Dried Blood Spots in Liquid Chromatography Mass Spectrometry-based Protein Analysis

Thesis for the degree Philosophiae Doctor

by

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Oslo, September 2017

Cecilie Rosting

LIST OF PAPERS

This thesis is based on the following papers which will be referred to by their roman numeral in the text:

I **Rosting, C.**; Gjelstad, A.; Halvorsen, T.G., Water-Soluble Dried Blood Spot in Protein Analysis: A Proof-of-Concept Study. *Anal Chem* **2015**, 87 (15), 7918-7924 doi: 10.1021/acs.analchem.5b01735

II **Rosting, C.**; Sae, C.O.; Gjelstad, A.; Halvorsen, T.G., Evaluation of water-soluble DBS for small proteins: a conceptual study using insulin as model analyte. *Bioanalysis* **2016**, 8 (10), 1051-65 doi: 10.4155/bio-2016-0002

III **Rosting, C.**; Gjelstad, A.; Halvorsen, T.G., Expanding the knowledge on dried blood spots and LC-MS-based protein analysis: two different sampling materials and six protein targets. *Anal Bioanal chem* **2017**, 409 (13), 3383-3392 doi**:** 10.1007/s00216-017-0280-3

IV **Rosting, C.**; Tran, E.V.; Gjelstad, A.; Halvorsen, T.G., Determination of the low-abundant protein biomarker hCG from dried matrix spots using immuocapture and nano liquid chromatography mass spectrometry. Manuscript submitted to *J Chromatogr B.*

V Andersen, I.K.L.; **Rosting, C.**; Gjelstad, A.; Halvorsen, T.G., The spotlight on volumetric absorptive microsampling vs. other sampling materials in LC-MS-based protein analysis. Manuscript submitted to *Bioanalysis*.

VI **Rosting, C.**; Yu, J.; Cooper, H.J., High field asymmetric waveform ion mobility spectrometry in non-targeted bottom-up proteomics of dried blood spots. Manuscript in preparation for *Mol Cell Proteomics*.

Papers not included in the dissertation:

- **Rosting, C.**; Pedersen-Bjergaard, S.; Hansen, S.H.; Janfelt, C., High-throughput analysis of drugs in biological fluids by desorption electrospray ionization mass spectrometry coupled with thin liquid membrane extraction, *The Analyst*, **2013**, 138 (20), 5965-72 doi: 10.1039/c3an00544e

- Skjervoe, O.; **Rosting, C.**; Halvorsen, T.G.; Reubsaet, L., Instant on-paper protein digestion during blood sampling, *The Analyst*, **2017** doi: 10.1039/C7AN01075C

ABBREVATIONS

AA	Amino acids
ABC	Ammonium bicarbonate
CID	Collision induced dissociation
CMC	Carboxymethyl cellulose
CV	Compensational voltage
DBS	Dried Blood Spots
DDA	Data Dependant Acquisition
DMS	Dried Matrix Spots
DSS	Dried Serum Spots
DV	Differential voltage
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FAIMS	High Field Asymmetric Waveform Ion Mobility Spectrometry
FDA	Food and Drug Administration
hCG	Human chorionic gonadotropin
hct	Hematocrit
HPLC	High-performance liquid chromatography
ID	Inner diameter
IS	Internal standard
IU	International units
kDa	Kilo Dalton
LC-MS	Liquid Chromatography Mass Spectrometry
LESA	Liquid extraction surface analysis
LOD	Limit of Detection
MeCN	Acetonitrile

SRM	Selected reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
Mw	Molecular weight
m/z	Mass-to-charge ratio
PBS	Phosphate buffer saline
p*I*	Isoelectric point
PrEST	Protein Epitope Signature Tag
PSAQ	Protein Standards for Absolute Quantification
PTM	Post-translational modification
QconCat	Quantification concatamer
R^2	Coefficient of determination
RT	Room temperature
RSD	Relative standard deviation
TDM	Therapeutic drug monitoring
QqQ	Triple quadrupole detector
SIL	Stable Isotope Labelled
SPE	Solid phase extraction
SDC	Sodium deoxycholate
S/N	Signal-to-noise
VAMS	Volumetric Absorptive MicroSampling
WADA	World Anti-Doping Agency

ABSTRACT

Dried Blood Spots (DBS) has been used in newborn screening for decades, but has also shown potential as a sampling technique in any remote sampling situation, and in fields such as therapeutic drug monitoring and in detection and quantification of disease markers. Small molecular analytes have been the main target in DBS analysis. However, larger biomolecules such as proteins have increasingly been evaluated as disease indicators or pharmaceutical products, and proteins are hence interesting to analyse from DBS samples. Different tools for protein analysis are available, but Mass Spectrometry (MS) has been thoroughly investigated for this purpose as it offers many advantages over the methods currently used in the clinical laboratories.

The aim of this thesis was to explore DBS for both targeted and non-targeted LC-MS-based protein analysis.

Paper I was a proof-of-concept paper showing analysis of human chorionic gonadotropin (hCG) from water-soluble DBS (carboxymethyl cellulose, CMC) and a pure cellulose-based material (DMPK-C). The samples were prepared by using immunoaffinity sample clean-up prior to targeted SRM analysis. The main focus was on the water-soluble material which showed complete recovery of analyte during the dissolvation step of the material, and comparable recovery with the pure cellulose-based material. Stability was acceptable for 45 days when samples were stored ≤ 8 °C, while degradation was observed for analytes in samples stored at room temperature (RT). Quantitative performance was acceptable with RSD ≤ 22 % and accuracy of 116 % (of true concentration). However, the estimated Limit of Detection (LOD, S/N=3) was approximately 20 times higher than previously obtained by using 1 mL of serum or urine samples.

Paper II demonstrated LC-MS analysis of small proteins from DBS by using insulin as a model compound. Recovery of insulin from the water-soluble material was estimated to be 68±4 %, showing that analyte was lost during preparation of the sample. Pure cellulose material (DMPK-C) showed however comparable recovery regarding signal intensity, but the noise level in chromatograms from DMPK-C samples was increased compared to the water-soluble material. This was probably due to the increased clean-up achieved in the water-soluble DBS samples as CMC had a promoting effect on the protein precipitation. Stability of insulin in DBS samples stored at RT was acceptable for at least seven days.

Paper III explored LC-MS analysis of six proteins with different physicochemical properties by using both water-soluble (CMC) material and pure cellulose-based sampling material (DMPK-C) for DBS. CMC showed no influence on the digest and precipitation, except for slightly higher standard deviation in samples where CMC was present (RSDs from 40-62 % for some of the signature peptides). Slightly decreased signals were observed for most signature peptides when samples were stored for four weeks compared to one week. However, acceptable stability was in general demonstrated for the proteins in DBS stored for one week on both CMC and DMPK-C (> 65 % signal compared to reference).

Paper IV demonstrated analysis of the protein biomarker hCG in endogenous reference levels from DBS by using immunoaffinity clean-up together with state-of-the-art nanoLC-MS/MS. LOD was determined from four different matrices (blood, plasma, serum and urine) and by using two different sampling materials: CMC (the water-soluble sampling material) and DMPK-C (the pure cellulose-based material). LOD from 2.1-10.5 IU/L was estimated, similar to the LODs obtained by using 1 mL serum or urine in previous published papers. Quantitative performance in pM levels was demonstrated from dried serum spots (DSS) by using both CMC and DMPK-C, and the linearity ($R^2 \geq 0.930$), precision (RSD: 13-31%) and accuracy (95-106%) were considered as acceptable for the low sample volumes used in the present project. The potential of Dried Serum Spots (DSS) for disease monitoring was also shown by applying the method for estimation of hCG concentration from a patient serum sample spotted on both CMC and DMPK-C (estimated hCG concentration from the spotted serum samples: ~ 5000 IU/L).

Paper V was a continuation of *Paper III* and key parameters for DBS, not evaluated in *Paper III*, were evaluated using the protein mix from the previous paper. A Volumetric Absorptive MicroSampling (VAMS) device was evaluated together with the water-soluble material (CMC) and the pure cellulose material (DMPK-C). Isotopically labelled internal standards were in addition available for the study. VAMS showed decreased recoveries (75-78 %) compared to DMPK-C for four of the proteins while CMC showed lower recovery (60 %) compared to DMPK-C for one of the proteins. Hematocrit effect was evaluated for DMPK-C and VAMS. Both materials seemed to be influenced by the hematocrit; however, VAMS was probably less influenced by volume bias caused by the hematocrit.

The potential of DBS for discovery proteomics studies (non-targeted) were shown in *Paper VI* by using High Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS) as an online-separation technique prior to MS detection. Two different procedures were used for sample preparation of the DBS samples: the *punch and elute* procedure and the liquid extraction surface analysis (LESA) procedure. Both sample preparations were fairly simple with a minimum amount of manual steps. The samples were analysed with LC-MS/MS or with LC-FAIMS-MS/MS (by changing the compensation voltage (CV) from -55 to -25 using the external stepping approach). The total number of detected proteins was increased by 50 % for the *punch and elute* prepared samples when analysed with FAIMS compared to without FAIMS, and by 45 % with FAIMS for the LESA samples. In total was 173 non-redundant proteins found by combining the data (from *punch and elute* sample) from three different CV steps (CV -55, -37.5 and -25). However, only eight proteins (< 5 %) were observed in all the tree CV steps and demonstrated the filtration properties of FAIMS. LC-FAIMS-MS/MS was also shown to work complementary with LC-MS/MS as 30 % of the proteins (found in total from *punch and elute* samples) was detected with both LC-FAIMS-MS/MS and LC-MS/MS.

1. INTRODUCTION

The increase in sensitivity and specificity of analytical instruments has enabled analysis of more complex analytes from low sample volumes. The work described in this thesis was related to use of microvolumes of sample (Dried Blood Spots, DBS) for analysis of proteins by advanced analytical tools (mass spectrometry, MS). The section *1.1 Dried Blood Spots* in the *Introduction* will describe DBS and aspects of DBS. Both immunoassays and MS-based protein analysis will then be discussed in section *1.2 Protein analysis*, including advanced tools for MS-based protein analysis. The last section of the *Introduction*, section *1.3 MS-based peptide and protein analysis of DBS*, will highlight available literature on MS-based peptide and protein analysis of DBS samples.

1.1 Dried Blood Spots

1.1.1 Background, benefits and challenges

DBS is a sampling and storage technique for whole blood where a few drops of blood are deposited from the finger or heel and onto a piece of paper. Other matrices can also be sampled and dried on paper (e.g. saliva, plasma, serum, urine and cerebrospinal fluid)[1-5], referred to as Dried Matrix Spots (DMS)[6]. The focus throughout the rest of the *Introduction* will be on DBS samples, but most of the aspects are also relevant for DMS samples.

The idea of sampling biological fluids on paper was proposed by Ivar Bang nearly a century ago[7,8], but was first introduced in 1963 by Guthrie for screening of phenylketonuria in newborns[9]. Most commercially available sampling materials for DBS are designed as cards. The patient's finger or heel is pricked and a few drops of capillary blood are deposited and dried onto these sampling cards prior to storage or shipping. The samples are normally extracted from the sampling card and into solution prior to analysis using a mix of buffers and organic solvents. The lack of volume control during sampling is mainly solved by punching out a fixed part (typically diameter of 3-6 mm) of the spot.

The interest in DBS has increased tremendously the latest years as shown in Figure 1.1, and several advantages are associated with using DBS over the conventional blood collection method, venepuncture[8,10]. The infection risk is generally low when sampling capillary blood, enabling blood sampling without assistance from a phlebotomist. Only a small volume of sample is required (typically $\leq$ 50 µL), which is beneficial in sampling from certain patient groups and associated with minimal blood waste. The blood samples

are directly applied on to the paper and the need for centrifuges at the sampling locations is therefore eliminated. The samples are easy to store and ship due to increased stability in dried samples and the minimal of space occupied by the sample cards. Storage and shipping of DBS is therefore related to reduced cost compared to liquid samples. Many viruses and bacteria are also inactivated upon dehydration, decreasing the biohazard related to handling DBS samples.

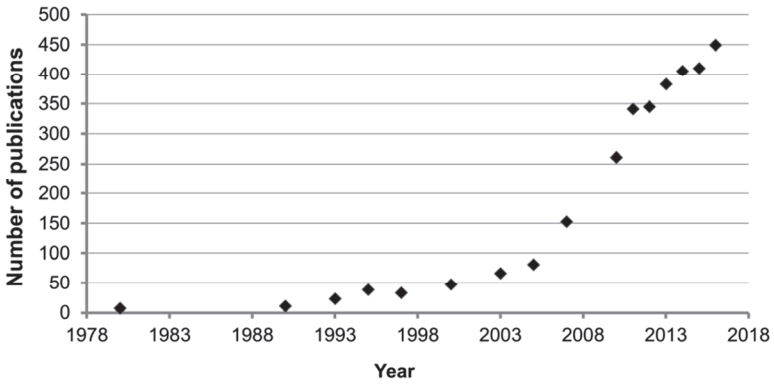


Figure 1.1: Number of publications that contain the term "Dried Blood Spot" from 1980-2016. *The data is obtained from a search in Thomson-Reuters Web of Science (21.08.2017).*

The advantages related to DBS could be beneficial in collection of biological samples in different fields. However, there are still challenges that need to be addressed before wide application of DBS is possible.

The sensitivity possible to obtain by using DBS samples is limited by the low volume of sample available, often difficult to preconcentrate or reanalyse[11]. Analysis of small sample volumes has partly been improved by introduction of more sensitive analytical instruments[12], but DBS analysis is still challenging in detection and quantification of trace amounts of analytes (where a high degree of preconcentration is necessary).

The DBS samples are normally transferred from the sampling material by punching out a part of the spot and eluting the sample with buffers or organic solvents. This introduces an additional preparation step, increasing both preparation time and the probability for additional variabilities[10,13,14]. Carry-over during the punch out of samples can lead to contamination hampering the quality of the analytical results, and cleaning of the puncher between every sample is often required. Another challenge is the incomplete elution of the sample together with the limited sample volumes available, which will be critical for the sensitivity. The efficiency of transferring analyte from the material (termed as the elution efficiency) can be influenced by factors such as the analyte affinity to the paper, the sample material and the preparation protocol used[15]. Additional time spent on evaluating and

optimizing the elution of sample from material and in solution is therefore expected. A few papers have described automatic and on-line elution of DBS to speed up the elution process[16-18], but most laboratories are still using the traditional "punch and elute" procedure.

