vivo (Kautiainen et al., 1986). This reduces the risk of false positives and makes quantifications of human exposure more reliable.

The time for preparation and analysis with the N-alkyl Edman procedure, has set practical limits for the number of samples to be analyzed in human studies. The method has though been applied for analysis of acrylamide

exposure in cohorts of about 300 (Wilson et al., 2009) to 1000 individuals (Kütting et al., 2009). The request of applying this methodology in epidemiological studies, particularly regarding acrylamide exposure, has initiated further developments to achieve faster analysis. Developments regarding analysis of pentafluorophenyl thiohydantoin (PFPTH) derivatives of Val adducts concern solid phase extraction (SPE) instead of liquid-liquid extraction (Jones et al., 2006), and automatization of work-up and application of LC/MS analysis (using atmospheric pressure chemical ionization) (Vesper et al., 2007). Other developments concern adaption to LC/MS analysis by using different Edman reagents (phenyl isothiocyanate (Fennell et al., 2005)). A successful development concerns the development of the FI*R*E procedure, described in the next section (Ch. 4.2.). This method applies another Edman reagent, fluorescein isothiocyanate, suitable for LC/MS analysis and direct derivatization in hemolysate of blood, and work-up using SPE (von Stedingk et al., 2010b).

4.2. The FI*R*E procedure

The method used for this project was the FI*R*E procedure, which was given its name because fluorescein isothiocyanate (FITC) is used as the reagent for the derivatization of adducts, denoted *R* (covalently bound modification), in a modified Edman procedure (Rydberg et al., 2009). The FI*R*E procedure was developed as a method for semi-high throughput LC/MS determination of Hb adducts. The motivation was the need for a faster alternative to the N-alkyl Edman procedure for GC/MS (cf. Ch. 4.1.), to meet requirements for application in epidemiological studies. To achieve a higher throughput method the derivatization of adducts should preferably be done directly in whole blood without prior isolation of globin. Furthermore the derivatives should be easily isolated from the derivatized blood. An additional advantage with LC/MS methods is the possibility to determine thermolabile, hydrophilic, and non-volatile compounds.

In the first steps of the development of the LC/MS method the suitability of several isothiocyanate Edman reagents were tested (Rydberg et al., 2009). Besides FITC, 4-*N*,*N*-dimethylaminoazobenzene 4'-isothiocyanate (DABITC) and 4-dimethylamino-1-naphthyl isothiocyanate (DNITC) were compared with phenyl isothiocyanate (PITC) and pentafluorophenyl isothiocyanate (PFPITC), the latter used in the GC/MS method. FITC was superior to the other reagents in LC/MS, in terms of detectability of the formed thiohydantoin derivatives and with the additional advantage of being soluble in whole blood at physiological pH.

With FITC chosen as the reagent the FI*R*E procedure was developed as a semi-high throughput method (von Stedingk, 2011). The method is described in detail by von Stedingk et al. (von Stedingk et al., 2010b) and will only be described briefly in the following. The derivatization is performed by adding FITC (normally 5 mg) to whole blood or lysate (normally 250 µL) and mixing the samples over-night at 37ºC. A solution of internal standards (deuterium-substituted standards corresponding to fluorescein thiohydantoin derivatives of Val adducts) is then added and the proteins precipitated with acetonitrile, followed by centrifugation of the samples. The acetonitrile phase containing the fluorescein thiohydantoin (FTH) derivatives is then purified using mixed-mode anion-exchange SPE columns, utilizing the carboxylic acid functionality of the FTHs to retain the analytes on the columns. The LC/MS/MS analysis is performed in the MRM mode, with positive ionization, to achieve a good detectability for adducts at low levels. Reversed phase (C18) columns work well for the separation of FTH derivatives, also for adducts with small differences in structure and elemental composition. Internal standard calibration is used for quantification, and the adduct levels are adjusted for the Hb concentration in the blood samples (measured separately using a spectrophotometric device). The procedure is summarized in **Figure 10**.

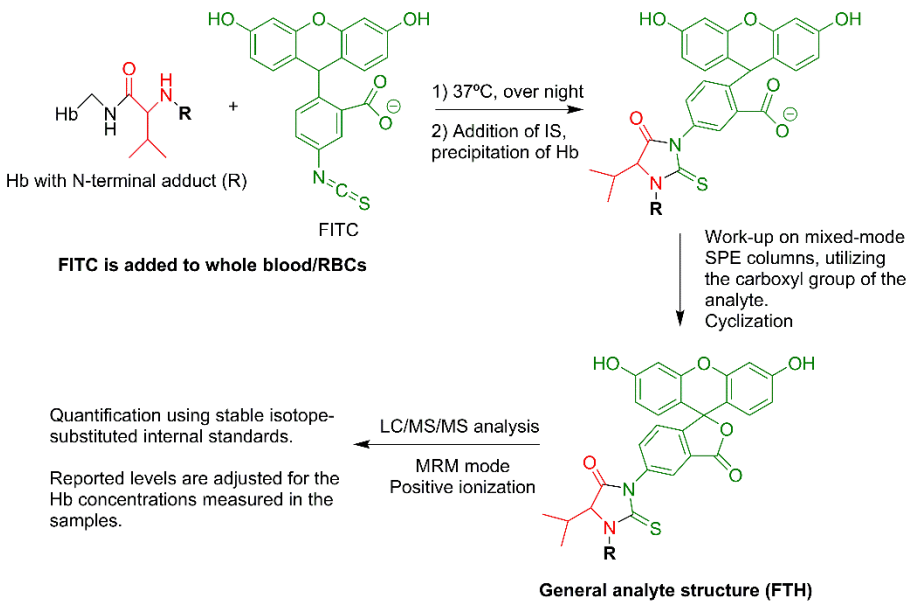

Figure 10. Illustration of the FI*R*E procedure: N-terminal Hb adducts are derivatized using fluorescein isothiocyanate (FITC), and fluorescein thiohydantoin (FTH) derivatives are formed.

The lowest LOQ reported for the FI*R*E procedure is ~1 pmol/g Hb (von Stedingk et al., 2011), but the value varies with instrumental and chromatographic column conditions. The RSD of the method is 5 – 10%, according to earlier studies, when having specific internal standards (von Stedingk et al., 2011). For some analytes the N-alkyl Edman procedure for GC/MS/MS provide lower detection limits. One example is an adduct from propylene oxide for which background levels of about 2 pmol/g globin were quantified with the GC/MS method (Törnqvist and Kautiainen, 1993). This adduct have so far not been detected with the FI*R*E procedure. Compared to the method for GC/MS/MS the excess of reagent is not removed prior to analysis when using the FI*R*E procedure, which may affect detection limits negatively.

Shortly after its development, the FI*R*E procedure was applied to measurements of adducts from AA, glycidamide (GA), and EO in large sets of samples from blood banks (>1000 samples from newborns) (Pedersen et al., 2012; von Stedingk, 2011). This demonstrated the applicability of the method for the measurement of background levels of adducts in large populations for studies of effects.

The FI*R*E procedure was originally applied for the simultaneous determination of adducts from AA, GA, and EO. The general fragmentation pathways observed for FTH derivatives of these Val adducts implied that the method could be useful for screening of a priori unknown Val adducts. An adduct from methyl vinyl ketone (MVK) was identified using the method (von Stedingk et al., 2010a).

The fast work-up of the FI*R*E procedure makes it suitable also for clinical applications. The method is developed for the measurements of adducts formed from phosphoramide mustard, a cytotoxic agent formed from the cytostatic drug cyclophosphamide (von Stedingk et al., 2014). The purpose is to allow for individualization of administered doses of cyclophosphamide, and thereby improve the efficacy of the drug and reduce side-effects.

4.3. Adducts for measurements of dose (AUC) in vivo

An important concept in the study of internal exposures to various chemicals and drugs is the concept of internal dose. The internal dose of a chemical is the effective concentration of the chemical over time as measured in blood, normally reported as the area under the concentration -versus- time curve (AUC, expressed in M × h). The AUC is dependent on absorption, distribution, metabolism and excretion of the chemical in vivo. Knowing the AUC is useful for toxicological and toxicokinetic evaluations of reactive

chemicals, since it reflects the net effect of absorption and metabolic rates in relation to exposure.

As mentioned previously it is not possible to reliably measure the concentrations of electrophilic compounds as free compounds in biological samples, due to their inherent instability and reactivity. Methods to measure protein adducts were in fact originally developed to enable determination of AUC in vivo of such short-lived compounds (Ehrenberg et al., 1974; Osterman-Golkar et al., 1976). The parameters needed for determination of the AUC of an electrophilic compound, based on protein adduct level measurements and assuming a constant exposure over a long period of time (i.e. background exposure), are the rate constant for adduct formation, the stability of the formed adduct (i.e. the rate of its disappearance), and the turnover of the protein (Ehrenberg et al., 1983).

Two recent examples of how the AUC of electrophiles in humans have been assessed from the measurements of Hb adducts are (1) the AUC of AA and GA after intake of AA-rich food (Vikström et al., 2011), and (2) the estimation of AUC of butadiene epoxides by using cob(I)alamin for in vitro enzyme kinetics (Motwani and Törnqvist, 2014). The AUC concept, based on protein adduct measurements, has also been applied in procedures for cancer risk estimation for a few compounds; butadiene (Fred et al., 2008), EO (Granath et al., 1999) and AA (DeWoskin et al., 2013; Törnqvist et al., 2008).

Adducts are formed in second-order reactions, with the rate of adduct formation depending on both the concentrations of the electrophilic compound, *RX*, and the nucleophilic compound, *Y*. The rate of formation, *v*, of adducts, *RY*, is determined by the second-order rate constant, k_Y, of the reaction and by the concentrations of *RX* and *Y* according to:

$$v = d[RY]/dt = k_Y \times [RX] \times [Y] \qquad (1)$$

The unit of k_Y is $M^{-1}h^{-1}$, or alternatively mol/g Hb per Mh when describing the rate constant for the reaction with Hb. The second-order rate constant can be calculated from the initial rate of adduct formation (cf. *Paper II*) (Ehrenberg et al., 1983).

During chronic exposure, stable adducts to proteins accumulate over the lifetime of the targeted proteins, to reach a steady-state adduct level ($[RY]/[Y]_{ss}$). The steady-state level depends on the daily adduct increments (*a*) (expressed in the unit pmol/g Hb per day, for Hb adducts), and is calculated differently for stable and unstable adducts.

Regarding adducts to Hb in a chronic exposure situation, stable adducts accumulate over the lifetime of the erythrocytes, t_{er} (about 124 days, (Furne et al., 2003)), and the steady state level is calculated according to:

$$\left(\frac{[RY]}{[Y]}\right)_{SS\ stable} = a\frac{t_{er}}{2} \qquad (2)$$

For unstable adducts the steady-state adduct level is attained more rapidly. In addition to the lifetime of the erythrocytes the instability of the adducts per se is an important factor. The steady state can be calculated according to Granath et al. (Granath et al., 1992):

$$\left(\frac{[RY]}{[Y]}\right)_{SS\ unstable} = a\frac{1}{k_{el}}\left[1 - \frac{1-e^{-k_{el}t_{er}}}{k_{el}t_{er}}\right] \qquad (3)$$

where k_{el} is the first-order rate constant for elimination of adducts due to their instability. This rate constant can be estimated from in vitro experiments where the disappearance of adducts are followed over time (cf. *Paper II*). In the case when the half-lives of adducts are much shorter than the life-time of Hb, the impact of t_{er} becomes negligible and $([RY]/[Y])_{ss}$ approaches a/k_{el}.