The introduction of internal standard is another challenge widely discussed for DBS. The best analytical precision would be achieved by adding the internal standard together with the sample during deposition on the sampling material, but is impractical to accomplish in field. The current "best practice" for addition of internal standard for DBS is by adding the standard to the elution buffer[19]. Hence, variation in recoveries during the elution step of the sample is not corrected for. Different approaches to incorporate the internal standard for DBS have been discussed[20,21], but all approaches have either analytical or procedural drawbacks.

Some of the common benefits related to DBS have also been questioned, among those the ease of sampling and the increased storage stability. There are a number of important requirements for a successful DBS sampling and good-blood-spotting-practice has been described in several papers from The European Bioanalysis Forum[19,22]. Training or written guidance will be necessary to fulfil these requirements. The increased storage stability obtained by DBS has also in some studies shown to be dependent on both low temperatures and low humidity[23], and controlled conditions during storing and shipping will therefore be essential to circumvent degradation of certain analytes in DBS samples.

Several challenges associated with DBS are related to the use of whole blood instead of plasma or serum. The concentration of an analyte will depend on the affinity of the analyte to plasma versus red blood cells, and the analyte concentration will therefore depend on the matrix analysed. Plasma and serum have predominately been used as matrices in bioanalysis and new reference values will therefore be required for whole blood samples. The composition of capillary blood and venous blood is also different (e.g. due to intracellular and interstitial fluids) and caution must thus be taken when comparing venous blood samples and capillary blood samples[24].

The blood hematocrit is one of the main challenges related to analysis of DBS and is defined as the volume fraction of red blood cells in a blood sample. Reference values are typically between 0.35-0.50, but age and diseases can influence the amount of red blood

cells resulting in hematocrit values outside the reference range[25]. The amount of red blood cells reflects the viscosity of the blood as higher hematocrit (amount of red blood cells) increases the blood viscosity. The viscosity of the blood will thus be important in DBS sampling as it influences the spread of the sample on the sampling material, as illustrated in Figure 1.2. A punch out (of a part) from a DBS sample with low hematocrit blood will consequently contain a lower volume of sample than a punch out from a high hematocrit DBS sample, introducing bias in the quantitative analysis of DBS[26-30]. Alternative sampling materials have been introduced to solve problems related to hematocrit and will be further discussed in section *1.1.3 Sampling materials and sampling devices.* Prediction of the sample hematocrit through markers such as potassium, hemoglobin or zinc has also been suggested[25,31], but has not been widely adapted. The blood hematocrit has in addition been reported to influence the recovery during the elution process[32] and has been proposed to contribute to different degree of matrix effects[10]. The analyte concentration in a blood sample will also be influenced by the hematocrit, especially if the distribution of analyte is highly skewed to either blood cells or plasma[33]. It is important that this effect is fully understood before using DBS for prediction of plasma concentration[10,34].

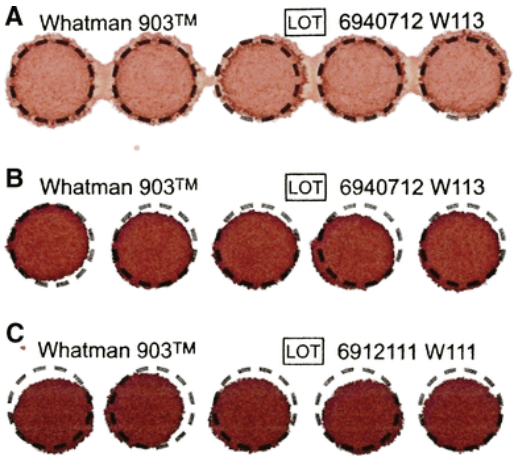


Figure 1.2: Spread of blood with different hematocrit (hct) on DBS sampling material Whatman 903™: *hct=0.18 (A), hct=0.35 (B) and hct=0.50 (C). Reprinted with permission from reference[46]. Copyright © (2014) Springer.*

Health authorities have by now not accepted DBS as a blood sampling technique for wide use in the bioanalytical field, and there is no official guidance available for handling of these samples. The acceptance criteria's for bioanalytical method validation, defined by health authorities such as the Food and Drug Administration (FDA)[35], will not be altered by using DBS samples instead of liquid samples. However, the experimental protocol used for DBS samples is different compared to the protocols used for liquid samples. The European Bioanalysis Forum has published several papers describing parameters which should be particularly monitored when using DBS[19,22], but standardized guidance will be essential for wide application of this sampling technique.

1.1.2 Selected application for DBS

Newborn screening

The primary use of DBS has been through the newborn screening program, and developments in instrumentations have been important for the expansion in the number of biomarkers included in the screening programs. Most countries are today screening between 20-50 diseases in newborns by using DBS samples[36-38].

Disease screening and monitoring

Although extensively used in newborn screening, DBS has also been demonstrated as a valuable sampling technique in any remote sampling situation as there is no need for phlebotomists during sampling or pre-treatment prior to storage. DBS has shown to be both a sensitive and specific method for sampling in HIV screening[39] and has been tested for in-field sampling in resource poor areas[40,41]. DBS has also been suggested as a useful technique in diagnosis of a range of other viral diseases, both due to the ease of sampling and storage, and due to reduced biohazard risk[42]. A patient's home is another example of a remote sampling area where DBS can be beneficial and DBS has shown to be a valuable technique in therapeutic drug monitoring (TDM)[43,44] for patient groups where frequent monitoring is required. The use of DBS for TDM is convenient for the patient themselves and will also reduce costs related to doctors or hospital visits[45-47]. DBS has also been suggested as an ideal sampling technique in large population-based research/epidemiological studies as it enables easy and economical collection of samples[34,48,49].

Doping analysis

Testing of substances listed on the World Anti-Doping Agency's (WADA's) prohibited list is required both in-competition and out-of-competition. Blood samples from athletes are typically obtained by venepuncture sampling and then archived for up to ten years[50]. DBS would be beneficial in sport doping control analysis as the samples can be taken without a phlebotomist and archived in small storage containers. DBS has so far not been implemented in routine testing, but the potential in this field has been demonstrated in several papers[51-55]. DBS has also shown to be useful in analysis of other banned substances, e.g. in monitoring of illegal drugs in drivers[56].

Pharmaceutical industry

The pharmaceutical industry has also shown interest for applying DBS sampling in research and development of new drugs. DBS as sampling technique can fulfil the three R's of animal welfare: reduce, refine and replace[50], and can also be feasible in sampling of blood from patients during pharmacokinetic study of new drugs. However, the use of DBS in the early-stage of a trial is questioned as controlled circumstances and accurate measurements of the drug are essential at this stage[11].

1.1.3 Sampling materials and sampling devices

Different cards and devices for sampling of microvolumes of biological matrices are either commercially available or have been described in the literature. The most frequently used DBS sampling materials are the Whatman® grade 903™ and the Ahlstrom grade 226 as these are the only sampling cards approved by FDA for clinical use[57,58]. These materials are made of pure cellulose, and are thoroughly tested for DBS sampling. Other cellulose sampling materials, only approved for research use, are also commercially available. Examples are the pure cellulose material FTA® DMPK-C and the impregnated cellulose sampling cards (FTA® DMPK-A and FTA® DMPK-B). The impregnated cards, which lyse cells and denature proteins, have shown to be beneficial for quenching the enzymatic activity in the blood samples. The chemical treated cards have also been related to increased background signals during the analysis[59]. Other commercially available cellulose-based cards are the indicating sampling cards (FTA® indicating cards) for clear/non-coloured samples (e.g. plasma, serum, and saliva)[60].

Several non-cellulose-based materials are also commercially available. The Bond Elute cards, made of glass fibres, have been claimed to reduce unspecific binding of analyte and hence be less influenced by hematocrit dependent recovery. However, this card has also (as other sampling materials) been related to concentration dependent recovery due to hematocrit[61]. Another example is Hemaform™: a fan shaped sampling material (patent pending) which is claimed to give less variability in the analyte recovery compared to traditional sampling cards. However, only a few published papers have demonstrated the use of this material[62,63].

Removal of red blood cells by membrane-based sampling cards has been described in several papers[64-66], and Noviplex® is a commercially available sampling card using this

concept and is shown in Figure 1.3. The card is built up by a blood deposition area, a spreading layer, membranes and a final plasma collection reservoir. The red blood cells are trapped in the membranes and a fixed volume of plasma is sampled onto the collection reservoir at the bottom of the membrane layers. Sampling with Noviplex circumvents challenges related to whole blood such as hematocrit and the need of converting blood concentrations to plasma concentrations. However, these sampling cards are expensive and only a few studies have so far applied these cards for DBS.

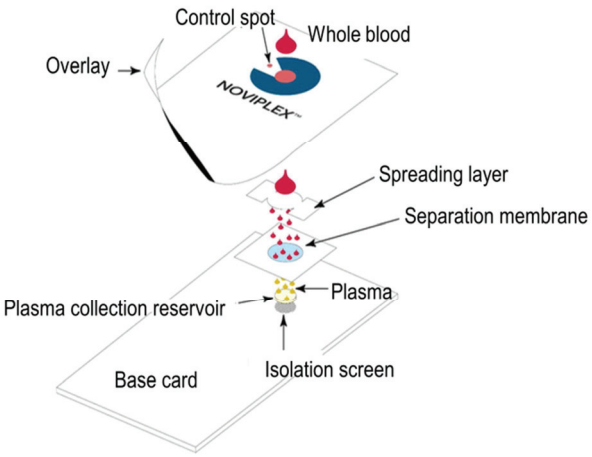


***Figure 1.3: Overview of the composition of the Noviplex sampling cards**. Reprinted with permission from reference[66]. Copyright © (2013) American Chemical Society*

Water-soluble materials for DBS sampling were introduced to solve challenges related to the elution of analyte from the sampling card[67,68]. The transfer of sample from the material was performed by dissolving the whole sampling material (including the dried sample) in buffer rather than eluting the sample from the material. This concept has been demonstrated by using soluble chitosan and alginate as DBS sampling materials. Increased analyte recovery compared to non-soluble commercially available materials was shown together with reduced preparation time. Only a few studies using these materials have so far been published and hematocrit effect is still to be evaluated.

Several other sampling materials and formats for sampling of whole blood have been developed to avoid problems related to hematocrit. Sampling of a fixed volume of sample (10 or 20 µL) by using an absorptive pad (attached to a plastic stick) has shown to reduce the volume bias related to hematocrit[69,70]. This sampling device is based on Volumetric Absorptive MicroSampling (VAMS^{TM}) technology and has been approved by FDA for sampling in clinical settings. The VAMS samplings stick, both before absorption of sample and after absorption of whole blood sample is shown in Figure 1.4.

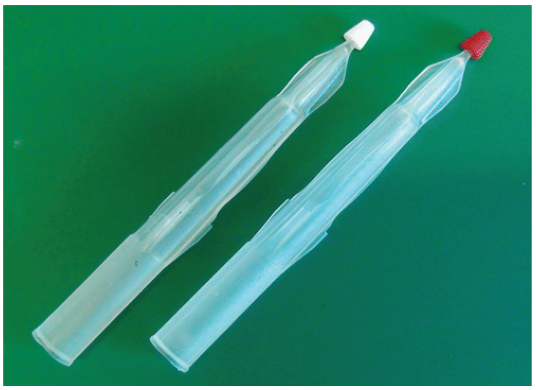


Figure 1.4 Shows the VAMS stick for sampling for accurate volumes of samples*. Sampling pad prior to sampling (left) and after sampling of blood (right). Reprinted with permission from reference[77]. Copyright © (2014) American Chemical Society.*

Challenges related to hematocrit and volume control have also been addressed by using microcapillaries for sampling of whole blood[71]. The capillaries draw a fixed volume of blood and can easily be centrifuged for obtaining plasma samples. An approach for integrating microcapillary on DBS cards has been shown for sampling of accurate volumes of whole blood directly onto regular DBS sampling cards[72]. However, microcapillary sampling will introduce new challenges in the sampling of microvolumes of blood such as poor recovery of large biomolecules (due to unspecific binding to the glass capillary) and the risk of glass breakage during handling of the samples[73].

1.2 Protein analysis

Proteins are important molecules for indication of biological states[74-81], and are of great research interest in proteomics studies. Proteomics is the large scale exploration of proteins and is an interdisciplinary field which includes different tools for either qualitative or quantitative protein studies[82]. Proteins are interesting as biomarkers for screening and monitoring of diseases and treatment response. Several proteins are also available as therapeutics (e.g. insulins and monoclonal antibodies) or listed on WADA's prohibited list (e.g. erythropoietin). The clinical value of proteins is tremendous and reliable methods for measuring these molecules are consequently needed. Two techniques for protein analysis will be described in the following sections: immunoassay-based techniques and LC-MS-based techniques.

1.2.1 Immunoassays

Immunoassays have been used for detection and quantification of proteins for decades and are considered the golden standard for protein analysis in clinical settings. Tailor made antibodies are used for detection of an antigen (the analyte) which upon binding generates a read back signal. Two main types of immunoassays are available: Liquid-phase-binding assays and solid-phase binding assays[83]. The liquid-phase-binding assays are based on binding between the analyte and the labelled reagent in solution, while the solid-phase-binding assays contain reagent immobilized onto solid support. Different labels for the

detection and quantification of the analyte are available such as radioisotope labels (radioimmunoassays), fluorophore labels (fluoroimmunoassays) or enzymatic labels (e.g. in enzyme-linked immunosorbent assay, ELISA).

The immunoassays are normally divided into competitive or non-competitive assays[83] and the principle of these two assays are illustrated in Figure 1.5.