When the daily adduct level increment and rate constant for the reaction are known the AUC (expressed as the average daily dose, AUC_d, e.g. in µMh/day) may be calculated according to

$$AUC_d = a/k_Y \qquad (4)$$

4.3.1. Estimation of daily dose of electrophiles in human blood

To calculate the AUC from an adduct level to N-terminal Val in Hb, the second-order reaction rate constant for adduct formation is required. In this project two approaches were used to estimate the second-order reaction rate constant for the reaction between electrophiles and N-terminal Val in Hb at physiological conditions.

The first approach involved incubation of whole blood with the electrophile at different concentrations for a defined period of time. The adduct levels formed at the different concentrations were then plotted against the incubation doses (concentration × time) and the rate constant extracted as the slope of the linear regression of the data (expressed as mol/g Hb per Mh). For AA and other electrophiles of similar low reactivity, where the change of concentration of the reactants during the time of incubation can be neglected,

this approach works well. For electrophiles with higher reactivity, e.g. MVK, the reaction was found to be too fast to be measured by this method.

The second approach used Val *p*-nitroanilide (Val-*p*NA, **Figure 11**) as a model of N-terminal Val in Hb. The comparable reactivity of Val-*p*NA and Hb-Val was confirmed in experiments with AA, giving similar reaction rates in both systems. The advantage with using Val-*p*NA as a model nucleophile of N-terminal Val is the possibility to choose suitable concentrations of both the nucleophile and electrophile, and thus to follow the reactions of both slow and fast reacting electrophiles. The reaction products can be detected by either UV detection or MS, making it a convenient model system to follow reactions in real-time. Using Val-*p*NA it is also possible to observe reaction products that would not be possible to detect using modified Edman procedures, such as ring-closed adducts that block the Val nitrogen for reaction with Edman reagents. *p*-Nitroanilides of amino acids have widely been used as chromogenic substrates for determination of the activity of proteolytic enzymes in body fluids (e.g. (Haverback et al., 1960; Masler, 2004)). Details for the method with Val-*p*NA are given in *Paper II* of this thesis.

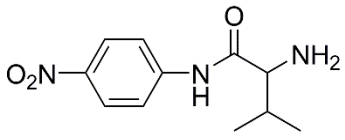

Figure 11. Valine *p*-nitroanilide

Formed adducts cannot always be assumed to be sufficiently stable for reliable determinations. To determine the AUC of unstable adducts both the reaction rate constant for adduct formation, and the rate of decay of the formed adduct need to be quantified. In this project the stability of adducts were studied by allowing adducts to form to completion in incubation experiments with human whole blood. The decay of adducts was then studied over a period of >24 h. Samples were taken at different times (and the reaction terminated) and derivatized and processed according to the FI*R*E procedure. The adduct levels were then plotted against time, and the rate of decay was extracted from the exponential function fitted to the data. Details for this procedure are given in *Paper II* of this thesis.

5. Screening of Hb adducts in human blood samples (Paper I)

5.1. The FI*R*E procedure for adductomic screening

The primary aim of this project was to develop a method for the screening of Hb adducts in human blood samples, hitherto known and a priori unknown, based on the previously reported FI*R*E procedure (von Stedingk et al., 2010b). In addition to qualitative information, the method should also give information about the adduct levels. The adductome data should be collected in a way that facilitates subsequent identifications. For the development and application of the screening procedure a total number of 12 human blood samples were used, six from smokers and six from nonsmokers.

From the development of the FI*R*E procedure it was clear that N-terminal Val adducts studied as FTH derivatives exhibit similar fragmentation pathways, resulting in at least three common fragments, **Figure 12**. The general fragmentation of FTH derivatives was used to set up the methods used for screening. For each incremental m/z unit within the screened m/z range of precursor ions, four diagnostic fragments, m/z 445, m/z 460, m/z 489, and m/z $[M+H]^+$ - 43 (**Figure 12**) were monitored (**Figure 13**). To qualify as an adduct candidate a compound should exhibit at least two of these fragments. The inclusion of several fragments is a necessity to obtain sufficient selectivity when screening for unknown FTH derivatives, since interfering ions of the same m/z may be present, occasionally at high concentrations. This is most probably often due to the formation of by-products in the derivatization reaction.

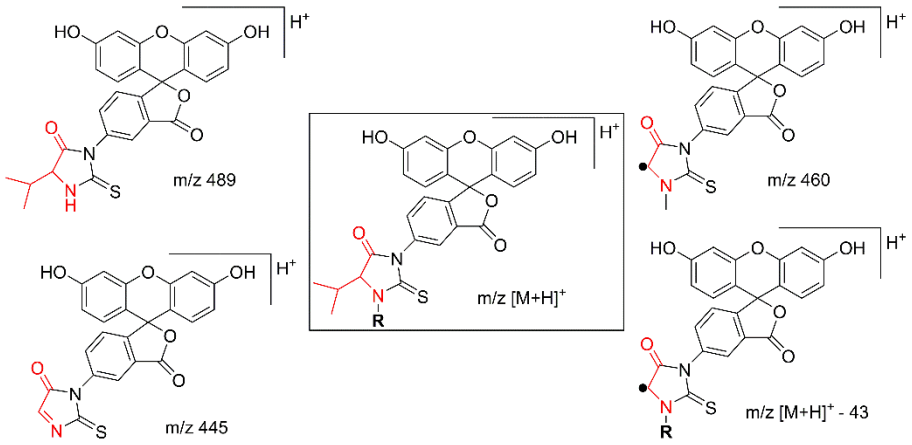

Figure 12. General structure of an FTH derivative of a Val adduct shown in the center, with the four common fragments shown in the corners. The red-colored parts represent the detached N-terminal Val. This figure originally appeared in *Paper I* of this thesis.

Q1 →	Q2 →	Q3

Q1: FTH analyte mass **Q3: Fragments**

$[M+H]^+$	445	460	489	$[M+H]^+ - 43$
503	445	460	489	460
504	445	460	489	461
505	445	460	489	462
506	445	460	489	463
.	.	.	.	.
.	.	.	.	.
638	445	460	489	595

Figure 13. The MRM method used to screen for N-terminal Hb adducts. The m/z range studied covered 135 units, from m/z 503 to m/z 638. For each precursor ion, four MRM transitions were included, corresponding to the fragments shown in *Figure 12*. This figure originally appeared in *Paper I* of this thesis.

A range of 135 m/z units (m/z 503 – 638) were screened for adducts, as FTH derivatives. All Hb adducts previously studied using modified Edman procedures are included within this m/z range[2]. The lowest m/z within the range corresponds to a methyl modification and the highest m/z corresponds to a modification of 149 Da.

For quantitative purposes the MRM method cycle was set to 1 s (50 MRM transitions with 20 ms dwell time for each transition) to allow for a sufficient number of data points over the chromatographic peaks. To cover the whole m/z range and the four monitored fragments each sample was injected 12 times. For each injection, transitions for AA and GA derivatives occurring as background adducts (von Stedingk et al., 2011), and corresponding deuterium-substituted ISs, were included as reference points. The AA IS (AA-d_7-Val-FTH) was used for semi-quantitative determination of adduct levels.

The FI*R*E procedure was used with minor adjustments for the derivatization and work-up of samples prior to the adductomic screening. In the MS analysis the parameters used had previously been optimized for the simultaneous determination of the FTH derivatives of adducts from AA, GA, EO, and MVK.

5.2. Selection of adduct candidates and control experiments

All chromatograms were individually evaluated and all peaks above 100 cps manually integrated. Compounds with precursor ions exhibiting two or more of the diagnostic fragments at the same retention time were considered as possible adducts. These compounds were further studied in product ion scan mode and their fragmentation patterns compared with those of known adduct analytes (cf. Table 1 and Figure 3 in *Paper I*). To control for possible artifactual formation of adducts or interfering compounds control experiments were performed. In the control experiments equine myoglobin and HSA (that do not have Val as the N-terminal amino acid) were derivatized and worked-up according to the FI*R*E procedure and analyzed (targeted analysis) for the adduct candidates. Some preliminary adduct candidate compounds could be excluded after detection in the control samples (cf. Supporting Information of *Paper I*). In total, 19 analytes, assumed to be unidentified adducts (unknowns), were detected, as well as 7 previously known adducts. Fourteen of the

[2] The highest molecular weight adducts to N-terminal Val in Hb that previously have been studied using modified Edman procedures are Michael addition adducts from 2-nonenal (only studied in vitro) (Kautiainen, 1992). The FTH derivative of this adduct would correspond to a quasi-molecular ion $[M+H]^+$ at m/z 629.

unknown adducts exhibited fragmentation patterns similar to those of previously studied Hb-Val adduct derivatives (cf. Table 1 in *Paper I*).

From the screening data and in vitro incubation experiments, an analyte corresponding to the ethyl adduct (m/z 517) could be confirmed. This modification was identified by matching retention times and fragmentation patterns with a standard that was generated in vitro by adding iodoethane to a sample of red blood cells. To my knowledge, the results from this screening was the first observation of ethyl adducts in human Hb. The ethyl adduct was thus the first Hb-Val adduct to be identified through this adductomic approach. Since the observation of the ethyl adduct was expected, and the adduct previously had been observed as a modification of DNA (cf. e.g. (Balbo et al., 2008)), it was added to the list of known adducts in *Paper I*.

5.3. Semi-quantitative assessment of adducts

As a simplification all FTH derivatives of Val adducts (at least the low molecular weight compounds within the studied m/z range) were assumed to have similar response factors, under the same LC/MS/MS conditions. Internal standard calibration, using the AA reference standard and the corresponding IS, was used to semi-quantitatively determine adducts levels of all modifications, both known and unidentified. The average of the integrated peak areas of the detected diagnostic transitions were used for the determinations. This was considered the best option since the relative intensities of the single different fragments vary between adducts. It would have been too difficult at this early stage to select optimal fragments for quantification.

For the studied adducts, both known and unidentified, the range of estimated adduct levels was 5 – 1200 pmol/g Hb. For the adducts from AA, GA, EO, and AN there were significant differences observed in adduct levels between the smokers and nonsmokers, as expected from earlier studies (e.g. (Bergmark, 1997; von Stedingk et al., 2011)). For the unidentified adducts there were no clear differences, except for the analytes with $[M+H]^+$ 547 m/z (later identified as an adduct corresponding to glyoxal/carboxymethylation, cf. Ch. 6.2.2. and *Paper III*, higher in nonsmokers) and 595 m/z (still unidentified, higher in smokers). To observe small differences between the groups much larger sets of samples would be needed. It is also possible that the majority of the observed adducts do not have any connection to smoking and reflect other sources of exposure. The semi-quantitatively determined adduct levels are presented in a relative scale in adductome map format in **Figure 14**.

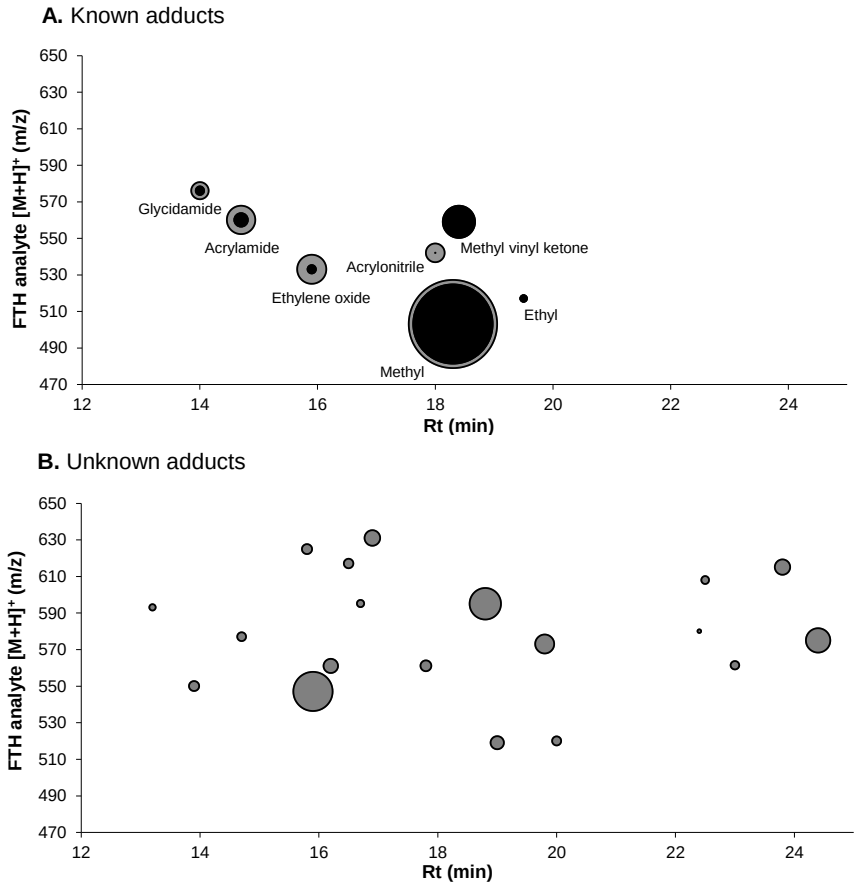

Figure 14. Adductome maps showing the relative average levels of: (A) seven previously identified adducts detected in smokers (n = 6, gray) and nonsmokers (n = 6, black); and (B) the 19 unknown adducts detected in smokers and nonsmokers (n = 12). The retention times (min) are shown on the x axis, and m/z of the FTH analyte precursor ion ($[M+H]^+$), on the y axis. The scale is the same in both A and B, and the range of estimated adduct levels was 5 – 1200 pmol/g Hb. This figure originally appeared in *Paper I* of this thesis.

5.4. Conclusions regarding the adduct screening in Hb

The screening procedure was successful since a rather large number of unidentified adducts were detected. Compared to the seven adducts that previously had been observed and confirmed with the method, the detection of 19 additional probable adducts showed that the FI*R*E procedure has a wide applicability, demonstrating the strength of adductomic approaches. Further considerations about the FI*R*E procedure as a basis for adductomic approaches are discussed in Ch. 8. Following adduct detection, the next step is then to

identify the adducts and the precursor electrophiles, and trace the exposure sources and prove their in vivo formation.

6. Identification of unknown adducts (Paper II and III)

6.1. Strategy for identification of detected unknown adducts

Adductomic approaches may greatly expand the number of adducts detected with various analytical methods. A challenge following adductomic experiments is the identification. Ideally, the adductome data should provide some parameters useful for identification. From the screening of adducts described in this thesis, based on the FI*R*E procedure, the information received on hitherto unknown adducts are the m/z of the precursor ions ($[M+H]^+$) and the retention times. From these two parameters adduct identities may be hypothesized.

The mass of the Hb modifications may be calculated by subtracting m/z 489 from the precursor ions. The m/z 489 fragment corresponds to the intact FTH derivative of unmodified Val (**Figure 15**). From the obtained masses the corresponding precursor electrophiles may be suggested. This process may be facilitated by the use of databases. During this project a database provided by the National Institute of Standards and Technology, "Search for Species Data by Molecular Weight" (National Institute of Standards and Technology, 2016), were used. This system searches for species with molecular weights within 0.5 units of the input value, based on single isotope atomic masses. In the resulting list of species, precursor electrophiles with a potential ability to form adducts were searched for. Such compounds should normally be reactive with functional groups such as activated double bonds, aldehydes and epoxides (cf. **Figures 2**–**5**).

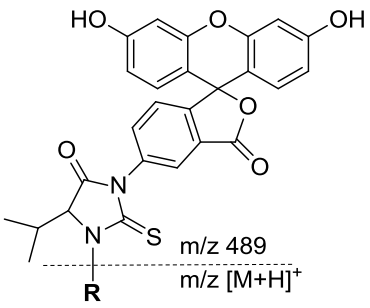

Figure 15. By subtracting m/z 489 from the precursor ion ($[M+H]^+$) of a fluorescein thiohydantoin derivative of an N-substituted Val, the mass of the modification, R, is obtained. This figure originally appeared in *Paper III* of this thesis.

Given that reversed phase chromatography (C18 HPLC columns) was used for the adductomic experiments the retention times are mainly correlating with the lipophilicities of the analytes. An advantage with the detection of Hb adducts as FTH derivatives is that they, even with similar or identical elemental composition, in general are separated relatively well. Since the seven previously identified adducts were included in the screening they were used as references in the next step of formulation of hypotheses on adduct identities.

The hypothesized adduct analytes (R-Val-FTH) corresponding to suggested precursor electrophiles, were drawn in ChemBioDraw Ultra 12.0 (CambridgeSoft). The theoretical Log P of the analytes are given by the software, and compared with the theoretical Log P values of the previously known adducts to conclude if the observed retention time of the unknown adduct could match that of the theoretical hypothesized analyte. The overall correlation between retention time and theoretical Log P is strong for the identified adducts (**Figure 16**), and with a few exceptions (adducts from glycidamide and methylglyoxal) the retention time can be suggested relatively well from the Log P value.

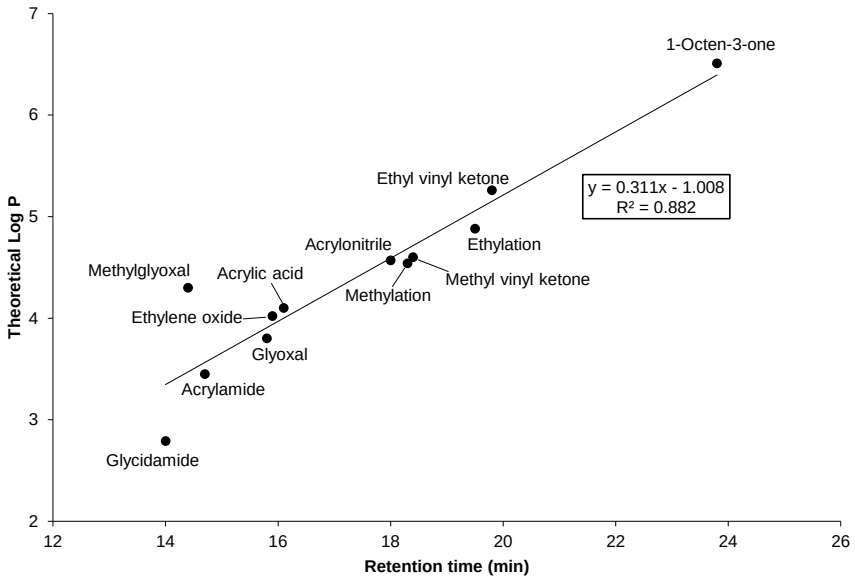

Figure 16. Correlation between theoretical Log P and retention time of fluorescein thiohydantoin derivatives of Val adducts. Each data point corresponds to an identified adduct/precursor. This figure originally appeared in *Paper III* of this thesis.

When a probable precursor electrophile has been suggested for a specific modification the hypothesis should be tested. In this project this was done by the generation of reference Hb modifications by performing in vitro incubations in human blood with the suggested electrophiles. The incubated blood was then processed and analyzed according to the FI*R*E procedure. The in vitro generated adducts, formed at high levels, were then compared with the so far unknown adducts in vivo, considering the precursor ions, retention times and fragmentation patterns in the LC/MS/MS analysis. An adduct was considered identified if those parameters were matching. An example of a matching in vitro generated adduct and a background adduct observed in vivo is shown in **Figure 17** (adduct from 1-octen-3-one shown as example).

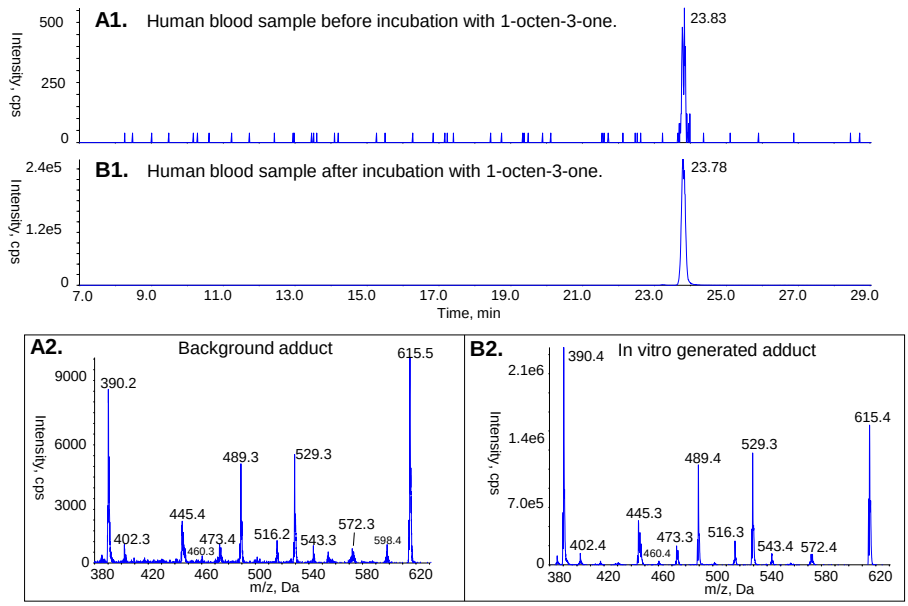

Figure 17. Example of identification through generation of a hypothesized adduct corresponding to an observed so far unidentified adduct, and matching of LC/MS/MS properties. The case of the adduct from 1-octen-3-one is shown as an example. A1 and B1 (upper) show the increase in peak area following incubation with 1-octen-3-one (the shown transition is m/z 615 to 489). A2 and B2 (lower) show the matching fragmentation patterns of the background adduct and the in vitro generated adduct. This figure originally appeared in *Paper III* of this thesis.

For the identified Hb adducts during this project, the precursor electrophiles were available commercially, which makes the generation of reference adducts straightforward. For adducts were the proposed precursors are not readily available the identification process will be more complex, and synthesis of either the precursors or the corresponding adducts will be needed. In cases were an origin of an unknown adduct cannot be easily hypothesized, the identification will be even more complex. Even when considering adducts of low mass several structural isomers may be possible, and in many cases synthesized standards and NMR spectroscopy will be needed for the characterization.