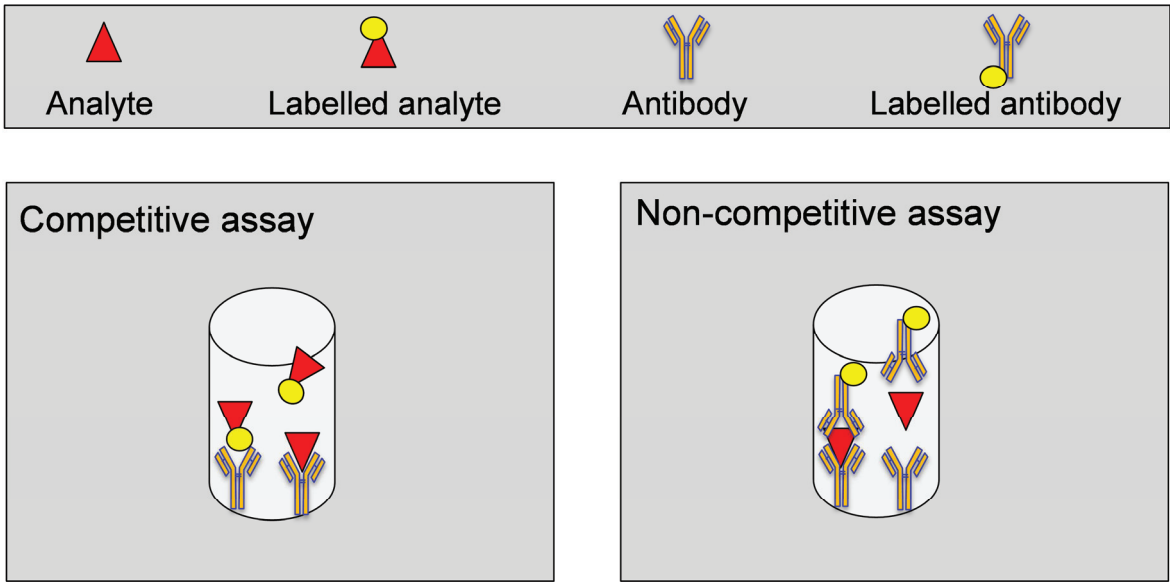


Figure 1.5: The principle of a non-competitive immunoassay (showed here as the antigen-capture immunoassay) and a competitive immunoassay.

In a competitive assay the measurement of the analyte will occur through either a labelled analyte (antigen-capture immunoassays) or a labelled antibody (antibody capture immunoassays). The labelled antibody or the labelled analyte will compete with the non-labelled analyte for the binding sides in the assay. The unbound antibody or analyte is removed after equilibration. The bound labelled compound is then measured and inversely correlated to the concentration of analyte. In a non-competitive assay (frequently referred to as "sandwich assay") the measured analyte must contain two binding sites (epitopes): One epitope that can bind to the immobilised antibody and one epitope that binds to a labelled reporter antibody. After equilibration and washing the labelled antibody is measured and correlated to the analyte concentration.

Immunoassays provide high analytical sensitivity and the samples can normally be added directly in the assay without any clean-up or pre-fractionation, enabling high-throughput workflows[84]. Drawbacks related to the assay-based techniques are expensive and time consuming development and validation, often the bottleneck in verification of a new biomarker candidate[85]. Immunoassays have also limited possibilities for multiplexing

(analysis of several molecules simultaneously) together with lack of specificity and accuracy. This is contradictory to the increasing amount of identified protein biomarkers and the clinical value associated with different protein isoforms. Another major challenge associated with immunoassays has been auto-antibodies in the sample, blocking the epitope of the protein. This has shown to influence the analytical measurements of e.g. thyroglobulin in cancer patients[86]. Reproducing the antibodies has also proven to be difficult and batch-to-batch variations are claimed to be the main cause of variability in laboratories using immunoassay-based techniques[87]. Cross-reactivity, unspecific binding and saturation (referred to as the "hook effect") are also typical challenges during immunoassay analysis[88,89], and can lead to either false positive or false negative results.

1.2.2 LC-MS-based protein analysis

The drawbacks related to immunoassays have been an important factor in the search for alternative methods for detection and quantification of proteins, and MS has proven to be an interesting and useful analytical technique for this purpose[90].

MS is based on gas-phase separation of molecules according to their mass and charge. The charged ions are introduced to the MS by an ionization source and separated according to their mass-to-charge ratio (*m/z*) using different mass analysers and modes. High sensitivity and specificity are often achieved by applying the MS^n mode, where the intact molecules are fragmented prior to detection. MS instruments are expensive to purchase and maintain, and trained staff is required for operating these instruments. However, MS can differentiate between isoforms of a molecule and is in addition less prone to false results. MS is also a versatile and flexible tool and can be used for analysis of several molecules simultaneously (multiplexing). Detection by MS can be difficult for molecules where no pre-knowledge on size and fragments are available, or for molecules exposed to unexpected structural changes[90]. The sensitivity offered by MS is generally poor compared to the assay-based techniques, but has been improved by new developments in instruments and sample preparation techniques.

Liquid chromatography (LC) is often coupled with MS for separation and preconcentration of compounds prior to detection[90]. LC is useful in MS-based protein analysis for separation of complex protein mixtures and for detection of low analyte concentration. Hydrophobic packing materials (e.g. C18) are most frequently used, but other materials for separation of large biomolecules are also available. Different dimensions of the separation column are

available and will be further discussed in section *1.2.9 Advanced analytical tools for MS-based protein analysis.* The Electrospray Ionization (ESI) source has been important in combining LC separation with MS detection as it generates a spray of the LC mobile phase, containing the ionized analytes. The spray is directed towards the inlet of the MS and desolvation of the ionized analytes is often achieved by high temperature and gas flow. ESI is a soft ionization technique which leaves the molecules intact prior to entering the MS. Multiple charge analytes are generally produced by the ESI process. This is a great advantage in analysis of large molecules (e.g. peptides and proteins). One major drawback with ESI is the possibility of matrix effects caused by co-eluting compounds. This can lead to ion enhancement or ion suppression of the analyte, and evaluation of the matrix effects is consequently important in method development[91].

1.2.3 Top-down and bottom-up approach

Protein analysis by MS is mainly performed by either the top-down or the bottom-up approach[84,92]. The top-down approach is based on intact analysis of the proteins and will reveal the whole mass of the molecule, which is typically useful in detection of post-translational modifications (PTMs). However, mass spectra from top-down analysis are complex as large proteins will appear with several charge states, leading to a "dilution" of the protein signal and hence limit the sensitivity. Fragmentation of large proteins is also difficult to achieve by collision induced dissociation (CID) and fragmentation techniques only available on high-end instruments are usually required for fragmentation in top-down analysis. Separation of intact proteins by LC is often impossible due to the slow diffusion rate of these large biomolecules and the top-down approach is therefore less suitable for analysis of complex samples.

The bottom-up approach is based on chemical or enzymatic cleavage of the proteins prior to analysis of one or several of the peptides generated from these proteins[84,93]. The main steps in this approach are shown in Figure 1.6. The cleavage of proteins increases the complexity of the sample and can lead to additional variability in the sample preparation. The protein cleavage will also generate peptides which are not suited for LC-MS analysis and full sequence coverage is therefore rarely obtained when using the bottom-up approach. The bottom-up approach is also considered time consuming and therefore less suited for high-throughput analysis. However, peptide analysis is related to increased sensitivity compared to protein analysis as the peptide signal is distributed on a few charge states and can be separated by LC prior to MS detection. CID fragmentation of the peptides cleaves

the amide bond into mainly two types of fragments: one from the C-terminal (y-ions) and one from the N-terminal (b-ions). The fragmentation of the amide bond is advantageous for elucidating the peptide composition and CID is frequently used for *de novo sequencing* of the peptides. The bottom-up approach is therefore useful in both elucidations of peptide amino acid composition and for analysing of complex samples were detection of low protein concentration is desired.

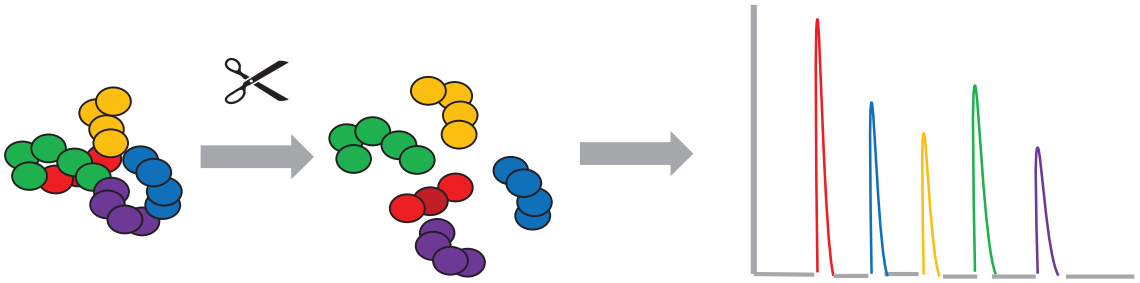


Figure 1.6: Illustration of a typical workflow for targeted bottom-up analysis*. Intact proteins are cleaved into peptides and one or several peptides are measured by LC-MS.*

1.2.4 Trypsin

Trypsin is the most commonly used enzyme for digestion of proteins into peptides[93]. This enzyme is a serine protease with a molecular size of 23.3 kDA. Trypsin selectively cleaves after the amino acids (AA) arginine (R) and lysine (K) at the C-terminal of the protein, if not followed by proline (P). Arginine and lysine are often distributed continuously throughout the whole protein sequence and trypsin will therefore generate peptides with a desired length for LC-MS analysis. Trypsin also generates peptides which have at least one basic amino acid (arginine and lysine), advantageous for the ionization process prior to the entering the MS. Temperature of 37°C and pH 7.5-9.0 are optimal conditions for the trypsin activity, and the tryptic digest is often performed for hours or overnight under these conditions[94]. The digest rate of trypsin is concentration dependent (first order kinetic) and consequently a lower digest rate is expected for low abundant proteins compared to high abundant proteins[95].

1.2.5 Non-targeted bottom-up protein analysis

Bottom-up LC-MS protein analysis can be performed by either targeted analysis where specific peptide(s) (corresponding to specific protein(s)) are measured or non-targeted. Non-targeted protein analysis is typically useful in discovery proteomics experiments where complex samples are analysed without any pre-knowledge of the protein[96]. Non-

targeted protein analysis is usually performed by high-resolution hybrid MS instruments (e.g. Ion trap coupled to Orbitrap) using the Data Dependent Acquisition (DDA) mode[97]. The instrument is operated to continuously switch between analysing peptide ions in full scan (MS1) followed by fragmentation (MS2) and detection of the peptide fragments. The ions are selected for fragmentation based on signal intensity, and re-fragmentation of the ion is circumvented by excluding (for a certain time) the measured mass for re-analysis. The acquired *m/z* values of the precursor ion obtained in MS1 together with the specific fragment pattern obtained in MS2 are used to elucidate the amino acid sequence of the peptide and hence the corresponding protein. Software and databases are often used to speed up this process.

1.2.6 Targeted bottom-up protein analysis

Targeted LC-MS protein analysis is a commonly used approach when pre-knowledge of the protein is available. Targeted MS-based analysis is usually performed by the Selected Reaction Monitoring (SRM) mode using a Triple Quadrupole MS (QqQ) due to the sensitivity and specificity offered by this instrument and the instrument mode[98]. One or several selected *m/z* values (precursor ions) are scanned in the first quadrupole of the instrument (MS1) followed by fragmentation (MS2) and scanning of selected fragments (product ions) in the last quadrupole (MS3).

One or several peptides are typically used for monitoring the protein of interest, commonly referred to as signature peptide(s). The signature peptides are chosen based on uniqueness of the protein analyte and by appropriate length (minimum 6-7 AA) for optimal ESI response and specificity[99]. Peptides with poor stability (e.g. with AA such as methionine and tryptophan), high likelihood for missed cleavages (peptides with double arginine or lysine) and peptides easily subjected for PTMs (e.g. with AA such as serine, threonine and tyrosine) should in general be avoided (unless these peptides are of particular interest).

1.2.7 Sample preparation of proteins and peptides prior to LC-MS analysis

The complexity of biological matrices is a great challenge in analysis of proteins and peptides as the amount of proteins in the sample can lead to matrix effects during the ESI process or blocking of instrument parts. The dynamic range of the proteins is also a challenge for detection and quantification of low abundant proteins by MS, illustrated by the fact that the twenty most abundant proteins contribute to 99 % of the protein amount in the human plasma[100,101]. Consequently high abundant proteins are present in mg/mL levels

while low abundant proteins, often interesting as biomarkers, are present in pg/mL levels[102,103]. Other compounds (e.g. phospholipids or salts) in the sample can in addition influence the analysis by e.g. blocking or matrix effects. Sample preparation is consequently required prior to LC-MS analysis of proteins, and ideally the sample preparation should improve sensitivity and specificity, as well as being simple and time-efficient.

Separation of proteins has traditionally been performed by using gel based techniques where the proteins are separated according to their molecular weight (SDS-PAGE), isoelectric point (isoelectric focusing)[104] or a combination of both (2D separation). Gel separation has successfully been combined with MS, but reproducibility and low throughput are common limitations related to these techniques.

Solid phase extraction (SPE) has frequently been used for preconcentration and desalting of the sample prior to LC-MS-based protein analysis. Sample clean-up is achieved by the different affinity between a stationary phase and a mobile phase, and the technique can be tuned by changing the functionalized groups of the stationary phase[105]. SPE has been extensively used for sample preparation (exclusively or in combination with other techniques) in LC-MS-based protein analysis, but is associated with challanges such as blocking and limited throughput.

Depletion of high abundant proteins is useful for reducing the dynamic range of a complex sample such as plasma or whole blood[106], and kits, LC columns, microcolumns and spin columns for depletion of one or several abundant proteins are commercially available[100]. The depletion methods are often combined with fractionation to further increase the coverage of the human plasma proteome[107]. Another approach for reduction of the dynamic range is obtained by the equalizer kits containing hexa-peptides. These kits will dilute high abundant proteins in the sample while enriching the low abundant proteins[108]. Drawbacks with the immunodepletion or equalizer techniques are the high cost of these products combined with the low sample capacity[109].

Matrix precipitation is an example of a simple and cost saving sample preparation procedure of biological samples. Precipitation with acids or organic solvents has shown to be a useful clean-up method of proteins or peptides from complex samples, either by removal of high abundant proteins[110], high molecular proteins[111] or by removal of salts and detergents[112,113]. Clean-up by precipitation is however related to lack of selectivity and

possibility for co-precipitation of the protein or peptide analyte. Another fairly simple technique for fractionation of proteins in the sample is by using cut-off filters for enrichment of proteins or peptides according to their molecular weight. Several papers have demonstrated successful use of these filters, but lack in precision in the molecules removed or retained has also been a concern when using these filters[114].

The most efficient clean-up of analytes from complex samples is achieved by methods utilising specific affinity for the analyte. Immunoaffinity clean-up prior to MS analysis, often referred to as Immuno-MS, is considered the most selective and sensitive clean-up procedure for proteins and peptides[115-118]. Selective antibodies are attached to beads or sample wells and are used for capturing either the intact protein or the signature peptide (the latter is often referred to as Stable Isotope Standards and Capture by anti-peptide antibodies, SISCAPA). The use of this clean-up technique has shown to provide LC-MS detections of low abundant proteins[119,120], but the need for specific antibodies for the enrichment is a drawback with the method[106]. Molecular Imprinted Polymers (MIP) is another sample preparation method based on specific affinity between analyte and polymer[121]. MIP is often referred to as *artificial antibodies,* but avoids the time and cost related to development of regular antibodies, and has also shown to be more robust than the antibodies[122,123]. A drawback related to MIP has been the possibility of unspecific binding to the polymers, reducing the specificity of this sample preparation method[121].