6.2. Identified electrophile precursors

During the project five precursor electrophiles corresponding to five previously unidentified adducts have been identified (in addition to the ethyl adduct identified in *Paper I*, cf. Ch 5.2.). Three of those adducts (formed from

glyoxal, methylglyoxal, and acrylic acid) have previously been reported by others as either protein or DNA modifications (cf. *Paper III*). The other two adducts (formed from ethyl vinyl ketone and 1-octen-3-one) have not been previously described in vivo. The identification of the adducts is described in detail in *Papers II* and *III*. The possible exposure sources and toxicological information concerning the precursor electrophiles are also extensively discussed in these papers. In the following sections the precursor electrophiles, and their corresponding Hb-Val modifications, are shortly presented. In **Table 2** the identified adducts and their estimated levels in the studied samples (the same 12 human blood samples as analyzed in *Paper I*) are shown.

Table 2. Precursor electrophiles corresponding to previously unknown adducts, and corresponding adduct levels.

Precursor electrophile	FTH $[M+H]^+$ m/z	Mean adduct level ± SD (pmol/g Hb) (n=12)	Range (min-max) (pmol/g Hb) (n=12)
Glyoxal	547	356 ± 148[a]	205-661[a]
Methylglyoxal	561	73 ± 82[a]	25-325[a]
Acrylic acid	561	41 ± 17[a]	15-76[a]
Ethyl vinyl ketone	573	40 ± 12[b]	25-68[b]
1-Octene-3-one	615	62 ± 15[a]	44-91[a]

[a] Adduct levels estimated using a semi-quantitative approach, cf. Ch. 5.3.

[b] Adduct levels determined using an authentic synthetic standard (EVK-Val-FTH).

6.2.1. Ethyl vinyl ketone

Ethyl vinyl ketone (EVK) is an α,β-unsaturated ketone and forms adducts by Michael addition (**Figure 18**).

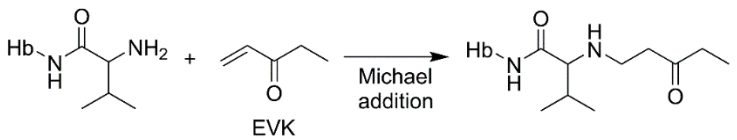

Figure 18. The amino group of Val reacts with EVK through Michael addition and yields an *N*-pentyl-3-one modification.

The identity of the EVK adduct was first suggested based on a comparison with the previously identified MVK adduct; the m/z of the precursor ion $[M+H]^+$ m/z 573 was 14 m/z (i.e. CH_2) higher than that of the MVK adduct and the compound was eluting 1.5 min later during the chromatographic run. The identity was confirmed with a reference obtained from an incubation

experiment where EVK was added to a blood sample, and the adduct was generated in vitro. To further verify the identity and allow for quantitative work a reference standard of EVK-Val-FTH was synthesized and characterized by NMR.

In addition to the identification of the modification, an extensive quantitative characterization of the adduct kinetics was performed to estimate the doses of the precursor electrophile in humans. This involved several kinetic experiments, described in the following.

Estimation of daily dose, AUC_d, of EVK in human blood

From incubation experiments with whole blood to determine the reaction rate between EVK and N-terminal Val in Hb, it was found that the reaction was too fast to allow accurate measurement (cf. Ch. 4.3.1.). The same was observed for MVK. The half-life of EVK in blood was estimated as < 1 min. Instead the approach using Val-*p*NA as a model of N-terminal Val in Hb, was used to determine the second-order reaction rate constant (cf. Ch. 4.3.1.). The rate constant, at 37 ºC, for the formation of the EVK adduct was determined to 200 $M^{-1}h^{-1}$. The corresponding rate constants for MVK and AA are 180 and 0.10 $M^{-1}h^{-1}$, respectively. This means that the two vinyl ketones react approximately 2×10^3 times faster than AA.

From incubation experiments with blood it was found that formed EVK-Val adducts to Hb are not stable. Incubation experiments with EVK in human whole blood were performed and adducts allowed to form to completion (~30 minutes at 37 ºC). The decay of adducts was then studied over a period of 26 h (cf. Ch. 4.3.1.). The half-life of EVK-Val-Hb adducts at 37 ºC was estimated to 7.6 h, which for Hb adducts is a very short half-life.

Knowing the mean adduct levels, the second-order rate constant of formation of adducts, and the first-order rate constant for elimination of adducts for EVK-Val adducts, the daily dose, AUC_d, could be calculated according to the equations given in Ch. 4.3. The AUC_d of free EVK in blood was thereby calculated as about 7 nMh. This is about 20 times lower than the estimated AUC_d of AA from intake via food (160 nMh).

The general sequence of work described here, and the experiments performed to estimate the AUC_d, illustrate the first steps in the evaluation of a recently identified adduct. The example of EVK-Val adducts is interesting because of the high reactivity of the electrophile and the instability of the formed adducts, demonstrating the importance of quantifying both of the parameters.

Potential sources of EVK exposure and concerns regarding toxicity

EVK is found as a naturally occurring and a synthetic flavouring substance in a wide range of foods and beverages. A major occurrence of EVK is in orange essence oil, commonly used in orange juice products (Moshonas and Shaw, 1990; National Toxicology Program, 1992). Other occurrences include soybeans, black tea, potatoes, tomatoes, grapes, kiwi fruit, banana, grapefruit juice, peach, fish oil, chicken fat, lovage leaf, endive, oysters, clam and boiled and cooked beef (cf. *Paper II* for references). The concentrations of EVK in individual food items is generally expected to be low, but the presence of EVK in such a diversity of frequently consumed food items indicates a significant human exposure. Most probably the diet is the major exposure source of observed EVK adducts, but the compound could possibly also be formed endogenously during metabolism and other cellular processes.

Regarding the toxicity of EVK, the knowledge is limited. Eder et al. have published several studies on the genotoxicity of EVK and concluded that the compound is clearly mutagenic in the Ames test (*Salmonella typhimurium*) and the SOS Chromotest (using *E. coli*) (Eder and Deininger, 2000). The National Toxicology Program performed short-term inhalation toxicity studies of EVK in rats and mice, and concluded that EVK is direct-acting upper respiratory tract irritant with few systemic effects (Morgan et al., 2001).

The observed instability of the formed Hb adducts of EVK is interesting, as the potential reversibility of the modifications might prolong the lifetime of EVK in vivo. The reversibility of adducts formed by Michael addition is an interesting topic that could have importance for the toxicity of, especially, fast reacting electrophiles forming unstable adducts.

6.2.2. Glyoxal

Glyoxal is a reactive aldehyde and proposed to form adducts by Schiff base formation (reversible) followed by Cannizzaro rearrangement (irreversible), **Figure 19** (Davies, 2009; Thorpe and Baynes, 2003). The corresponding modification, carboxymethylvaline (CMV), was the modification present at the highest level observed in the screening (cf. **Table 2**). The rate of Hb-Val adduct formation was observed to be low during incubation experiments, and the approximate rate was comparable to the rate of methylglyoxal and about 100 times lower than the rate of adduct formation from AA.

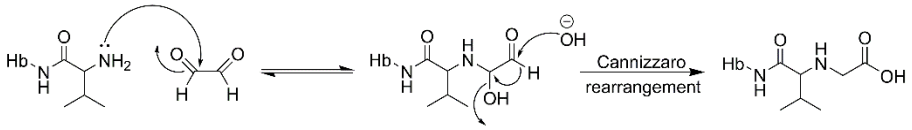

Figure 19. The amino group of Val reacts with glyoxal to yield an *N*-carboxymethyl modification, following a Cannizzaro rearrangement (proposed mechanism).

Glyoxal is formed both in vivo and in vitro, mainly in the autoxidation of glucose, from oxidation of lipids, and from degradation of Maillard reaction products (Poulsen et al., 2013; Thornalley, 2005). Food is considered the major exposure source of glyoxal, and concentrations are particularly high in sugar-rich foods. The carboxymethyl modification of N-terminal Val in Hb (CMV-Hb) is an example of an advanced glycation endproduct (AGE), and generated in the degradation of glycated Hb (HbA1c) (Shimada et al., 2005; Uchimura et al., 2001). Several percent of the N-terminals in human Hb are normally glycated, which gives an explanation to the high background levels of CMV-Hb. Of the observed adduct levels, only a small fraction is expected to stem from actual glyoxal exposure and the major part is believed to be the degradation product of glycated Hb (Glomb and Monnier, 1995; Poulsen et al., 2013).

CMV-Hb adducts have been studied by others a few times previously, and for instance found at elevated levels in diabetic patients (Shimada et al., 2005). Cai and Hurst studied CMV-Hb adducts using the N-alkyl Edman procedure (cf. Ch. 4.1.) and observed a mean adduct level 14 nmol/g globin in human blood samples (n=10) (Cai and Hurst, 1999). This adduct level is considerably higher than the levels measured during this project (about a factor 40 higher than the mean adduct level given in **Table 2**).The adduct levels reported by Shimada, determined by an immunoassay, 15 nmol/g Hb for non-diabetic patients (n=486), are also in agreement with the higher adduct level reported by Cai and Hurst. The cause for the lower level observed with the FI*R*E procedure should be investigated in future studies. For quantitative work a synthetic standard should be prepared to confirm if a possible underestimation of the adduct level is due to the semi-quantitative approach.

Carboxymethyl modifications of proteins are among the most frequently studied AGEs (Poulsen et al., 2013), and there are several methods available for the study of the modification to various sites. The method presented here, using the FI*R*E procedure, could be an alternative method to measure carboxymethyl modifications in humans.

6.2.3. Methylglyoxal

Methylglyoxal (**Figure 20**) is a reactive aldehyde, similar to glyoxal, and proposed to form N-terminal Hb adducts by the same mechanism, following a Cannizzaro rearrangement (Davies, 2009). As for glyoxal, the reaction rate towards N-terminal Val in Hb was observed to be low during incubation experiments.

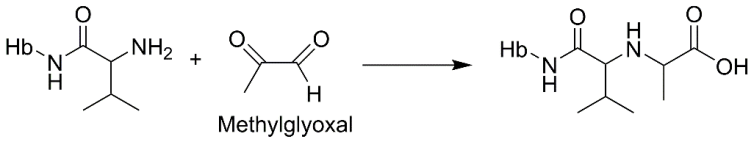

Figure 20. The amino group of Val reacts with methylglyoxal to yield an *N*-1-carboxyethyl adduct.

The modification corresponding to methylglyoxal, 1-carboxyethyl, was not included in the original set of unidentified adducts detected in the screening. This was because of the deviating fragmentation pattern of the compound, only exhibiting one of the four screened characteristic fragments (m/z 489). Instead, it was first observed to occur as a background adduct in human blood in a control experiment to verify that a suggested modification from acrylic acid was not really formed from methylglyoxal, as both electrophiles form adducts with the same elemental composition (the adduct formed from acrylic acid is a 2-carboxyethyl modification). From those experiments it was concluded that the two electrophiles form adducts for which the derivatives are well-separated and exhibit different fragmentation patterns. The detection of the methylglyoxal adduct implies that there might be several FTH derivatives corresponding to so far unknown modifications which do not fragment as expected, and thereby not qualify according to the criteria set here at adduct screening.

Methylglyoxal is formed both in vitro and in vivo (Poulsen et al., 2013), as a sugar fragmentation product, and through oxidation of lipids and degradation of Maillard reaction products. The major exposure source for humans is food, and as for glyoxal the concentrations are higher in sugar-rich foods. Methylglyoxal reacts mainly with arginine groups to form a hydroimidazolone adduct (Rabbani and Thornalley, 2010). This adduct is often the most abundant AGE observed in vivo, and levels are higher in persons with diabetes, renal failure, cirrhosis, Alzheimer's disease, arthritis, Parkinson's disease and during ageing (Han et al., 2009; Poulsen et al., 2013; Rabbani and Thornalley, 2010). As for glyoxal, N-terminal Hb adducts could be an alternative choice for the measurement of methylglyoxal exposure in vivo.