1.2.8 Internal standard for quantitative targeted bottom-up protein analysis

Variation in ionization efficiency during the ESI process will influence the accuracy and precision of the MS analysis. In addition sample preparation steps prior to analysis can introduce variation in the quantitative measurements. Hence, internal standards (IS) are often needed for quantitative LC-MS-based protein analysis.

Isotopically labelled standard, identical to the analyte of interest, has been extensively used as internal standards for quantification of small molecules. These standards have similar behaviour as the analyte during the sample preparation, LC separation and ESI process, but are separated from the analyte during the MS detection due to differences in molecular weight. Isotopically labelled standards have also been shown useful in quantification of proteins and different isotopically labelled internal standard approaches for protein analysis are available[124,125] as illustrated in Figure 1.7.

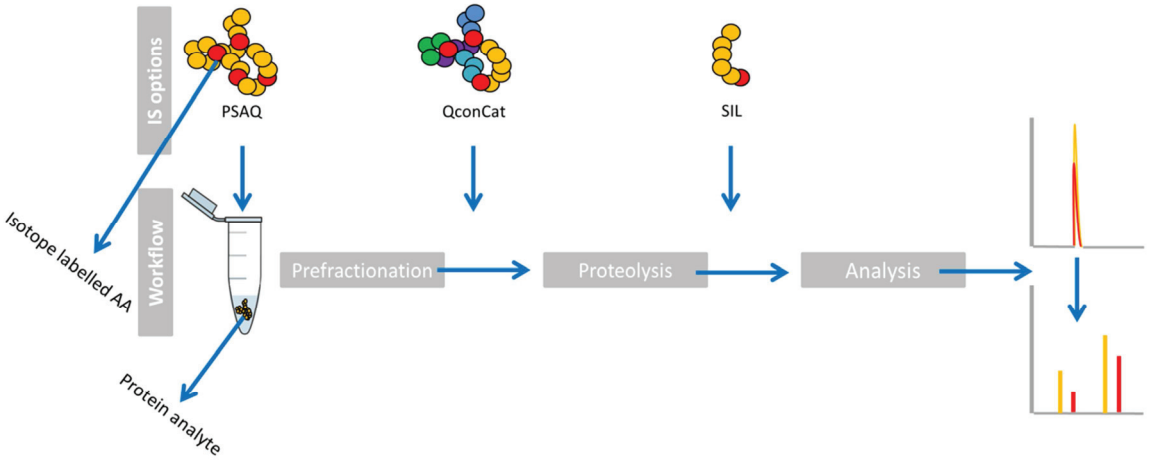


Figure 1.7: IS approaches in LC-MS-based protein analysis. *Red dots represent the labelled amino acid (AA) in the internal standard. The IS (red chromatogram) is separated from the peptide of interest (yellow chromatogram) in the MS by different m/z values.*

The IS is preferably added to the sample early in the preparation procedure, and QconCAT (quantification concatamer) and PrEST (Protein Epitope Signature Tag) are two examples of ISs which are added in the sample prior to the proteolysis of the protein. QconCAT is an artificial protein containing a set of labelled peptides. QcontCAT is useful in multiplexed protein analysis as the protein internal standard contains several labelled peptides. PrEST is another approach where shorter fragments of the protein are produced. QconCat and PrEST are not structural analogues with the protein analyte and differences in protein digest or in preparation steps prior to the digest (e.g. fractionation) may therefore not be corrected for. A labelled full-length equivalent to the protein is favoured for optimal correction of variations during all preparation steps, and this can be achieved by using Protein Standards for Absolute Quantification (PSAQ)). However, the use of PSAQs is limited due to the expensive production of these standards.

A commonly applied IS for bottom-up protein analysis is Stable Isotope Labelled peptides (SIL peptides) as these are easy to use and commercially available from several companies[124]. A SIL peptide is an isotopically labelled peptide analogue to the peptide analyte (the signature peptide), and has been successfully applied in quantitative bottom-up protein analysis in several papers[126,127]. However, SIL peptides will only correct for preparation steps performed after the proteolysis and highly reproducible proteolysis is therefore required when using this internal standards.

1.2.9 Advanced analytical tools for MS-based protein analysis

Sensitivity and selectivity are always a driving force in development of an analytical method. Two techniques, integrated with the LC-MS instrument, for increasing sensitivity or selectivity are presented in this thesis and will be discussed in the following sections.

NanoLC-MS

The inner diameter (ID) of the analytical column (used for separation in LC) is crucial for the sensitivity. Downscaling the column ID reduces the radial diffusion of chromatographic bands in the column, and hence increases the analyte concentration reaching the detector[128] as illustrated in Figure 1.8. NanoLC is often defined as columns with ID less than 0.1 mm and flow rates in nanoliter per minutes[129]. Changing the column from a ID of 2.1 mm to a ID of 0.1 mm reduces the dilution of the sample more than 400 times. However, lower sample volumes are injected on a nano-system compared to a conventional system and the theoretical gain in sensitivity is therefore rarely achieved. Trap columns are often integrated in the nano-system to partly compensate for this decrease in sensitivity gain as injection of larger sample volumes is possible (in addition to desalting and preconcentration of the sample)[130,131]. Other advantages related to the nanoLC is more efficient ESI process as low flowrates produce small droplets (which increases the ion transfer to the MS)[132] and less consumption of sample and reagents compared to the conventional systems. NanoLC is today an important tool in protein analysis due to the increased sensitivity compared to conventional systems.

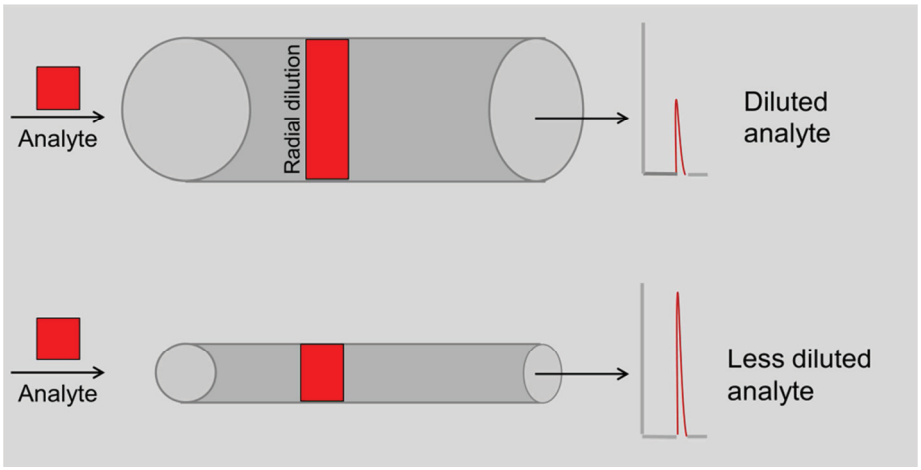


Figure 1.8: Effect on downscaling the ID of the analytical column: *Reduced radial dilution of the chromatographic bands increases the analyte concentration reaching the detector.*

High Field Asymmetric Waveform Ion Mobility Spectrometry

Improved analytical selectivity can be achieved by using High Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS) prior to MS detection. FAIMS is a separation tool which is coupled between the ion source and the inlet of the MS instrument, and has shown to be a useful tool in protein analysis. FAIMS was introduced in 1991 as an alternative ion mobility technique[133], and differs from the regular ion mobility techniques as an asymmetric electric field is applied rather than a constant electric field. Figure 1.9 illustrates the asymmetric waveform applied to the electrodes (a) and the path of an ion through the electrodes in the FAIMS device (b). The FAIMS device consists of two planar or curved electrodes (an inner and an outer electrode) and the ions are carried through the electrodes by a carrier gas[134]. An asymmetric oscillating electric field (dispersion voltage, DV) is applied on the electrodes, and due to this oscillating field the ion will be displaced against one of the electrodes depending on the mobility in low and high electric field. The ion will eventually get neutralized upon collision with one of the electrodes, but a superposition of a direct current (compensation voltage, CV) can be applied to prevent this collision. The DV is usually operated at a fixed negative or positive value, while the CV can be optimized to transmit only selected ions through the electrodes and in to the MS. The CV can be changed by internal stepping (scanning through different CVs within one analytical run) or by the external stepping (changing CV between each run).

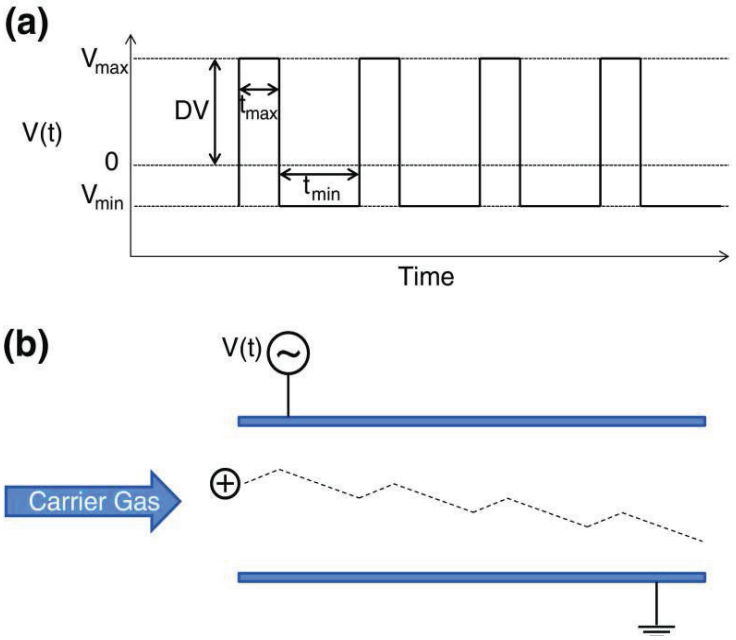


Figure 1.9: The principles of FAIMS: *The asymmetric waveform applied to the electrodes (a) and the path of an ion through the electrodes in the FAIMS device (b). Reprinted with permission from reference[133]. Copyright © (2016) Springer.*

FAIMS has been used for separation of a range of different analytes such as enantiomers[135], lipids[136] and phosphorylated peptides[137,138]. This technique has also shown to increase

proteome coverage[139,140], to work complementary with other separation techniques[141] and to be beneficial for separation of proteins from complex samples such as tissue or DBS samples[136,142].

1.3 MS-based peptide and protein analysis of DBS

Different classes of substances have been analysed from DBS, e.g. organic acids, steroids and peptides[143], but mainly small molecules have been targeted both in the clinical laboratory and in research. A few large biomolecules have been analysed from DBS sample in the newborn screening program by using assay-based detection and quantification techniques. However, there has been an increase in papers describing DBS and MS-based protein analysis lately. MS analysis of proteins from DBS has been demonstrated for peptides with molecular size of 3 kDa to proteins with molecular size above 100 kDA[51,144-147], and mostly high abundant endogenous proteins (e.g. hemoglobin, albumin or ceruloplasmin) have been analysed from DBS by MS[145,148-150]. Detection of low abundant protein biomarkers by LC-MS has also been achieved in a few published papers by using immunoaffinity clean-up[151,152]. MS-based peptide and protein analysis of DBS have been demonstrated as useful in detection of the use of prohibited substances[51,52,153] and for analysis of therapeutic proteins[144,154,155]. DBS has been evaluated for LC-MS protein analysis in biomarker measurements, either by the bottom-up approach[145,148-152,156-159] or by the top-down approach[160-162]. DBS has also been used for non-targeted discovery protein analysis[163,164]. DBS has been tested in combination with immobilized-enzyme reactor for rapid digestion of proteins from these samples[165,166] and in automatic liquid surface extraction (LESA) for rapid elution of the sample prior to MS detection[162,164]. MS-based protein analysis and DBS has so far proven to be a powerful combination for different applications, but further evaluation is needed before routine use is possible[167].

2. AIM OF THE PROJECT

The overall aim of the present project was to investigate the use of Dried Blood Spots (DBS) in Liquid Chromatography Mass Spectrometry-based (LC-MS-based) protein analysis. The focus was on both method development for LC-MS analysis and evaluation of key parameters of DBS such as recovery, stability, hematocrit and quantitative performance in targeted protein analysis. The potential of DBS in non-targeted protein analysis was also evaluated with the focus on different sampling materials and tools for improving proteome coverage.

The following aspects of combining DBS and LC-MS-based protein analysis were examined:

Targeted LC-MS protein analysis and DBS:

- Different DBS sampling materials (*Paper I-V*)
- Model proteins with different physicochemical properties (*Paper I-III* and *Paper V*)
- Reaching low endogenous protein levels from DBS (*Paper IV*)
- Alternative biological matrices for DBS (*Paper IV*)

Non-targeted LC-MS protein analysis and DBS:

- Different blood sampling materials in non-targeted protein analysis
- High Field Asymmetric Waveform Ion Mobility Spectrometry as a tool in non-targeted protein analysis of DBS (*Paper VI*)

3. RESULTS AND DISCUSSION

The main results from *Paper I-VI* are described and discussed in the following sections. Most of the experiments were performed by using blood as sample matrix (DBS), but a few experiments were conducted with other matrices (DMS) and will be discussed when relevant. Materials used for sampling of biological matrices are described in section 3.1. Section 3.2 and section 3.3 are summing up and discussing the results from targeted and non-targeted protein analysis of DMS samples.

3.1 DBS sampling materials

Different materials for sampling of microvolumes of biological matrices were examined throughout the work presented in this thesis, both for targeted and non-targeted analysis. The sampling materials used in the six papers are listed in Table 3.1.

Table 3.1: The sampling materials and devices used in Paper I-VI*: Product name, manufacturer, material, comments and the paper it was used in are listed.*

Product	Manufacturer	Material	Comments	Used in
FTA® DMPK-C	Whatman	Cellulose	For research only	*Paper I-V*
Ahlstrom grade 226 filter	ID Biological systems	Cellulose	For clinical use	*Paper VI*
Mitra®	Neoteryx	Unknown[a]	VAMS™ technology	*Paper V*
Noviplex™	Novilytic	Cellulose, spreading layer and membrane	Volumetric plasma sampling	Ud[b]
Aquacel® hydrofiber®	Convatec	Carboxymethyl cellulose, CMC	Water-soluble	*Paper I-V*

[a] *Patent pending*
[b] *Unpublished data*

Different commercially available DBS materials were addressed in several of the papers. A pure cellulose-based material (DMPK-C sampling card, for research use only) was tested in *Paper I-V* (targeted protein analysis), while another type of pure cellulose-based sampling card (Ahlstrom grade 226 filter, approved by FDA for clinical use) was used in

Paper VI (non-targeted protein analysis). Two other commercially available materials for blood sampling were also tested: The volumetric plasma sampling card Noviplex™ (unpublished data) and the Volumetric Absorptive MicroSampling (VAMS) device, Mitra® (*Paper V*). VAMS is not, by definition, a DBS material (as the sample is absorbed rather than spotted), but will for the sake of simplicity be referred to as a DBS material in the rest of this thesis.