6.2.4. Acrylic acid

Acrylic acid is an α,β-unsaturated carboxylic acid and reacts with N-terminal Hb-Val through Michael addition (**Figure 21**). The reactivity was found to be about 20 times higher than that of glyoxal and methylglyoxal and 6 times lower than that of AA.

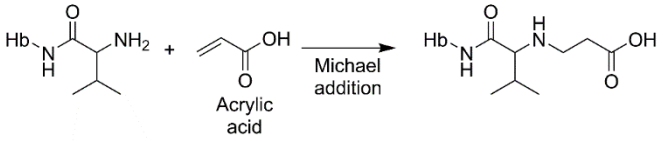

Figure 21. The amino group of Val reacts with acrylic acid through Michael addition and yields an *N*-2-carboxyethyl adduct.

Similar in structure to AA, acrylic acid has also been identified as a Maillard reaction product (Stadler et al., 2003). In the Maillard reaction acrylic acid is formed mainly through degradation of aspartic acid, but also via thermolytic deamination of alanine (Stadler and Studer, 2016). Oxidation of acrolein, formed as a product of thermal degradation and/or oxidation of free fatty acids or glycerol, is another source of acrylic acid (Mottram et al., 2002). The main exposure source of acrylic acid is therefore expected to be from the diet. Exposure to acrylic acid from food is expected to be lower than 1 µg/kg bw and day (Stadler et al., 2003).

Acrylic acid is a carboxylic acid with a pK_a of 4.25, which means that the deprotonated form (acrylate ion) will predominate at physiological pH values. In its deprotonated form acrylic acid is inert, while the neutral form is reactive (Frederick and Reynolds, 1989). The small fraction of the reactive form present at physiological conditions is believed to be the main reason for the low toxicity observed of the compound, with no observed genotoxicity or carcinogenicity (Stadler et al., 2003). Since the adduct levels are relatively high there might be other sources forming the same adduct in vivo. Wang et al. have observed 2-carboxyethyl modifications of DNA in human liver samples and explored various sources to those (Wang et al., 2013). They found endogenous nitrosation of dihydrouracil, an abundant metabolite formed from uracil, to be a likely major source to the observed adducts. *N*-nitrosodihydrouracil may also be a plausible source to the observed Hb modifications.

6.2.5. 1-Octen-3-one

1-Octen-3-one is an α,β-unsaturated ketone and forms Hb adducts through Michael addition (**Figure 22**). The reactivity of 1-octen-3-one is high and likely comparable with the reactivities of the structural analogues MVK and EVK (which were studied in more detail, cf. Ch. 6.2.1. and *Paper II*).

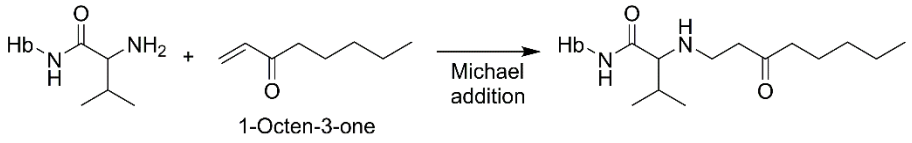

Figure 22. The amino group of Val reacts with 1-octen-3-one through Michael addition and yields an *N*-octyl-3-one adduct.

1-Octen-3-one has been reported as the main compound responsible for the typical metallic odor when metals or blood touch the skin (Glindemann et al., 2006). The compound is formed as a degradative reduction product in the reaction of skin lipid peroxides with ferrous ions (Fe^{2+}) formed in the sweat-mediated corrosion of iron. It is likely that 1-octen-3-one is also formed within the body, from reactions of lipid peroxides with Fe^{2+}. If this is the case, levels of 1-octen-3-one adducts could be related to lipid peroxidation.

It is suggested that the main exposure source of 1-octen-3-one is from endogenous decomposition of lipid peroxides. There are also exogenous exposure sources since the compound has been detected in different foods, e.g. dairy products (Hammond and Hill, 1964; Stark and Forss, 1962), mushrooms (Combet et al., 2006), cooked meat (Konopka et al., 1995), and fresh fish (Josephson et al., 1984). Oxidative degradation of arachidonic acid has been suggested as a pathway for the formation of 1-octen-3-one in food (Josephson et al., 1984; Kihara et al., 2014). To my knowledge there is no available information on the possible toxicity of 1-octen-3-one.

6.3. Conclusions regarding the identification of unknown adducts

A strategy for the identification of unknown adducts detected through adductomic screening was formulated and applied for the successful identification of five adducts. Some of the identified adducts had previously been observed by others using other methods, which add strength to results. The successful identification of unknown adducts as their FTH derivatives of Val adducts, further demonstrates the applicability of the FI*R*E procedure for

such approaches. The general strategy outlined here should be useful for future identification work.

7. Screening of adducts in larger sample sets (Paper IV and unpublished studies)

The studies so far described in this thesis concerned analysis of blood samples from a small number of individuals (smokers/nonsmokers, n=12, plus control and reference samples). To obtain a basis and statistical foundation for future studies and applications of the methodology, the approach has to be applied to larger sample sets. Therefore, monitoring of adducts by a targeted screening approach has been applied in pilot studies. In total, approximately 250 samples from three collaborative projects have been analyzed for both the known and the detected, unidentified adducts.

Our latest study is described in this thesis chapter (Ch. 7.1.) as well as in *Paper IV*. The overall aim of the study is to characterize dietary habits and associated exposure to unwanted substances in children of school age. Dietary habits were characterized, and blood samples collected to be analyzed for different biomarkers/metabolites. In our part of the study Hb adduct levels and the frequency of micronuclei (fMN), a marker of genotoxic effect, were measured.

In two other collaborative studies unidentified adducts were studied. One of these studies concerned effects of dietary intake of antioxidantia in intervention studies of smokers, with repetitive sampling, in about 20 individuals with monitoring of adducts related to tobacco smoking. The other concerned the functional role of CYP2E1 polymorphism in the metabolism of AA, studied in cancer cases and paired controls (n=120). In both these studies screening of the known and the detected unidentified Hb adducts was also performed. The results from those studies are unpublished and so far not fully evaluated. The two studies are discussed in Ch. 7.2. and 7.3., with focus on the Hb modifications.

7.1. Studies of exposure in children of school age

The sample material discussed in the following sections represent a collaborative study with the Swedish National Food Agency of school-age children. Out of 300 individuals in the SLV study 51 were selected, based on previously measured fMN, with fMN values ranging from the lowest to the highest within the cohort. The objectives in our study of this group were: to screen for unidentified Hb adducts to N-terminal valine by Orbitrap MS in a smaller number of the samples; screen for the earlier detected identified and unidentified Hb adducts in all samples and assess adduct levels; to evaluate possible association between adduct levels and measured genotoxic effect (fMN).

7.1.1. Adductomic screening using Orbitrap MS

Adductomic screening by HRMS was performed prior to the targeted analysis. The aim was to observe additional adducts to N-terminal Val in Hb in an extended m/z range (500 – 700 m/z, compared to the previous range 503 – 638 m/z), as well as to confirm the presence of the previously observed adducts in this set of samples from school children. The screening was done using an Orbitrap MS (Orbitrap Q Exactive HF) in the data independent acquisition (DIA) mode, with subsequent follow-up experiments in the parallel reaction monitoring (PRM) mode. The advantages of HRMS and Orbitrap instruments, compared to triple quadrupole MS, is further discussed in Ch 8.1. Six samples from the total 51 blood samples were randomly selected for adductomic screening.

Besides the different modes of scanning, the main advantage with Orbitraps compared to triple quadrupoles is the high resolving power and accuracy of the measurements. Accurate masses are often helpful for identification purposes, since the elemental composition may be proposed by the instrument software. The high resolution data also provide a better foundation for qualification and disqualification of adduct candidates, compared to the unit-resolution data obtained with triple quadrupole instruments. The analytes with precursor ions m/z 520, 550, 561 (late retention time; rt 23.0 min in *Paper I*), 575, 580, 608, previously considered adduct derivatives after the original screening (*Paper I*) were considered less likely as true adducts following the Orbitrap experiments. This is because of inconsistent fragmentation, exhibiting fragments that are close to the diagnostic fragments in m/z but not accurate (e.g. m/z 446 compared to m/z 445, and m/z 459 compared to 460 m/z, as for the late-eluting analyte with $[M+H]^+$ m/z 561). Those analytes will not be prioritized for future identification work, compared to more probable candidates. They will though not be fully excluded from future considerations as adducts, since there might be exceptions from the expected fragmentation

patterns, as found for the adduct from methylglyoxal (cf. Ch 6.2.3. and *Paper III*).

During the Orbitrap screening five probable adducts, that had not been detected previously, were observed. The precursor ions ($[M+H]^+$) of the five analytes are: m/z 519 (rt 12.3 min), m/z 519 (rt 15.7 min), 651 (rt 12.3 min), 659 (rt 12.3 min), and 686 (rt 12.3 min). Those unidentified adducts were observed in the majority of the samples, in the subsequent targeted screening.

7.1.2. Targeted screening results

The 51 samples from the cohort of school-age children were analyzed using a targeted approach by LC/MS/MS in the MRM mode. For both the known and hitherto unidentified adducts previously observed the two most abundant fragments were monitored, and for the five so far unidentified modifications observed in the previous Orbtitrap experiment (cf. Ch. 7.1.1.) three fragments were monitored to strengthen their detection.

The same semi-quantitative approach as used previously was used to obtain estimates of adduct levels (cf. Ch. 5.3.). Considerations about the semi-quantitative approach are discussed in Ch. 8.3. The estimated adduct levels are presented in Table 1 in *Paper IV*.

Large variations in adduct levels were observed within the group of studied samples. Comparing the lowest and highest levels in the 51 samples, the levels of most of the determined adducts vary more than by a factor ten. This is illustrated in an adductome map format were the lowest and highest adduct levels are compared for all adducts measured in the study, in all subjects (**Figure 23**).

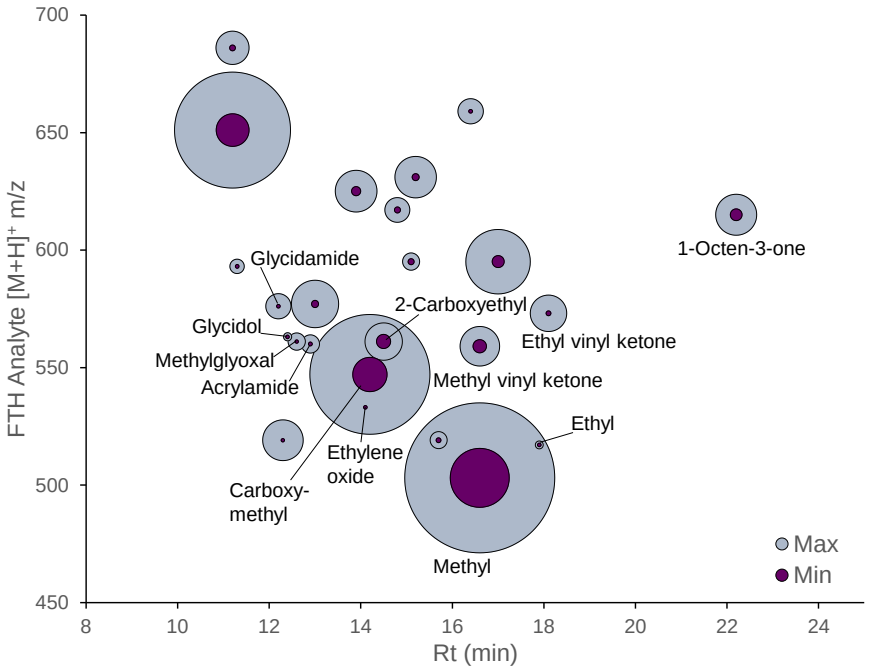

Figure 23. Adductome map illustrating the range of levels of the measured adducts (purple corresponds to the lowest measured level, blue to the highest). The range of estimated adduct levels range from approx. 5 – 3700 pmol/g Hb. Identified adducts/precursors observed in the samples are written out in the figure.

That the majority of the adducts (both identified and unidentified), that previously had been observed, were detected in all analyzed samples in this targeted screening study, is a valuable observation that confirms and strengthen the previous observations. What the large variations in adduct levels mean in the context of exposure and metabolism is so far unknown. After identifications and proposals of exposure sources focused targeted studies may be performed, in order to study the relevance of specific Hb modifications.

7.1.3. Association between measured Hb adduct levels and genotoxic effect

The measurement of micronuclei (MN) is an established end-point for genotoxicity, and one of the most sensitive for in vivo studies. If there is a break of the chromosome/chromatide during mitosis it might result in malsegregation of chromosomes/chromatides. This will result in the formation of a micronucleus, containing a piece of or a whole chromosome. For instance, after an exposure of the bone marrow to a genotoxic agent, an increase of the frequency of micronuclei (fMN) could be observed in young erythrocytes in

the peripheral blood. A highly sensitive method for measurement of fMN in humans, based on restriction of the measurement to the very youngest erythrocytes and analysis by a flow cytometer (Abramsson-Zetterberg et al., 2000), was applied in this collaborative study with the Sw. National Food Agency. The fMN reflects the exposure a few days prior to the sampling of blood (Abramsson-Zetterberg et al., 2008; Grawé et al., 2005).

Multivariate data analysis (partial least squares regression) was performed to compare the estimated adduct levels with the determined fMN in the 51 samples. The analysis showed that the adduct levels significantly correlated to the fMN, and that 40% of the fMN variation could be explained by the adduct levels. This observation require further evaluation, but is promising for future studies concering comparisons of adduct levels and fMN. Whereas fMN provides a measure of genotoxicity, individual adducts probably have a very small contribution to the observed genotoxic effect. However, when considering many adducts collectively a stronger correlation could possibly be observed, as in this study. Both known and unidentified adducts may reflect various processes influencing genotoxicity, even if they are not genotoxic per se. The significance of the totality of adducts observed in blood samples from individuals is a topic deserving future investigations.

7.2. Intervention studies with dietary antioxidantia in smokers

The first study in which targeted screening of known and unidentified adducts to N-terminal Val in Hb was applied was a collaborative study included in the EU project Funcfood (short for "functional food"; website: www.funcfood.eu). The study concerned the effects of dietary antioxidantia (lycopene, chlorophyllin, astaxanthin, gentiana and blueberry) in smokers during an intervention period.[3] Dietary intervention studies were performed for about 20 participants. Blood samples were taken repeatedly before and after intervention, wash-out period, or placebo period, every third weeks. Ethicalapproval was obtained from the Regional Ethical Review Board in Stockholm.

MN frequencies in peripheral blood reticulocytes were measured to determine genotoxic effect. The levels of Hb modifications from AA, GA, EO, and acrylonitrile (AN) were measured (with the FI*R*E procedure) primarily as a measure of the participants' smoking habits during the study (these compounds are known to be related to tobacco smoking, cf. e.g. Bergmark,

[3] This study was a collaboration with C. Frostne, D. Vare, M. Törnqvist and D. Jenssen (to be published).

1997). A further aim was to study whether adduct levels were affected by the intake of antioxidantia. As an additional aim, the same samples were analyzed for the previously detected unidentified adducts (from *Paper I*) using the same targeted screening approach as described above (Ch. 7.1.2.). From these results it was clear that the majority of those adducts were present in all analyzed samples (n=95, from 27 individuals, including nonsmoker controls).

From the results it could be concluded that the levels of the measured adducts (from AA, GA, EO and AN) are suitable to monitor smoking habits, showing relatively stable adduct levels for the individual participants over the study. There was no clear influence by the antioxidantia on the levels of any of the known adducts (evaluation not fully completed).

From the screening, to measure the previously detected unidentified adducts (from *Paper I*) it was clear that the majority of those were present in all analyzed samples (n=95, from 27 individuals, including nonsmoker controls). This was the first targeted screening of the unidentified adducts following their detection in the adductomic screening described in *Paper I*. The observation of the modifications in this much larger sample set confirmed the previous observations and provided information about variations in levels. There was no clearly observed influence by the antioxidantia on the levels of any of the studied adducts (not completely evaluated). The results from analysis of samples from one individual taking part in several of the interventions are shown in **Figure 25**. The levels over time of adducts from AA, GA, EO, and AN, monitored to measure smoking habits of this individual are shown in **Figure 25A** The estimated levels of the six adducts identified during this thesis project, are shown in **Figure 25B**. The cause for the variability of those six adducts are so far not known. This study will be further evaluated.

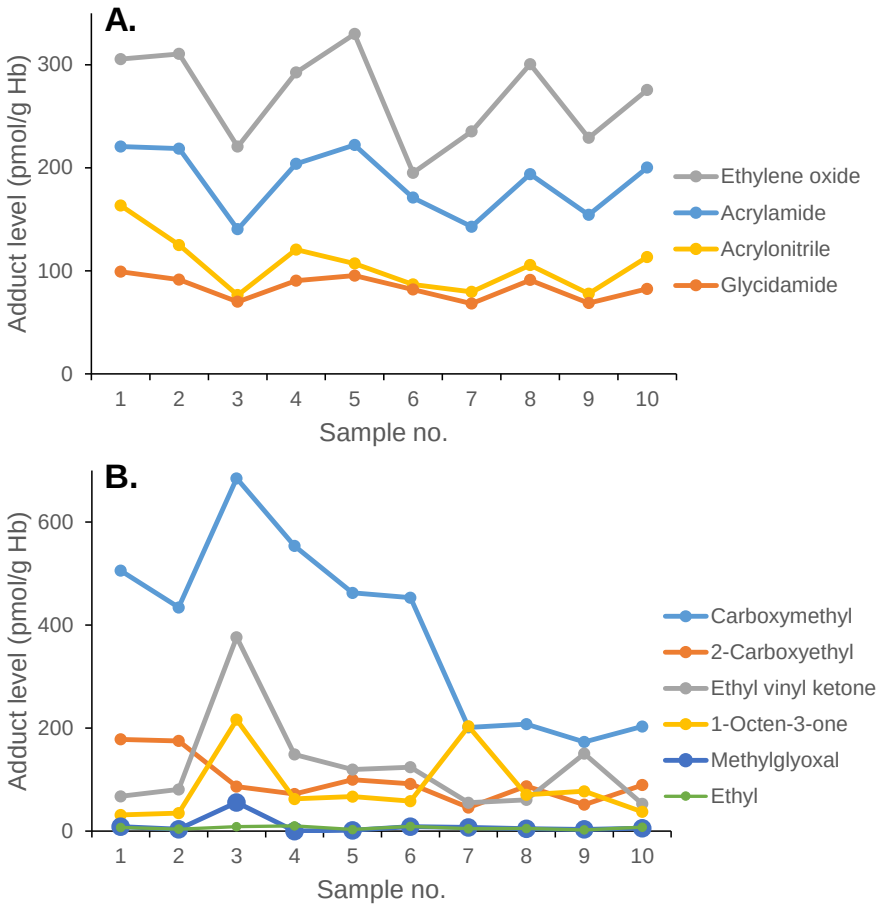

Figure 25. The variability in adduct levels over time for one individual (smoker). Samples were taken with about three-week intervals (including different interventions of antioxidantia). The four adducts used to measure smoking habits are shown in A. The six adducts identified during this thesis project are shown in B (carboxymethyl and 2-carboxyethyl correspond to the adducts formed from glyoxal and acrylic acid, respectively).

As no clear difference in adduct levels was observed with regard to the intake of antioxidantia, the levels for each individual adduct could be used to get an idea about the variation over the time of the study. Such information is valuable in the evaluation of adducts, and their corresponding exposures/ internal doses, over time (cf. (Vikström et al., 2012)). In studies of the exposome, the exposure over time of various chemicals will be an important parameter to assess (Wild, 2012).

7.3. CYP2E1 polymorphism and adduct levels

The second study in which targeted screening of known and unknown adducts was applied was a collaborative study with the research group of Stefano Landi at the University of Pisa, Italy. In this study, blood samples from patients with differentiated thyroid carcinoma (DTC) and paired healthy controls were studied. The research group had previously observed that a common mutation, a single nucleotide polymorphism (SNP), in the *CYP2E1* gene, was associated with the risk of DTC (Pellé et al., 2016b). The encoded protein is a cytochrome P450 enzyme, a class of enzymes important for the metabolism of xenobiotics. It was found that the variant allele was associated with reduced activity of this enzyme. A large portion of AA is metabolized to the carcinogenic GA, by CYP2E1 and thus AA was considered a suitable substrate for investigation of the functional role of the observed SNP. Furthermore, it has been discussed whether AA exposure could be associated with thyroid cancer (cf. Pellé et al., 2016b).

Adduct levels of AA and GA were measured in blood samples from 60 DTC patients and 60 paired healthy controls. Within each group, half of the samples were from patients with the mutant allele and the other half with the wild-type genotype. The ratio between the levels of the modifications from GA and AA was calculated as an index of metabolic rate of biotransformation of AA. These ratios were compared between the groups by statistical analysis (e.g. by multifactor analysis of variance), and a significant difference was observed between the two genotypes. The variant allele, observed to have lower CYP2E1 activity, showed a reduced rate of biotransformation of AA to GA. This observation was in agreement with the hypothesis of the study, as well as with previous observations (Doroshyenko et al., 2009; Huang et al., 2012). No significant statistical differences were observed between cases and controls. These results are under preparation of a manuscript (Pellé et al., 2016a).

For the same samples, known adducts other than AA and GA as well as unidentified adducts, were determined using the same targeted screening approach as described above (Ch. 7.1.2.). The majority of the earlier detected unidentified adducts were observed in all of the analyzed samples. Statistical analysis were performed to evaluate the estimated adduct levels in regard to the two genotypes and cases/controls, using the same statistical methods as for the AA and GA adducts. For some of the adducts some statistically significant differences have been indicated between the groups, but the results are not completely evaluated. Further adjustments and corrections may be required, prior to any conclusions are drawn (to be published). In general, no large differences in adduct levels were observed between the groups (cases/controls; genotypes).