Water-soluble DBS sampling materials were previously evaluated for small molecules[67,68], but these materials had not been demonstrated for larger molecules prior to the work presented in this thesis. The two biopolymers (alginate and chitosan) used for analysis of small molecular analytes were not available during the work with this thesis and alternative water-soluble materials, suitable for DMS sampling in protein analysis, were initially tested. Different commercially available wound dressings were initially evaluated as water-soluble materials and the solubility in trypsin compatible buffers was essential for the choice of material. The wound dressing product Aquacel®, made of carboxymethyl cellulose (CMC) fibres, was chosen due to acceptable water-solubility at physiological pH and the declared composition of this product. The material dissolved relatively fast (approximately 20-30 min) in ammonium bicarbonate (ABC) buffer and phosphate buffer saline (PBS) which were desired buffers for sample preparation of proteins. Drawbacks to this material were uneven spreading of sample (resulting in variation in spot shape and size), in addition to occasional observation of some non-dissolved fibres (after the dissolution). Despite of these shortcomings, Aquacel® (CMC) was used as water-soluble DBS material and the performance of the material is demonstrated in *Paper I-V*. Figure 3.1 shows spotting of blood sample on CMC and the dissolution of the CMC in the buffer.

Mainly blood was sampled throughout this work, but other dried biological matrices (Dried Matrix Spots, DMS) were also tested in LC-MS protein analysis. The materials CMC and DMPK-C were demonstrated in sampling of urine, plasma and serum (in addition to blood) in *Paper IV*.

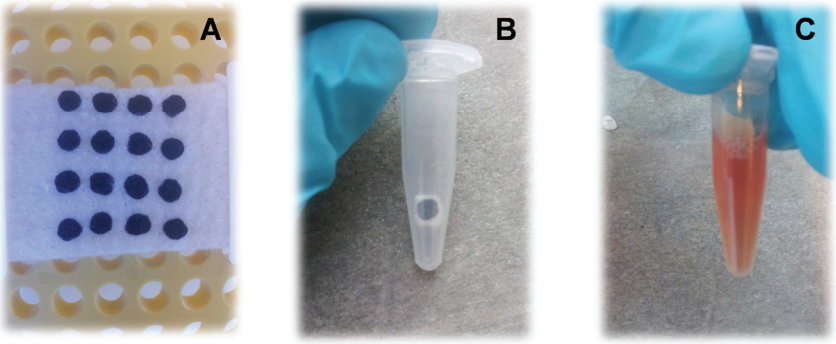


Figure 3.1: Blood spotted on CMC (A) prior to punch out (B) and dissolution in ABC buffer (C). *Modified and reprinted with permission from Paper I © (2015) American Chemical Society.*

3.2 Targeted protein analysis of DBS

DBS for targeted protein analysis was addressed in *Paper I-V,* and the results from these papers will be discussed in the following sections.

3.2.1 Model proteins

All proteins used for the targeted analysis in the present project are listed in Table 3.2, including information on the protein origin, the molecular weight (Mw), number of amino acids (AA) and isoelectric point (p*I*). The peptide sequences and the fragments monitored are listed in Table 3.3.

Table 3.2: Model proteins used in Paper I-V: *Protein, origin of protein, molecular weight (Mw), number of amino acids (AA), isoelectric point (pI) and which paper the protein was monitored in.*

Protein	Origin	Mw[a] (kDa)	AA[b]	p*I*[c]	Used in
Chorionic gonadotropin	Human	37.5[d]	237	8.7	*Paper I* and *IV*
Insulin	Human	5.8	51	5.3	*Paper II*
Carbonic anhydrase II	Bovine	29.1	260	6.4	*Paper III* and *V*
Catalase	Bovine	59.9	527	6.8	*Paper III* and *V*
Cytochrome c	Bovine	11.7	105	9.5	*Paper III* and *V*
β-lactoglobulin	Bovine	19.9	178	4.9	*Paper III* and *V*
Myoglobin	Horse	17.1	154	7.4	*Paper III* and *V*
Transferrin	Bovine	77.8	704	6.8	*Paper III*
Albumin	Bovine	69.3	607	6.2	*Paper V*

[a] *Mw, from www.uniprot.org (13.07.17)*
[b] *Number of AA, from www.uniprot.org (13.07.17)*
[c] *pI, from http://web.expasy.org/compute_pi/ (13.07.17).*
[d] *Mw of hCG from reference*[168]

***Table 3.3: The peptide sequence of the monitored signature peptide(s)**: Information on sequence, precursor ions (m/z), fragment ions (m/z) and in which paper the peptide was monitored.*

Protein	Peptide sequence	Precursor (charge)	Fragments	Used in
hCG	VLQGVLPALPQVVCNYR	964.2 (+2)	1036.3; 1317.8	*Paper I* and *IV*
Insulin	Intact protein	1162.5 (+5)	1159.0	*Paper II*
		968.6 (+6)	966.0	
Carbonic anhydrase II	QSPVDIDTK	501.8 (+2)	394.4; 591.3: 787.4	*Paper III* and *V*
	YGDFGTAAQQPDGLAVVGVFLK	1127.1 (+2)	1036.3; 1214.7; 1342.7	
Catalase	NFSDVHPEYGSR	704.3 (+2)	708.4; 845.5; 944.5	*Paper III* and *V*
	FNSANDDNVTQVR	740.3 (+2)	503.3; 1060.6; 1218.6	
Cytochrome c	TGQAPGFSYTDANK	728.8 (+2)	798.4; 1099.5; 1170.5	*Paper III* and *V*
	TGPNLHGLFGR	584.8 (+2)	505.9; 549.3; 686.4	
β-lactoglobulin	VLVLDTDYKK	597.3 (+2)	769.4; 882.5; 981.5	*Paper III* and *V*
	VYVEELKPTPEGDLEILLQK	771.8 (+3)	912.3; 976.6; 1026.5	
Myoglobin	VEADIAGHGQEVLIR	536.3 (+2)	654.4; 689.9; 754.4	*Paper III* and *V*
	LFTGHPETLEK	636.3 (+2)	716.4; 910.5; 1011.5	
Transferrin	GYLAVAVVK	919.1 (+1)	575.3; 674.3; 773.4	*Paper III*
	ELPDPQESIQR	656.3 (+2)	429.4; 535.4; 857.4	
Albumin	DAFLGSFLYEYSR	784.4 (+2)	334.3; 717.6; 1121.6	*Paper V*

Human chorionic gonadotropin (hCG) was chosen as the model protein for the proof-of-concept study in *Paper I* and was further investigated in *Paper IV*. hCG was both analysed from DBS (*Paper I and* IV) and from other dried biological matrices (*Paper IV*). The protein hCG has been thoroughly investigated for LC-MS analysis previously[127,169-171] and methods for both sample preparation and LC-MS analysis were available. This protein is interesting from a diagnostic point of view due to increased expression in cancer patients and in abnormal pregnancies[168]. hCG is also listed on WADA's prohibited list[168].

An evaluation of LC-MS-based protein analysis of DBS for smaller proteins was investigated in *Paper II* using insulin as a model protein. Insulin is also of diagnostic value due to the therapeutic effect, anabolic effect (listed on WADA's prohibited list) and the toxicity related to this protein[172-175]. Several papers describing LC-MS analysis of insulin are available[150,176-182], but none of these describes LC-MS analysis of insulin from DBS samples.

Six exogenous proteins were used as model proteins in *Paper III* and *Paper V*. The six proteins were chosen based on their difference in weight, hydrophobicity and isoelectric points rather than the clinical relevance. These proteins were used for comparison of different sampling materials and devices in DBS (*Paper III* and *Paper V*), examination of relative recoveries (*Paper III* and *Paper V*), stability (*Paper III*), hematocrit (*Paper V*) and quantitative performance (*Paper V*).

3.2.2 Sample preparation of proteins from DBS

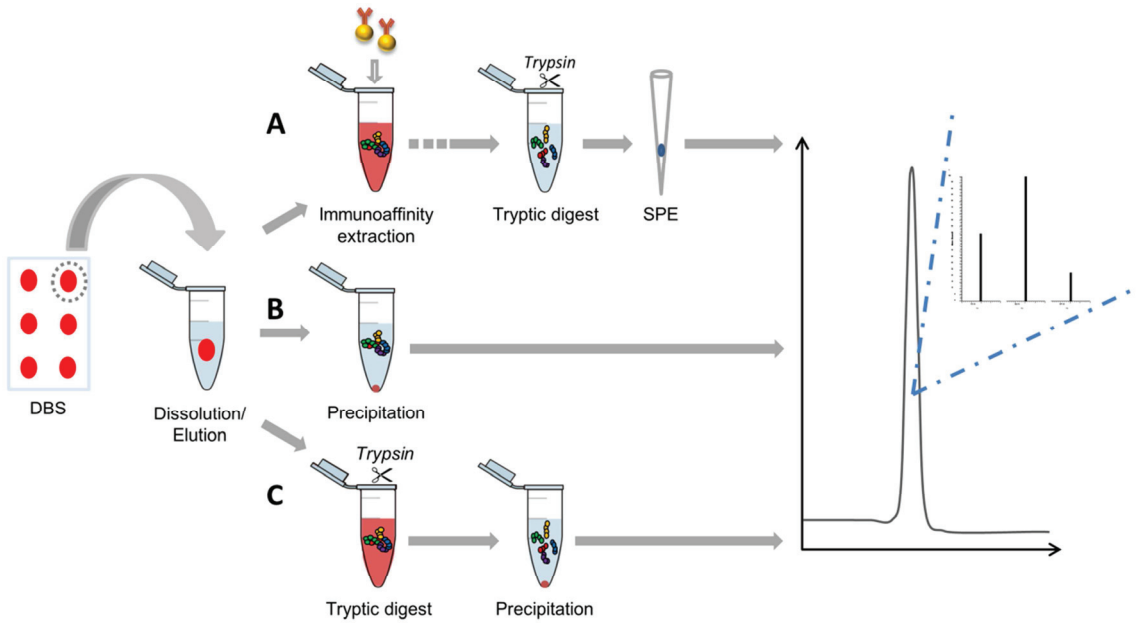
The work in this thesis demonstrated that both highly specific sample preparation procedures (*Paper I* and *IV)* and non-specific preparation procedure (*Paper II, III* and *V)* could be used in preparation of DBS prior to LC-MS analysis. The sample preparation was chosen and optimized based on the model protein(s) and the aim of the paper.

For sampling with DMPK-C and CMC a defined volume of sample (5-15 µL) was deposited on the materials and the whole spot was used for analysis (except for evaluation of hematocrit, described in *section 3.2.5*). For sampling with VAMS a defined sample volume of 10 µL was absorbed onto the material by dipping the sampling pad into the sample until the sampling pad turned completely red.

The sample was eluted (DMPK-C and VAMS) or dissolved (CMC) prior to further sample preparation. The elution/dissolution step was followed by either immunoaffinity extraction

(*Paper I* and *Paper IV*) or tryptic digest (*Paper III* and *Paper V*), and buffers compatible with these preparation steps were therefore required. The sample was eluted/dissolved in PBS pH 7.4 (*Paper I* and *IV*) or in ABC buffer pH ~ 8 (*Paper III* and *Paper V*). The model analyte insulin (*Paper II*) was not digested prior to analysis and ammonium acetate (25 mM) buffer pH 5.5 was chosen as this buffer is compatible with MS (volatile) and that the buffer pH showed to be optimal for the subsequent sample preparation steps. An overview of the workflows used for targeted protein analysis of DBS in *Paper I-V* is shown in Figure 3.2. Workflow A was used for preparation of the protein analyte in *Paper I* and *IV*, workflow B was used for preparation of the protein analyte in *Paper II* and workflow C was used for preparation of the protein analytes in *Paper III* and *V*.

The protein hCG (*Paper I* and *IV*) was cleaned up from DBS with a sample preparation method (Figure 3.2, workflow A) previously developed for clean-up of serum samples[127,171]. The same procedure was also used for preparation of hCG from different dried matrices (DMS) in *Paper IV*. The preparation of hCG included immunoaffinity extraction (prior to tryptic digest) which removed both the dissolved CMC (for CMC samples) and other endogenous components from blood sample. The sample was digested overnight, desalted and pre-concentrated with SPE prior to analysis. A SIL peptide (internal standard) of the signature peptide was added prior to the SPE step. Ion enhancement was observed during evaluation of matrix effects and the type of anticoagulant (EDTA or heparin) showed to influence the degree of enhancement. This demonstrated that the matrix could influence the assay, despite that a very specific sample preparation was used (immunoaffinity extraction). Precision was however acceptable (RSD $\leq$ 20 %) at both high and low hCG concentration when using EDTA blood and adjusting the peptide signal with the signal from the IS.



Figur 3.2: Main steps in the workflows used for targeted protein analysis*. In all workflows was the DBS sample punched out prior to dissolution or elution of the sample from the material. Workflow A shows the main steps in preparation of hCG (Paper I and IV). Workflow B shows the main steps in preparation of insulin (Paper II). Workflow C shows the main steps in the preparation of the six proteins with different physicochemical properties (Paper III and V).*

Insulin (*Paper II*) is a small protein (5.8 kDA) and tryptic cleavage of the protein was therefore not necessary. Insulin was cleaned up (Figure 3.2, workflow B) from the DBS by precipitation of larger proteins and matrix components with acetonitrile (MeCN) subsequent to the elution/dissolution step. Bovine insulin was used as internal standard and was added prior to the precipitation. The precipitation step removed both large proteins, intact cells (if any) and the dissolved CMC material (for CMC samples, as also previously demonstrated[183]). CMC was in the present study shown to have a promoting effect on the protein precipitation with approximately 30 times more proteins removed with CMC present compared to without CMC (5.4 µg protein v.s.141 µg protein respectively left in the sample after precipitation). The enhanced effect on protein precipitation by CMC has also been described by others[184]. Matrix effects were not observed in the retention window for the protein or the internal standard, except for whole blood from one of the five donors as shown in Figure 3.3 (matrix effects are indicated with arrows in the figure). This highlighted that examination of different matrix sources were important. The internal standard would probably correct for variation caused by the matrix, and this should be thoroughly tested if the method is to be used in a clinical setting.

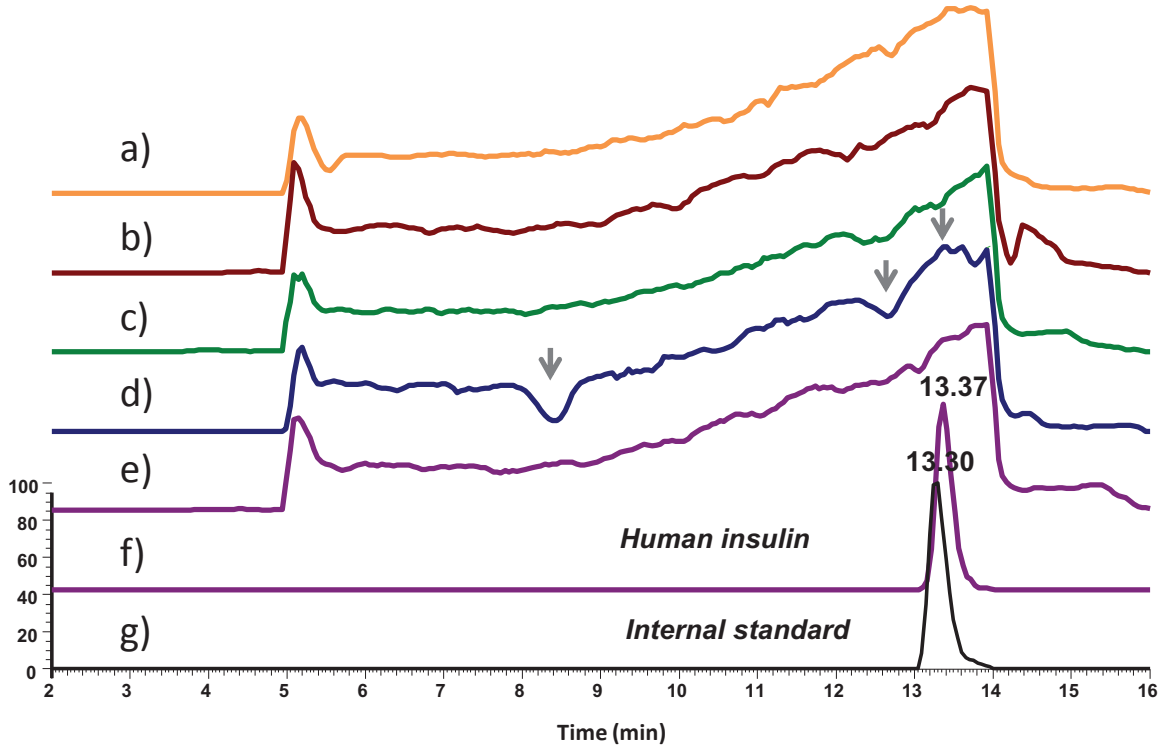


Figure 3.3: Qualitative evaluation of matrix effects*: a)-e) shows monitoring of the insulin signal after injection of five different matrices (cleaned-up as previously described for insulin). f) and g) show the retention times for insulin and the internal standard respectively. Matrix effects are indicated with arrows. Reprinted with permission from Paper II © (2016) Future Science group.*

An analyte specific sample preparation of the six model proteins used in *Paper III* and *Paper V* was difficult to obtain due to the differences in physicochemical properties of these proteins. The focus was to develop a simple and fast sample preparation method rather than an analyte specific method. The six proteins were therefore cleaned-up from DBS (Figure 3.2, workflow C) by using matrix precipitation with MeCN. The sample was tryptic digested prior to precipitation to circumvent co-precipitation of the analyte proteins. The precipitation was expected to be less efficient for the clean-up of samples as all proteins in the DBS sample were digested. However, the precipitation removed CMC (for CMC samples) and undigested proteins (if any), and the internal standards (SIL peptide) showed to correct for the matrix effects (*Paper V*) for most of the proteins, except for β-lactoglobulin (ion suppression) and albumin (ion enhancement). The internal standards were not compensating for the matrix effects for these two proteins, but acceptable precision for the two proteins (RSD ≤ 6 %) was shown by using the IS.

3.2.3 Recovery from DBS material

Recovery from water-soluble material

Water-soluble sampling material (CMC) is intended to release the whole sample and hence all analyte from the DBS material. The recovery of protein analyte from water-soluble material was therefore evaluated in *Paper I-III* by comparing analyte signals from normal DBS samples (spiked blood was deposited and dried on CMC prior to dissolution) with reference samples (spiked blood was added directly in the buffer).

The recovery of analyte from CMC was first evaluated in in *Paper I* for the protein hCG. The relative peak areas obtained from DBS samples (blood deposited on CMC) were comparable to reference samples (spiked blood sample added directly in the dissolution buffer) as shown in Figure 3.4. The results indicated that the dissolution of the water-soluble DBS gave complete recovery of hCG.

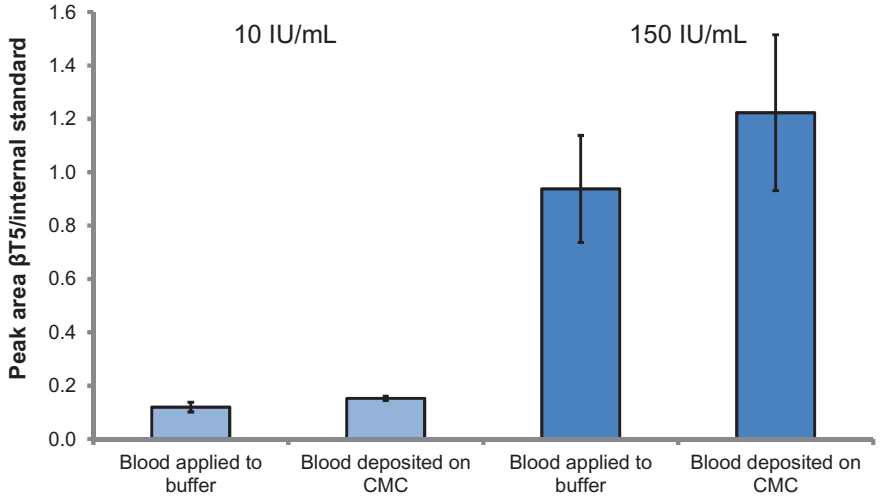


Figure 3.4: Recovery of hCG from water-soluble material (CMC) shown for two different concentrations: *Peak area ratio from spiked samples (10 IU/mL and 150 IU/mL) applied directly in buffer (reference sample) and samples deposited on CMC prior to dissolution. Reprinted with permission from Paper I © (2015) American Chemical Society.*

However, incomplete analyte recovery (68 ± 4 %) from the sampling material was seen for the small protein, insulin, in *Paper II*. Co-precipitation of analyte during the matrix precipitation step could explain the reduced recovery for insulin. Insulin has a higher molecular weight than most tryptic peptides (insulin was analysed intact) and could therefore be more subjected to co-precipitation than tryptic peptides. Insulin is also known to be a *sticky protein* and loss of insulin during preparation and analysis has been described

by others[150]. The recovery of insulin from pure cellulose material (DMPK-C) was comparable to the recovery from CMC (as will be discussed in the next section) and sticking to material is therefore also expected during analysis of insulin from commercially available non-soluble materials. The insulin recovery from CMC was however shown to be reproducible (n=6, RSD < 6 %).

The recovery of analyte from CMC was also evaluated for the six model proteins in *Paper III*. As reduced recovery was observed for insulin (*Paper II*), the influence of CMC during precipitation and digest was evaluated using three different samples: 1) reference sample (spiked blood with no CMC present during digest or precipitation), 2) spiked blood sample digested without CMC (CMC was added prior to precipitation) and 3) normal DBS samples (spiked blood deposited on CMC). No significant difference was observed between the three different samples, indicating complete analyte recovery from the material. However, the CMC seemed to increase the variation in the signal to some degree as higher standard deviations (RSDs from 40-62 % for some of the signature peptides) were observed in samples where CMC was present. Internal standards were expected to decrease the overall variations, but were not available in the work presented in *Paper III*.

Although CMC showed complete recovery for most of the proteins addressed in this thesis, challenges were observed regarding both recovery and reproducibility. Optimization of sample preparation or alternative water-soluble materials should be further examined to circumvent these challenges.

Relative recovery from different blood sampling materials

The relative recovery of the model proteins from different blood sampling materials was evaluated in *Paper I-III* (DMPK-C and CMC) and in *Paper V* (DMPK-C, CMC and VAMS).

In *Paper I* the relative recovery of hCG from DMPK-C and CMC was compared, and the relative peak areas obtained from these materials are shown in Figure 3.5. No significant difference was observed between the two materials, indicating that DMPK-C and CMC gave comparable analyte recovery.

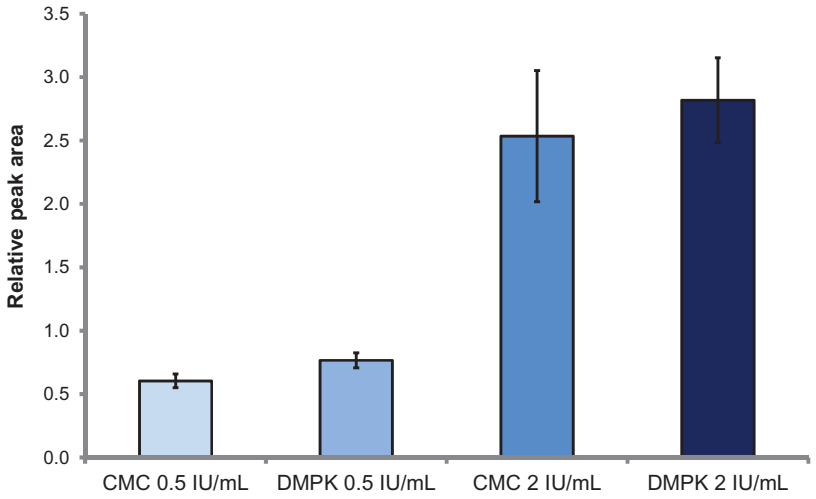


Figure 3.5: Relative peak area for hCG from CMC and DMPK-C using two different hCG concentrations (0.5 IU/mL and 2 IU/mL). *Reprinted with permission from paper I © (2015) American Chemical Society.*

The relative recovery from CMC and DMPK-C was evaluated for insulin in *Paper II***.** The insulin signals from the two different sampling materials were not significantly different. However, increased noise levels were observed for insulin analysed from DMPK-C, resulting in lack of baseline separation from a co-eluting peak as shown in Figure 3.6. The reduced noise levels observed for CMC were probably due to the increased clean-up effect as the material promotes precipitation of proteins (as described in section *3.2.2 Sample preparation of proteins from DBS)*. The sample clean-up was optimized for CMC and reduction in noise level from DMPK-C samples could be expected if sample preparation was optimized for the latter material.

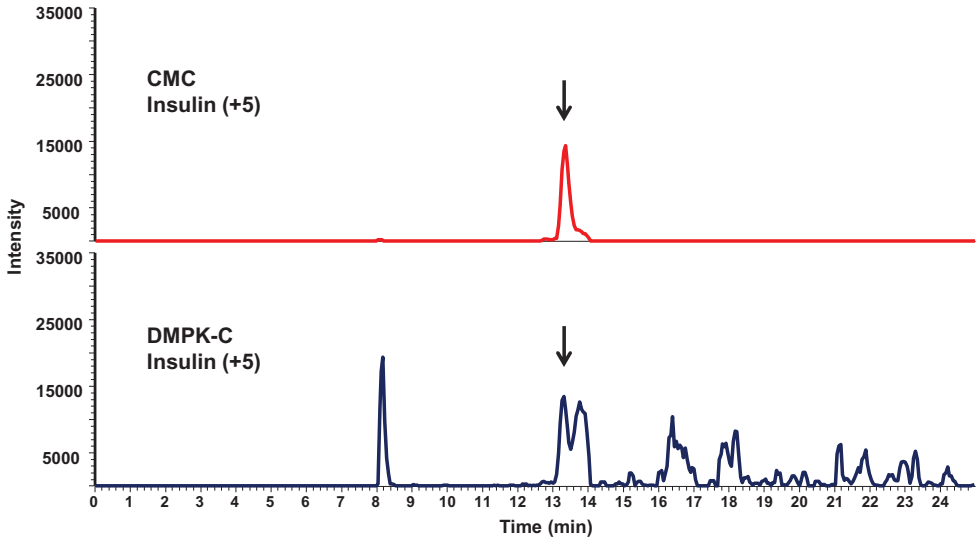


Figure 3.6: Chromatograms from insulin (charge +5) cleaned up from DBS deposited on CMC or DMPK-C. *Reprinted with permission from Paper II © (2015) American Chemical Society.*

Relative recovery from CMC, DMPK-C and VAMS was evaluated for the six model proteins of different physicochemical properties in *Paper V*. Decreased recoveries were observed for four of the proteins (catalase, cytochrome c, β-lactoglobulin and myoglobin) from VAMS (75-79 % relative to the recovery of DMPK-C) and for one of the protein (cytochrome c) from CMC (76 % relative to DMPK-C) as shown in Figure 3.7. Decreased recoveries of hydrophobic low molecular analytes have been demonstrated by others when using VAMS as sampling material[185]. This could explain the lower recoveries observed for β-lactoglobulin and myoglobin analysed from VAMS as these proteins have the highest hydrophobicity of the six model proteins. However, cytochrome c has the lowest hydrophobicity and could therefore not be explained by this property.