The study was conducted over a period of about two months, with the 120 samples randomly divided in six groups with 20 samples in each. In order to ensure to quality of the data, various quality control experiments were implemented in the study. Triplicates of the same sample (from a smoker) were derivatized and analyzed for each sample series, demonstrating a between-day variation (expressed as coefficient of variation, CV) of 12% for AA and 14% for GA (n = 3). The within-day variation for a sample from a nonsmoker was 4% for both AA and GA (n=6). The within- and between-day variations observed in this study is in agreement with previous observations, when using specific internal standards (von Stedingk et al., 2011), and similar variabilities should be expected for hitherto unknown adducts, at least with good internal standards.

7.4. Conclusions regarding screening of adducts in larger sample sets

Targeted screening of previously detected adducts, known and unidentified, were performed in three pilot studies of human blood samples. The majority of the adducts were observed in all analyzed samples. Small variations in adduct levels, e.g. caused by differences in genotype, could be observed with the FI*R*E procedure. Differences in adduct levels between individuals can be observed with the method. Adduct screening in combination with other sensitive methods for studies of e.g. genotoxic effect and health status is a promising tool for detection of risk factors associated with electrophilic agents.

8. Discussion

In this chapter the state of adductomics today is discussed in connection to the work described in this thesis. Problems and obstacles necessary to overcome for the methodology to reach its full potential are discussed.

8.1. Advances in mass spectrometry for adductomics

Recently, high resolution MS (HRMS), which can be offered by Orbitrap instruments, has been used for adductomic experiments (Balbo et al., 2014; Chung et al., 2014; Hemeryck et al., 2015). HRMS instruments have the ability to resolve isotopic peaks of ions and measure their masses with high accuracy (reviewed by e.g. Zubarev and Makarov, 2013). Accurate mass data can be used to propose elemental compositions of analytes, which may aid in the identification of analytes.

Orbitrap instruments offer classic modes of MS analysis such as full-scan and selected-ion monitoring (SIM), but also provide additional scanning modes to screen for hitherto unknowns compared to triple quadrupole instruments. Several different modes may be used in the same analysis. This mean that analytes, in principle, may be identified, quantified and confirmed in one single analysis.

Chung et al. performed adductomic screening of human serum albumin adducts using Orbitrap MS in the data-dependent acquisition mode, a full-scan experiment where the most abundant ions are selected for fragmentation by the instrument software (Chung et al., 2014). To avoid repeated fragmentation of the most abundant ions, and thereby risk to miss ions of lower abundance, exclusion criteria may be defined. Data-dependent acquisition is a good approach for analysis of clean samples that do not contain contaminants or interfering compounds at high concentrations. In the case of samples analyzed with the FI*R*E procedure, in which the concentrations of reagent by-products are high, it is however not a suitable approach.

To use the FI*R*E procedure and adapt the previously used MRM screening method for Orbitrap MS, the data independent acquisition (DIA) and parallel reaction monitoring modes (PRM) were used. Using DIA, all precursor ions

within specified m/z ranges are fragmented, regardless of abundance. For adductomic screening the m/z ranges should be relatively narrow; in the case of the present method the ranges covered 6 m/z, with ten consecutive ranges, in a single MS method. Using DIA for unknown screening requires several injections, to cover wide m/z ranges. Since the mass spectra recorded with DIA are combined from a range of precursor ions, PRM was used to confirm possible adduct candidates. PRM only fragments precursor ions of specific m/z (with unit resolution), and is suitable for targeted analysis. Details for the MS methods are given in *Paper IV* of this thesis.

Another useful Orbitrap experiment for adduct screening, which has been used for screening of DNA adducts (Balbo et al., 2014), is the data-dependent CNL scan, which triggers fragmentation (MS^2 or MS^3, depending on instrumentation) of compounds exhibiting the accurate mass CNL specified in the method. This is a good approach when all adduct analytes share a similar characteristic fragmentation pathway, as is the case for DNA adducts.

Nanospray ionization is another technical improvement that should improve the detection limit of many methods for adduct measurements (Balbo et al., 2014). Nanospray ionization utilizes lower flow rates compared to conventional electrospray, which leads to smaller droplets and usually increases the sensitivity of the analysis.

8.2. Qualitative adductomics: Identifying unknown adducts

The qualitative assessment, i.e. identification of unknowns, is the most important aspect of adductomics and also the most difficult. Even with accurate mass measurements and detailed fragmentation patterns there will likely be several possible hypothetical adduct identities. If the adduct analytes are well-separated in the chromatographic step the retention times (representing e.g. relative lipophilicity for reversed phase chromatography) can be used to discriminate between different candidate compounds. It is clear that several tools are needed in combination for successful identification of unknown adducts.

The work described in this thesis is unique in how adductome data were used to identify several unknown adducts. To the author's knowledge, the Rappaport group is the only other group that have reported identification of unknown adducts in connection to adductomic experiments. Using accurate mass data they identified several Cys34 adducts in HSA (Chung et al., 2014). These adducts were e.g. oxidation products, and none represented covalent

modifications from electrophiles. In comparison, several covalent modifications at relatively high concentrations have been observed, and identified, to the N-terminal in Hb in the present studies. Cys34 in HSA and N-terminal Hb are complimentary, and an adductomic approach that use both methods, for analysis of the same samples, could be useful to assess broader ranges of adducts. The identification of unknown adducts will be strengthened if the same adducts can be detected using several methods, which further motivates the implementation of different adductomic methodologies. When aiming to assess the whole adductome, several methods covering different nucleophilic sites over several biomolecules, will be needed.

For identification purposes, suggestions of probable exposure sources are of great importance. In general, a specific adduct could not be considered as reliably identified until a precursor and a probable exposure source/origin has been proposed, and has been related to the adduct quantitatively. The proposal of a source/origin increases the credibility of the suggested adduct, as well as its degree of priority in future studies. In this context there should always be an awareness of the risk that particularly low molecular weight adducts could be formed as artefacts, for instance during storage (Törnqvist et al., 1988) and work-up (Koc and Swenberg, 2002). When measuring adducts one should keep in mind the low levels studied (about 1 per $10^6 – 10^9$). Stored samples should be analyzed in comparison with fresh samples, before proposing adduct candidates.

The work described in this thesis, exemplifies a work-flow in the early qualitative assessment of hitherto unidentified adducts, tracking precursors and exposure sources. This has successfully led to the identification of six adducts, of which four (corresponding to acrylic acid, ethylation, ethyl vinyl ketone, and 1-octen-3-one) have not previously been reported as Hb adducts.

8.3. Quantitative adductomics

Ideally, the data generated from adductomic experiments should be useful both for qualitative and quantitative assessments. The quantitative assessment of unidentified compounds is always problematic. As a rule, accurate quantification can never be accomplished without synthetic standards. Adductomic assays give quantitative information in the form of chromatographic peak areas. Since those in general are proportional to the concentrations of the corresponding compounds there is often a wish to express the relative concentrations in a format more relevant than areas to the reader. A common assumption, in published adductomic studies, is that all adducts of the same type will exhibit the same response in the MS analysis.

In their work with DNA adductomics, Kanaly et al. assumed that all DNA adducts would have similar responses when monitoring the loss of 2'-deoxyribose in MRM mode (see e.g. (Kanaly et al., 2007, 2006)). As a basis for adductome maps, the peak areas for the observed unknown DNA adducts were normalized by the peak area of the internal standard (dideoxyinosine). The normalized peak areas were referred to as "area responses", and used as a basis for discussions on relative abundance of adducts. This was a good approach since it clarified that the measurements were too inaccurate to allow any levels to be estimated.

If all adducts can be assumed to exhibit the same response, their levels may also be estimated. Such estimates will never be accurate, but is the only option if there is a wish to compare and communicate levels of unidentified modifications. For researchers within the field, an estimated adduct level will be more relevant, instead of using peak areas, for comparisons with other, known adducts. Estimated levels of unidentified adducts should be considered as a basis for future, accurate quantitative work, once the adducts have been identified.

In their work with HSA Cys34 adductomics, Li et al. assumed that all tryptic peptides containing Cys34 adducts would exhibit the same response when monitoring the same fragment in MRM (Li et al., 2011). The quantification was done by comparing the peak areas of different suggested adducts with the peak area of the internal standard (corresponding to a carboxyamidomethyl modification), and the determined levels reported in pmol/mg HSA.

During this project a semi-quantitative approach similar to the one described above was used. The approach, described in Ch. 5.3., is based on the assumption that the response in the MS analysis will be approximately the same for all FTH derivatives of Val adducts. The average peak area of the observed fragments is then used for semi-quantification using the calibration curve of AA-Val-FTH/AA-d_7-Val-FTH. The assumption that all adduct derivatives exhibit the same MS response is considered a reasonable approximation, especially for the low molecular weight adducts included in the studied m/z range. It should however be remarked that large biases may occur when assuming that unidentified compounds of the same general structure exhibit similar fragmentation. For the determination of adducts according to the FI*R*E procedure, when using triple quadrupole MS, no other mode of analysis than MRM is however suitable, considering detectability and ability to resolve analytes from contaminants and reagent by-products. The usage of the average peak area for the semi-quantitative assessment can be debated, but was chosen to be the best option at this stage, since that implies that all available data are used. Another approach could have been to use the

most intense fragment, but the relative intensity of that varies more than the average fragment when comparing adducts (cf. Table 1 in *Paper I*). The semi-quantitative method was later confirmed to be a good approximate approach for estimating levels of EVK adducts, when the results from the screening were compared with the later quantification using a synthesized reference standard, giving values in the same range (*Paper II*).

When performing targeted screening in the MRM mode, several fragments should be monitored for so far unidentified adducts or adducts lacking internal standards, in order to confirm that the correct analytes are being assessed. In the targeted screening studies described in this thesis (*Paper IV*) two intense and characteristic fragments were used for each adduct analyte. The average peak area of these fragments was then used for semi-quantification.

To achieve the best possible results for semi-quantitative determination of adducts levels in the MRM mode, I suggest the following sequence of experiments. Following detection of suggested adducts during adductomic experiments, the MS/MS spectra of those should be collected (requires analytes of relatively high concentrations, and may not be applicable to low-level adducts). The fragmentation patterns should then be compared with the fragmentation pattern of the reference analyte constituting the calibration curve, recorded using the same parameters. Fragments of comparable relative abundance as the fragments used for quantification of the reference adduct should then be selected for semi-quantification, if possible. Furthermore, ideally several calibration curves and internal standards could be used, spanning over a range of modifications of different mass and lipophilicity, representing different classes of electrophiles.

The detection limit of the FI*R*E procedure could still be improved, as there is a relatively high signal interference from reagent by-products. Some background adducts at low levels that have previously been observed with the modified Edman method for GC/MS/MS, such as modifications from propylene oxide (Törnqvist and Kautiainen, 1993), have so far not been detected with the FI*R*E procedure. If the LOD could be decreased a large number of additional adducts with adduct levels below a few pmol/g Hb could be observed. So far there are no practical suggestions on how to decrease the LOD, while still keeping the procedure fast and simple.

8.4. Evaluation of adductomic data

Besides the difficulties with identification of previously unknown adducts, the most severe bottleneck for adductomic approaches is the time-consuming and complicated evaluation of experiments and data. Theoretically, adductomics

is a great tool to compare adduct patterns for instance when studying exposures in different populations, or when studying large groups of patients to search for biomolecule modifications significant for health status. However, to my knowledge, there is no commercially available software that allows for straight-forward evaluation of untargeted adductomic experiments.