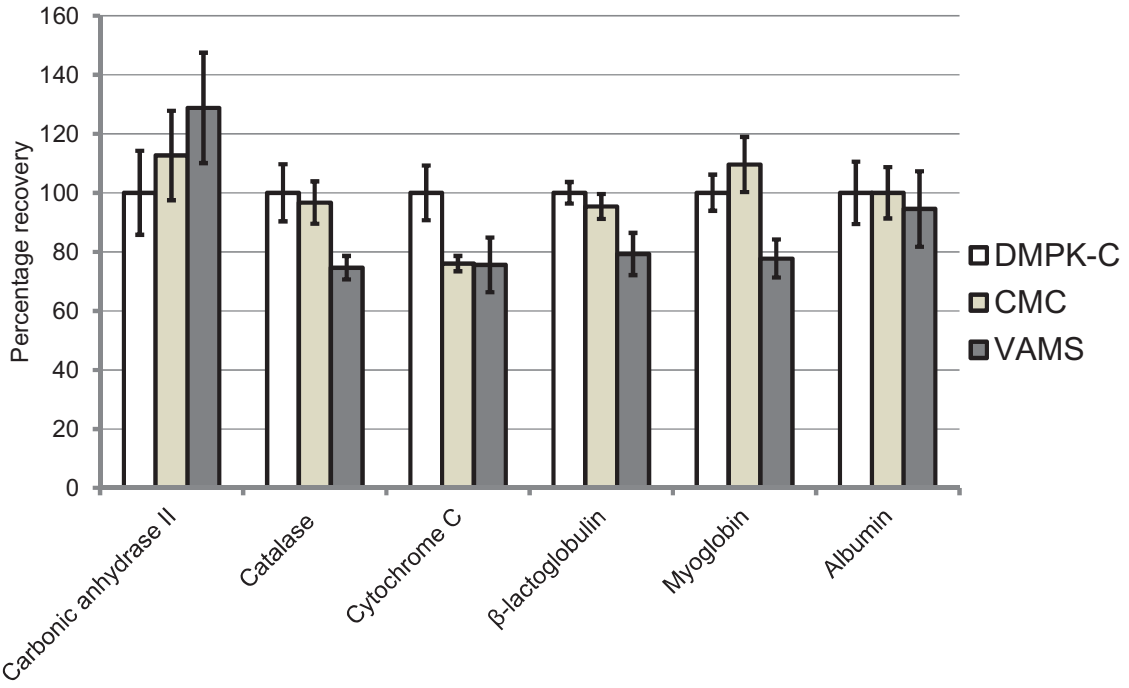


***Figure 3.7: Percentage recovery for the six proteins from CMC and VAMS relative to DMPK-C (100 %)**. Reprinted from Paper V.*

Proteins and peptides have also in other publications shown relative high recoveries (50-104 %) from the elution step of non-soluble sampling materials[51,52,148,186], and proteins may in general be easily eluted from DBS materials. To conclude, CMC (water-soluble material) did not improve analyte recoveries over DMPK-C (non-soluble material) as discussed in *Paper I-III* and *V*. This could partly be explained by co-extraction of DMPK-C (*Paper III* and *V*) during the overnight tryptic digest, resulting in complete recovery due to both lengthy elution time and conversion of proteins to peptides during the elution[144,187]. However, CMC showed to be beneficial for analysis of insulin (*Paper II*) with the applied

sample clean-up procedure. VAMS (*Paper V)* showed decreased recoveries (for the applied extraction time) for some proteins relative to DMPK-C and CMC. The format of the VAMS was hindering sealing of the vial during the overnight tryptic digest and VAMS was therefore extracted for 30 minutes and removed from the sample prior the tryptic digest. The short extraction of the sample from VAMS can also be a reason for the reduced recoveries (relative to DMPK-C) observed for some of the proteins.

3.2.4 Stability

The storage stability of proteins in DBS samples was addressed in *Paper I-III*, and different sampling materials, storage temperature and storage lengths were evaluated.

Stability of insulin in DBS (stored on CMC) was evaluated for one week at room temperature (RT) in *Paper II,* and was shown to be stable in water-soluble DBS during this storage period. A more comprehensive study on protein stability in DBS was performed for the six high abundant proteins of different physicochemical properties in *Paper III*. The stability of these proteins was evaluated for one and four weeks on both CMC and DMPK-C at 40 °C, 25 °C (RT) and -25 °C. The results from this study can be seen in Figure 3.8 (shown as change from reference samples, freshly prepared). The type of sampling material did not seem to influence the stability of the six proteins and the proteins were also shown to be relatively stable after one week of storage (> 65 % signal compared to reference). However, slightly decreased analyte signal was in general observed for sample stored for four weeks compared to reference (both sampling materials). Some of the proteins showed major degradation during storage: Decrease in analyte signal was observed after storage for four weeks for β-lactoglobulin (DMPK-C) and for one of the signature peptide from transferrin (DMPK-C), showing recoveries of 48 % (40 °C) and 50 % (-25 °C). Both signature peptides from myoglobin (both sampling materials), transferrin (both sampling materials), catalase (CMC) and β-lactoglobulin (DMPK-C) showed an increased signal after storage, without any clear link to the temperature used or the protein properties. Increase in peptide signal after storage was also shown in *Paper I* and has been reported by others[150] as caused by protein denaturation, improving the protein digest.

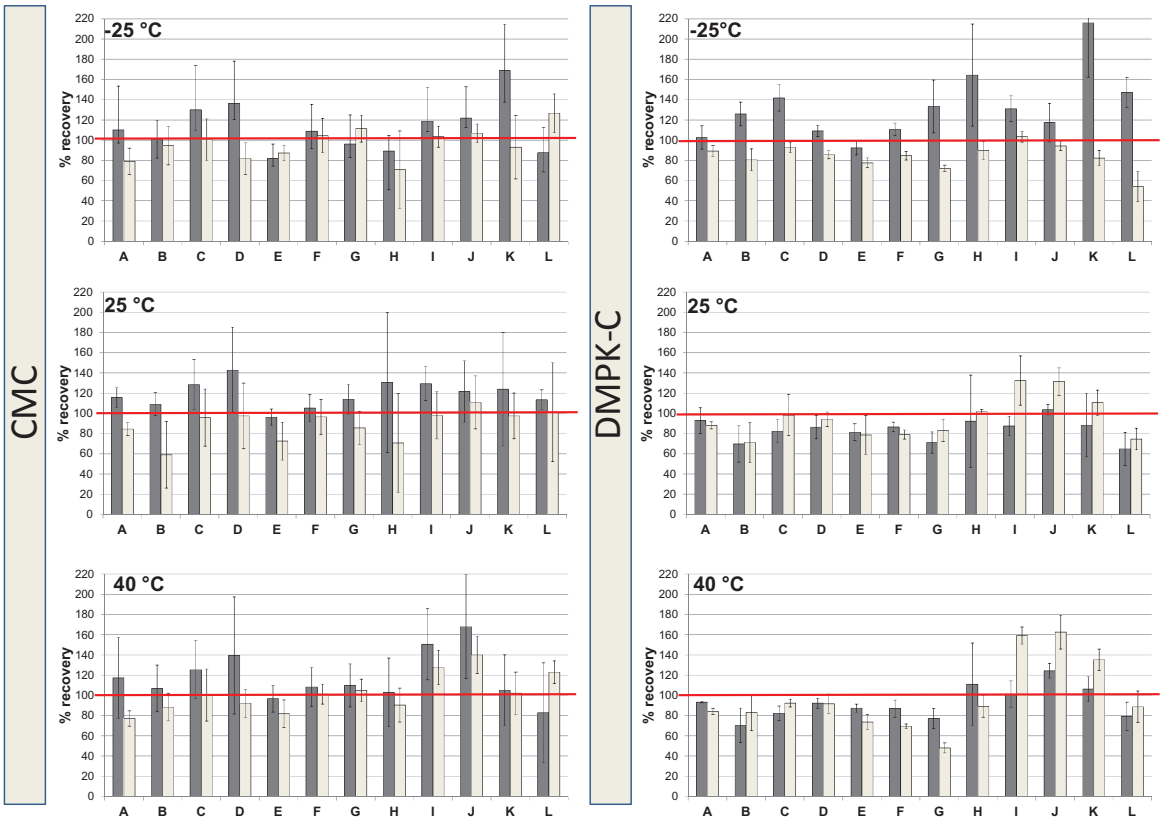


Figure 3.8: Percentage recovery of the signature peptides relative to reference sample (100 % samples shown by the red line). *The samples were stored at one week (dark grey) and four weeks (light grey) at different temperatures and on different sampling materials. The peptide A and B are generated from carbonic anhydrase II, peptide C and D are generated from catalase, peptide E and F are generated from cytochrome c, peptide G and H are generated from β-lactoglobulin, peptide I and J are generated from myoglobin and peptide K and L are generated from transferrin. Reprinted with permission from Paper III © (2017) Springer International Publishing AG.*

Paper II and *III* showed that the stability of high abundant proteins in general was acceptable for DBS stored in both CMC and DMPK-C at RT for one week. However, the stability of low abundant proteins may be more labile during storage as shown for hCG (*Paper I*). Degradation of hCG (two different concentrations) was shown for both concentrations (-23 % and -41 % of reference respectively) when sample (blood spotted on CMC) was stored in RT for 45 days. Degradation of this protein was also shown during storage for shorter periods in RT (7 and 14 days). Except for this observation, no clear link was seen between the stability and the protein property (*Paper III*). Another important observation was that the analyte stability was not negatively influenced by the water-soluble material (CMC) compared to non-soluble material (DMPK-C).

3.2.5 Hematocrit

The effect of blood hematocrit was evaluated in *Paper V* for six proteins with different physicochemical properties. Hematocrit (hct) bias was evaluated for the sampling material DMPK-C and the VAMS. The water-soluble material (CMC) was excluded from the hct evaluation as the blood sample was not uniformly spread on this material. Blood with high (60 % red blood cells), mid (40 % red blood cells) and low (20 % red blood cells) hematocrit values were evaluated in DBS sampling on DMPK-C and with VAMS, and the result are shown in Table 3.4.

Table 3.4: Hematocrit bias for DMPK-C and VAMS: *percentage recovery (± standard deviation) of protein for hematocrit (hct) of 20 % and 60 % relative to hct of 40 %. Reprinted from Paper V.*

Protein	**DMPK-C**		**VAMS**	
	20 % hct	60 % hct	20 % hct	60 % hct
Carbonic anhydrase II	72±5 %	100±5 %	125±5 %	115±4 %
Catalase	63±3 %	105±12 %	120±6 %	123±14 %
Cytochrome c	47±5 %	127±18 %	94±8 %	123±10 %
β-lactoglobulin	62±5 %	132±18 %	116±15 %	133±7 %
Myoglobin	134±8 %	106±46 %	203±23 %	126±12 %
Albumin	23±4 %	128±28 %	59±3 %	104±7 %

VAMS is claimed to sample a fixed volume of blood independent on the blood hematocrit, but in the present study both DMPK-C and VAMS were shown to be influenced by the blood hct. For DMPK-C samples low hct (20 %) blood resulted in decreased analyte signal compared to 40 % hct, and some of the proteins also showed significantly increased signal for the 60 % hct compared to the 40 % hct. A positive correlation between analyte signal and hct has also been shown in several other studies where pure cellulose-based sampling materials were used[26,27,61]. For the VAMS samples, no clear trend was seen between the blood hct and the analyte signal. Three of the proteins (carbonic anhydrase II, catalase and myoglobin) showed significantly increased signal for low hct (20 %) samples compared to the mid hct (40 %) samples. A significant increased signal for four of the proteins (carbonic anhydrase II, catalase, cytochrome c and β-lactoglobulin) was also observed for the high hct (60 %) compared to the mid hct (40 %). VAMS has previously been proven to be unaffected by volume bias related to hct[188]. However, hct can in addition to volume bias influence both the analyte recovery and the amount of matrix effects. The influence of hct

on VAMS samples (as demonstrated in *Paper V*) could therefore be due to recovery or matrix effects (or combination of both) rather than different blood volume absorbed.

3.2.6 Measuring low abundant protein from DBS

hCG was in *Paper I* analysed on a triple quadrupole coupled to a microbore LC (column ID of 1 mm). Limit of detection (LOD) for hCG was in this paper estimated to be 100 IU/L (0.1 IU/mL), twenty times higher than the previously established LOD for analysis of hCG from 1 mL serum sample[127]. The volume of the DBS samples (15 µL) was 67 times lower than 1 mL of serum and a more sensitive instrument was hence needed for measuring hCG at reference levels from DBS. The LC-MS method for hCG was in *Paper IV* transferred to a new triple quadrupole instrument, coupled to a nanoLC (column ID of 0.075 mm). An increase in the signal intensity of the signature peptide from hCG was expected due to both new MS instrument (with nanoESI) and the reduction in column ID. With the new instrument (combined with immunoaffinity clean-up) a LOD of ~ 5 IU/L was established from DBS samples using both CMC and DMPK-C as sampling material. Comparable LOD with the published paper[127] using 1 mL serum was therefore obtained with only 15 µL sample. This is promising results for using LC-MS in detection of low abundant proteins from microvolumes of samples such as DBS.

3.2.7 Evaluation of LOD in different matrices used for DMS

A small evaluation of different matrices (Dried Matrix Spots, DMS) possible to sample in detection of hCG was performed in *Paper IV,* with both DMPK-C and CMC as sampling material. Serum, plasma and urine were evaluated together with whole blood (*as described in section 3.2.6 Measuring low abundant protein from DBS*) for determination of LOD of hCG. Chromatograms from blank matrix and spiked matrix (hCG concentration at 20 IU/L (58pM)) were used for calculation of the LODs. Examples of chromatograms for both hCG (20 IU/L) and blanks are shown in Figure 3.9.