The qualitative and quantitative evaluation of chromatograms from adductomic experiments is in general very time-consuming, demanding a large extent of manual adjustments and repeated evaluation. Those difficulties are clearly reflected by the fact that most adductomic studies to-date only involve a very limited number of samples (cf. **Table 1**). For this field to take the step from method-development to larger-scale applications, a development of powerful software to greatly reduce the time of sample evaluation is required. At the present stage untargeted adductomic studies concerning large number of samples are not realistically feasible. In cases where unidentified adducts have previously been detected in a smaller number of samples, targeted screening in large populations is a more realistic approach.

9. Future perspectives

It is now ten years since the adductome concept was introduced by Kanaly et al. (Kanaly et al., 2006). Since then, the potential of adductomic approaches has been clearly demonstrated. Although a small field, several important publications have set the stage for adductomics. During this early phase much focus has been on method development. For several research groups with previous experience in targeted adduct determination, it has been relatively straight-forward to adapt previously developed methodology towards untargeted screening. The potential and capability of those methods have been demonstrated, but so far there have been few studies published involving large sample series (cf. **Table 1**). For adductomics to reach its full potential, it is necessary that the methodology is applied on a larger scale, and that continued efforts are done to identify adducts and track their probable exposure sources.

Once an adductomic method has been set-up, it is relatively straight-forward to observe large numbers of possible adducts. The subsequent identification of those suggested adducts is often a greater challenge than their observation. Few research groups have attempted to identify adducts in their publications on adductomics. To my knowledge only the Rappaport group have attempted identification (Chung et al., 2014), besides the work described in this thesis (Ch. 6, *Papers II* and *III*).

One of the major driving forces in the formulation of the adductome concept was the prospect to use it for the discovery of possible biomarkers for exposure and/or health status. Such studies would involve large scale studies were adductome profiles would be compared between populations of interest, e.g. case/control studies. By comparing adductome profiles, adducts of significance should ideally be possible to pinpoint. Due to the large inter-individual variabilities in adduct levels, large number of samples would in most cases be needed to observe significant differences between populations. This set a practical limit to such experiments, since no computer software available today allows convenient evaluation of untargeted adductomic experiments.

At the present stage, the most realistic application of adductomics is to screen a small sample series for possible adducts. With a good selection of samples, relatively few samples may be used to obtain a good foundation for

future studies and identifications of the adducts. Following the proposal of adducts in a small sample series, targeted screening approaches may be applied for the determination of those in larger sample series. Adductomic methods should be developed to obtain results which facilitates the identification of suggested adducts. HRMS in combination with MS/MS should be combined with LC methods giving good chromatographic separations. The inclusion of reference compounds, either present in the samples or added as standards, may facilitate the formulation of hypotheses on adduct identities based on comparisons of retention times (cf. 6.1. and *Paper III*).

The work described in this thesis has given some frames for future adductomic studies of N-terminal Hb adducts. Of the unknown adducts observed in *Papers I* and *IV*, many remain to be identified. In general, each subsequent identification is expected to be more complex than the last, and different approaches will be necessary for successful identification. With a decreased LOD of the FI*R*E procedure, several additional adducts not possible to observe today should be observed. Efforts should be made to improve the analytical procedure, to achieve cleaner samples. There may also be other methods that could be investigated for the screening of Hb adducts, for instance using tryptic digests of globin, to broaden the applicability to ring-closed adducts at the N-terminal or adducts to other sites within the protein.

Goel et al. developed an alternative approach that could potentially be used for adductomic screening of N-terminal Hb adducts (Goel et al., 2013). The method was based on a previously established immunoaffinity liquid chromatography method for the enrichment of adduct-containing tryptic peptides from Hb (Boysen et al., 2004). For a general procedure for enrichment of the tryptic peptide containing the alkylated Hb N-terminal, two different immunoaffinity columns were used in sequence. The first column was prepared with antibodies raised against the N-terminal of the unmodified peptide to allow depletion of those, the second column was prepared with antibodies raised against the C-terminal to retain modified peptides. The applicability of the method was demonstrated with the enrichment of a few in vitro generated peptides with modifications from low molecular weight alkylating agents. Measurements were done by LC/MS/MS in the MRM mode. So far, the method has only been applied for measurements of adducts at high levels, following acute exposure of mice. One advantage with this method is that adducts to N-terminals, that would not be detached by Edman reagents, could also be detected.

To assess the whole adductome, different methodologies and approaches are needed in conjugation. By screening several nucleophilic targets a larger range of adducts will be observed. There are presently suitable methods

available to screen for adducts to DNA, Cys34 in HSA, and N-terminal Val in Hb. These methods are complementary and it would be valuable to see the outcome if the samples from the same individuals were to be analyzed using all three methodologies.

The previous identification of background adducts, for instance from AA (Tareke et al., 2002), has shown the large range of studies that possibly follow upon identification. To assess the possible contribution to health risks from the identified precursor electrophiles, studies of toxicokinetics and genotoxic potency should be relevant.

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11. Summary in Swedish

Människor och djur är konstant exponerade för kemiska föreningar. Exponeringskällor kan vara kemiska ämnen i exempelvis mat och dryck, cigarettrök och luftföroreningar m.m. (yttre miljö) eller kemiska ämnen som bildas naturligt i kroppen i olika cellulära processer (inre miljö). En individs exponering under hela livet definieras som exposomet. Reaktiva föreningar anses vara en viktig del av denna totala exponering. Om dessa föreningar reagerar med biomolekyler som DNA och proteiner m.fl. kan stabila reaktionsprodukter bildas, s.k. addukter, vilka kan orsaka toxiska effekter. Exempelvis kan icke- reparerade addukter till DNA orsaka mutationer, vilka kan leda till cancer. En stor andel av de reaktiva föreningar människor exponeras för antas vara okända.

Under senare år har det visats att icke-genetiska faktorer (d.v.s. miljö-relaterade) bidrar till en större andel av cancer och andra kroniska sjukdomar jämfört med genetiska faktorer. Detta har ökat efterfrågan på metoder som förutsättningslöst ger möjlighet att identifiera och mäta exponering från kemiska föreningar. Konceptet adductomics innebär metodik som möjliggör studier av samtliga addukter till en viss position i en biomolekyl, genom screening med masspektrometri (en analytisk teknik).

Människans exponering för reaktiva föreningar mäts lämpligast i blodprover. Reaktiva föreningar är dock, i sin fria form, mycket svåra att bestämma i blod, p.g.a. kort halveringstid. För att mäta halten av sådana föreningar i blod och vävnader kan istället de stabila reaktionsprodukter, d.v.s. addukter, som bildas med biomolekyler mätas. Sedan 1970-talet har addukter från olika elektrofilt reaktiva föreningar, till olika biomolekyler, använts för att bland annat uppskatta exponering och den interna dosen i kroppen av dessa ämnen. Ett känt exempel är hemoglobinaddukter från akrylamid, en elektrofil förening som bildas vid upphettningen av kolhydratrik föda. De flesta hittills utvecklade metoder för adduktmätning har varit specifikt inriktade på en eller ett fåtal reaktiva föreningar. Därför har de flesta studier varit inriktade på att studera enskilda addukter.

Detta avhandlingsarbete behandlar utvecklingen och tillämpningen av en adductomics-metod för att detektera och identifiera tidigare okända addukter till proteinet hemoglobin i blodprover från människor. Addukterna som

studerats är kovalent bundna till en ändgrupp, den N-terminala aminosyran valin, i hemoglobin. Tekniken som huvudsakligen har använts är vätskekromatografi kopplat till masspektrometri (LC/MS/MS).

Avhandlingen består av tre publikationer (*Paper I - III*) och ett icke-publicerat manuskript (*Paper IV*). *Paper I* berör utvecklingen av en metod för att möjliggöra screening av addukter till hemoglobin. För att åstadkomma detta modifierades en tidigare etablerad metodik, tidigare tillämpad för bestämning av ett fåtal specifika addukter. Analysen sker genom separation av de olika föreningarna i proverna, främst baserat på deras fettlöslighet, genom vätskekromatografi, följt av masspektrometrisk analys där föreningarna detekteras utifrån förhållandet mellan molekylvikt och laddning. För att öka känsligheten och specificiteten i analysen fragmenteras föreningarna i en kollisionscell, i kollision med kvävgas, före detektionen. I studier av tidigare kända addukter karaktäriserades flera gemensamma egenskaper i den masspektrometriska analysen. Dessa egenskaper antogs vara gemensamma för alla tänkbara addukter, inräknat okända föreningar, vilket utgjorde den teoretiska grunden för screeningmetoden. Screeningen utfördes på tolv blodprover från människa och utöver sju tidigare kända addukter detekterades 19 okända addukter. Resultaten av screeningen ger information om de okända addukternas molekylvikt och relativa fettlöslighet (genom kromatografisk separation), vilket är användbart för identifiering. Analysen har genomförts så att också ett kvantitativt mått på nivåerna av de olika addukterna erhålls.

Paper II och *III* berör identifiering av några av de okända addukter som detekterades i screeningen. För identifiering av dessa addukter formulerades hypoteser rörande deras identiteter genom att jämföra föreningarnas egenskaper med tidigare kända addukter, samt utnyttjande av databaser och enklare kemiska beräkningar. Hypoteserna testades sedan genom att generera de föreslagna addukterna för att kunna jämföra med de okända addukterna. Hittills har fem addukter identifierats och deras sannolika ursprung spårats. Dessa addukter bildas från de elektrofila föreningarna etylvinylketon (*Paper II*), glyoxal, metyglyoxal, 1-okten-3-on och akrylsyra (*Paper III*). Av dessa har addukterna från etylvinylketon och 1-okten-3-on inte rapporterats tidigare, medan övriga tre addukter tidigare har rapporterats som proteinaddukter eller addukter till DNA. Viktigt att poängtera är att observationen av dessa addukter inte säger något om eventuella toxiska effekter, utan snarare bör ses som en motivation för vidare studier där eventuella bidrag till hälsorisker kan studeras närmare.

För att få ett rikare statistiskt underlag för vidare studier och bekräfta de okända addukter som observerades i den ursprungliga screeningen, har flertalet studier genomförts där ett större antal blodprover har analyserats med

avseende på tidigare detekterade kända och okända addukter. En av dessa studier är inkluderad i avhandlingen (*Paper IV*). Studien genomfördes på blodprov från skolbarn i samarbete med Livsmedelsverket och blodprov från 51 individer analyserades. Syftet var att studera skolbarns kostvanor och eventuell exponering för relaterade toxiska ämnen. Majoriteten av de addukter som tidigare observerats, detekterades i samtliga prover, med kraftigt varierande halter mellan individer.

Med hjälp av screeningtekniken har kunskapen om bredden av elektrofila ämnen som kan detekteras som hemoglobinaddukter i blodprover från människa ökat och uppslagen till fortsatt forskning är många. Flertalet av de addukter som detekterats inom detta arbete återstår att identifiera. Efter identifieringen uppstår nya frågeställningar, framförallt för att utreda eventuella hälsorisker från exponering för de adduktbildande föreningarna, vilket kräver vidare studier. Forskningsområdet adductomics är i en tidig fas och även om flera metoder för screening av addukter till olika biomolekyler har publicerats har de flesta studier hittills handlat om metodutveckling för detektion av okända addukter. Detta avhandlingsarbete är ett av få arbeten inom adductomics där flera av de detekterade okända addukterna har identifierats och metoden tillämpats på ett större antal individer.

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