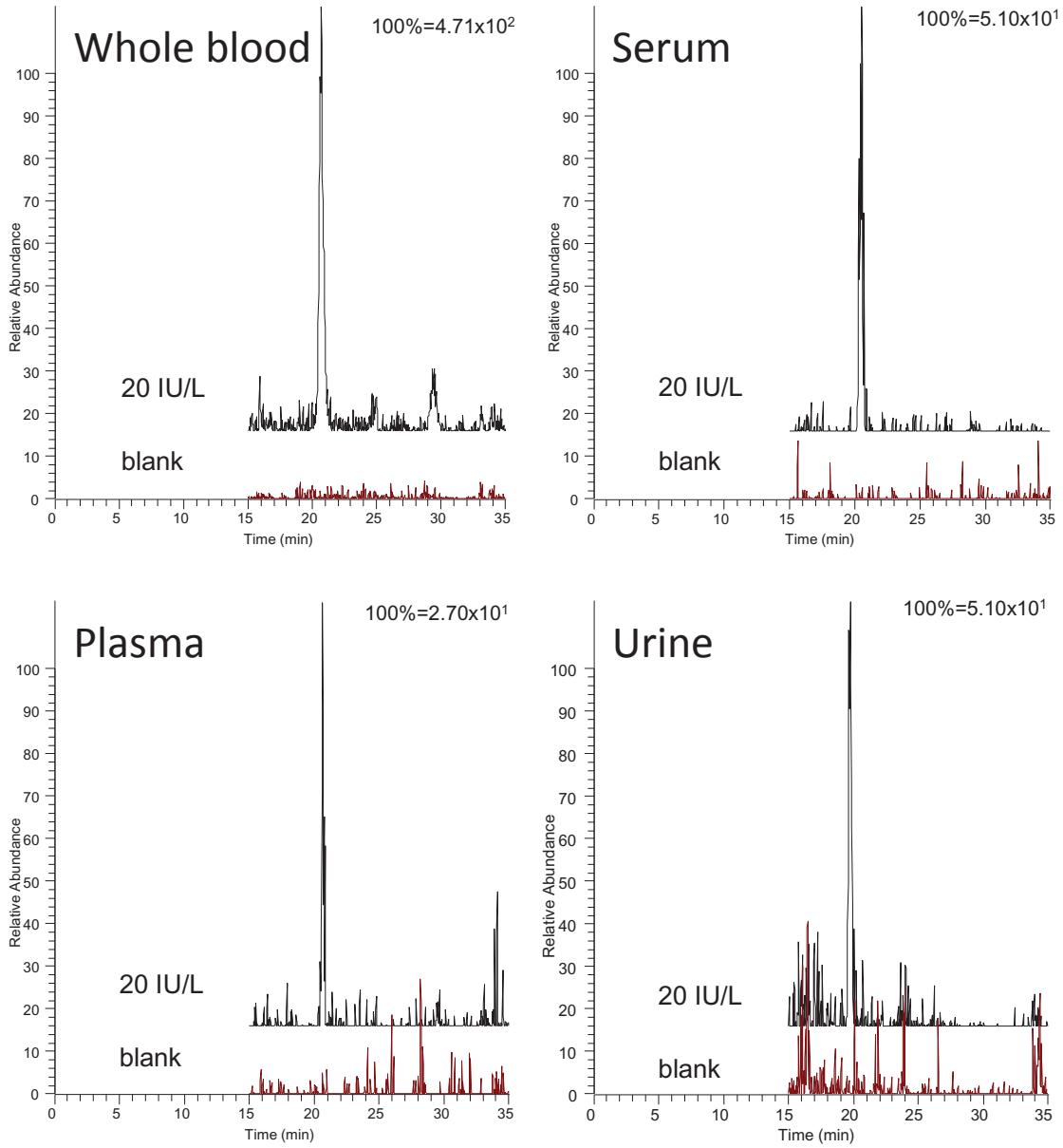


Figure 3.9: Chromatograms of the signature peptide of hCG from whole blood, serum, plasma and urine spotted on DMPK-C. *Preparation of blank (non-spiked) DMS (lower), and DMS spotted with samples spiked at low levels (20 IU/L (58 pM), upper). Reprinted from Paper IV.*

The calculated LODs for hCG (from both DMS on CMC and DMPK-C) are shown in Table 3.5 and were between 2.1-10.5 (6.1-30.5 pM). CMC gave in general lower LODs than DMPK-C except for whole blood. Challenges related to the precision of the signal from the plasma samples were observed and were proposed to be due to the anticoagulant used as also observed in *Paper I*. Apart from these observations, no major differences were observed between the two materials and the different matrices. The lower LODs obtained with CMC may be explained by the water-solubility of the material increasing the recovery of sample (probably more important when analysing low protein concentrations). The

lowest LOD (2.1 IU/L (6.1 pM)) was seen for urine (sampled on CMC) and was comparable with the LOD obtained for 1 mL of urine as previously shown[127].

Table 3.5: The detection limits (IU/L (pM)) obtained using the different sampling materials and sample matrices*. Reprinted with permission from Paper IV.*

Sampling material	Sample matrix	Limit of detection[1] (IU/L (pM))
DMPK-C	Whole blood	5.0 (14.5)
	Plasma	8.9 (25.8)
	Serum	7.8 (22.6)
	Urine	10.5[2] (30.5)[2]
CMC	Whole blood	4.9 (14.2)
	Plasma	6.4 (18.6)
	Serum	3.0 (8.7)
	Urine	2.1 (6.1)

[1] *n=5 and based on S/N=3*
[2] *n=3 and based on S/N=3*

3.2.8 Evaluation of linearity, precision and accuracy

The potential for using DMS in quantitative determination of proteins by LC-MS was briefly evaluated in *Paper I-II* and *Paper IV-V*. The results are summed up in Table 3.6.

Acceptable linearity (R^2=0.959-0.999), precision (RSD ≤ 22%, n ≥ 5) and accuracy (71-123% of true concentrations, n ≥ 3) were in general demonstrated for the different protein analytes in *Paper I-II* and *Paper V*. These data were all obtained for mid- to high abundant proteins. *Paper IV* demonstrated the quantitative performance at low pM-levels (10-1000 IU/L) of hCG using serum as matrix for DMS, immunoaffinty sample clean-up and analysis by nanoLC-MS/MS. Linearity was obtained for both DMPK-C (R^2=0.930) and CMC (R^2=0.940) in the applied concentration area, and precision and accuracy was estimated as ≤ 31 % (RSD) and 95-106 % (% of true concentration) respectively. The RSDs observed here were slightly increased and the linearity lower compared to the method where 1 mL serum (R^2 =0.997 and RSD ≤ 19 %)[127] was used. This was probably due to the lower sample volume (15 µL) used in the present study. A patient serum sample (from a patient diagnosed with testicular cancer) was also deposited (15 µL) on both materials and analysed with the immunoaffinity nanoLC-MS/MS method. The hCG concentration of the patient sample was estimated to be ~ 5000 IU/L from both CMC (5060±430 IU/L) and DMPK-C (5280±595 IU/L). These results demonstrated the possibility of using both commercially available DMS sampling materials and water-soluble DMS sampling materials for quantification of low-endogenous biomarkers from only 15 µL of sample. However, improvement of the quantitative performance and

analysis of a large set of patient samples are required to verify the potential for use in clinical settings.

Table 3.6: Quantitative data from analysis of proteins from DMS: *Linearity (R^2), precision as percentage (RSD) and accuracy (as percentage of true concentration) are shown for the proteins analysed from CMC, DMPK-C and VAMS at low and high concentrations (C).*

Protein	Material	Matrix	R^2	Precision		Accuracy	
				Low C	High C	Low C	High C
hCG (Paper I)	CMC	Blood	0.959	22 %	14 %	116 %[a]	
hCG (Paper IV)	CMC	Serum	0.940	31 %	29 %	96 %	95 %
hCG (Paper IV)	DMPK-C	Serum	0.930	13 %	29 %	106 %	97 %
Insulin (Paper II)	CMC	Blood	0.980	15 %	6 %	122 %	123 %
Carbonic anhydrase II (Paper V)	VAMS	Blood	0.983	14 %	16 %	71 %	96 %
Catalase (Paper V)	VAMS	Blood	0.999	18 %	4 %	101 %	98 %
Cytochrome c (Paper V)	VAMS	Blood	0.999	3 %	6 %	91 %	97 %
B-lactoglobulin (Paper V)	VAMS	Blood	0.998	ND[b]	7 %	ND[b]	89 %
Myoglobin (Paper V)	VAMS	Blood	0.999	ND[b]	15 %	ND[b]	87 %
Albumin (Paper V)	VAMS	Blood	0.985	19 %	13 %	79 %	98 %

[a] *Average accuracy*
[b] *Not determined*

3.3 Non-targeted protein analysis of DBS

DBS is a rich source of endogenous proteins and can therefore be useful for non-targeted proteomics-based studies. A small evaluation of different materials in non-targeted analysis of DBS was initially performed (data not published). Non-targeted analysis of DBS was further demonstrated in *Paper VI* using an ion mobility technique for additional online fractionation of the peptides in the sample. Both studies examined the amount of protein information possible to achieve from DBS samples and the results are discussed in the last part of this thesis.

3.3.1 Different sampling materials

Different sampling materials were evaluated in non-targeted analysis of DBS (data not published). In addition to DMPK-C, CMC and VAMS (as also tested in targeted analysis), the plasma sampling card Noviplex was included in this study. A published sample preparation method used for clean-up of DBS for non-targeted protein analysis was used in the present work[163], including elution/dissolution with sodium deoxycholate (SDC),

digestion, precipitation (removal of SDC and CMC) and SPE followed by DDA (fragmentation of the 15 most abundant ions). Table 3.7 shows the number (both average and total) of unique peptides and corresponding proteins found using the four different DBS materials. Around 300 peptides corresponding to only 70 high abundant proteins (both average and total) were seen from the DBS samples, independent of the sampling material used. This was a reduction in the number of detected proteins compared to the number of detected proteins found by others, using approximately the same sample preparation procedure[163]. No differences were either observed regarding the type of proteins detected from the different materials. The reason for the lower number of observed peptides and proteins compared to the published paper is unknown, but differences in sample preparation and the type of analysis instrument used could be possible explanations.

Table 3.7: The average number and the total number of non-redundant peptides and proteins found from different DBS materials.

	Average		Total non-redundant[a]	
Material	**Peptides**	**Proteins**	**Peptides**	**Proteins**
DMPK-C	336	63	422	72
VAMS	291	58	413	78
Noviplex	358	58	436	78
CMC	358	68	434	81

[a]*Combining data from all replicates (n=3)*

The results from this experiment highlighted the need for further preparation of the sample to increase the number of detected proteins. Increasing the number of detected peptides and proteins may also reveal differences in performance between the four sampling materials.

3.3.2 FAIMS as a tool in non-targeted protein analysis

High Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS) was demonstrated for non-targeted bottom-up protein analysis of DBS in *Paper VI*. Two different sample preparation procedures were used for DBS (sampled on Ahlstrom grade 226 filter cards) in FAIMS analysis; both methods were fairly simple and contained a limited number of manual handling steps (and a minimum of sample clean-up). One of the preparation techniques for DBS was a simple *punch and elute* of the sample followed by overnight tryptic digest, centrifugation and analysis. The other sample preparation technique was based on a previously published paper[164] where liquid extraction surface analysis (LESA) was used for surface extraction of the DBS samples. The DBS samples were analysed with

both LC-MS/MS (referred to as without FAIMS in the rest of the thesis) and with LC-FAIMS-MS/MS (referred to as FAIMS or with FAIMS in the rest of the thesis). For the FAIMS analysis external CV stepping was applied from CV -55 to -25 using 2.5 stepping (13 runs in total). The sample was re-analysed seven times without FAIMS (eight runs in total). The average number and the total number of peptides and corresponding proteins detected (with or without FAIMS) from both preparation methods are shown in Table 3.8.

***Table 3.8**: **Average number and the total number of unique peptides and proteins detected with and without FAIMS:** detected from DBS using A: punch and elute procedure and B: LESA procedure. Reprinted from Paper VI.*

	Average		Total non-redundant[a]	
Experiment	**Peptides**	**Proteins**	**Peptides**	**Proteins**
A: Punch and elute				
Without FAIMS	191	68	536	162
With FAIMS	124	80	1065	350
B: LESA				
Without FAIMS	288	81	784	187
With FAIMS	119	68	1077	272

[a]*Combining data from all CV-steps (FAIMS, n=13) or data from all runs without FAIMS (n=8)*

The average number of detected peptides and corresponding proteins where not increased by using FAIMS. However, the total number of non-redundant peptides and corresponding proteins was doubled when combining the data from the *punch and elute* sample analysed with 13 different CV steps (FAIMS) compared to re-running (n=8, analytical runs) the sample without FAIMS. For the LESA sample the total number of proteins was increased with 45 % by combining the data from the 13 different CV steps compared to data from a sample run without FAIMS (n=8, analytical runs). Figure 3.10 shows base peak chromatograms of DBS analysed with FAIMS (using three different CV steps) and a chromatogram from a run without FAIMS. The figure illustrates that different information was obtained from the DBS samples when FAIMS was applied, and also indicated that different information was obtained from the different CV steps.

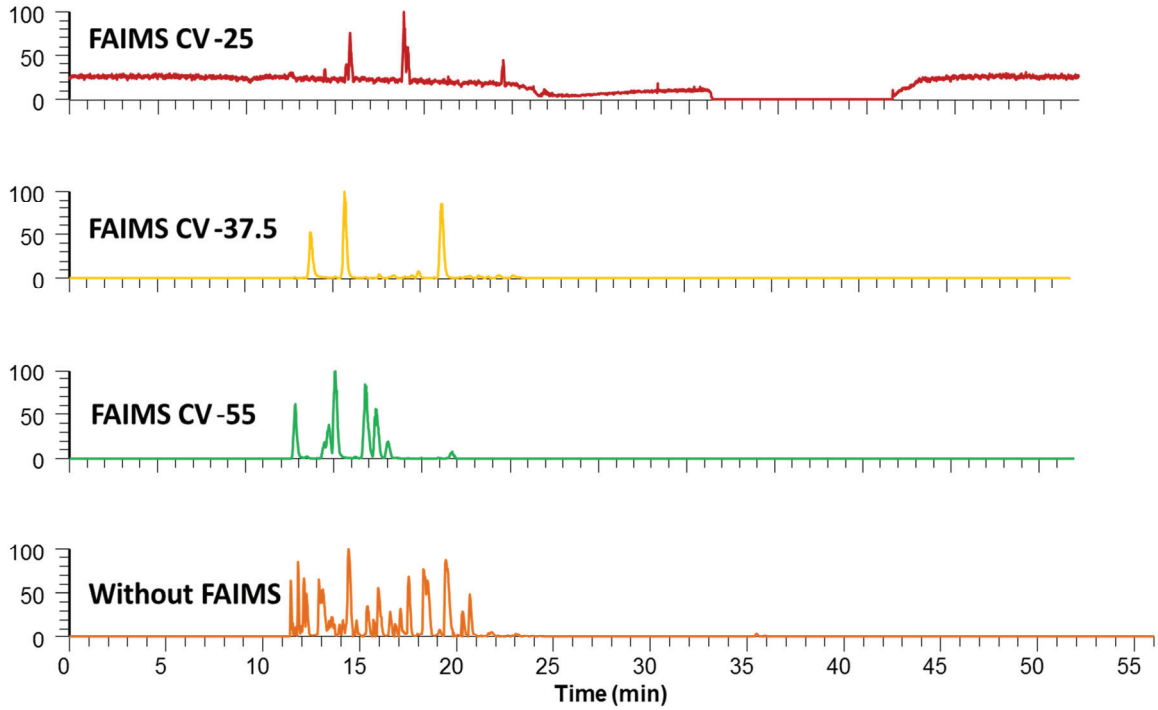


Figure 3.10: Base peak chromatogram of a LESA prepared sample*: Fullscan from analysis with FAIMS (CV-25, -37.5 and -55) and a run without FAIMS. Normalization level CV -25: 4.75E5, CV -37.5: 1.45E7, CV -55:3.80E6 and without FAIMS: 1.60E9. Reprinted from Paper VI.*

Examination of the redundancy between proteins detected with three different CV steps (CV -55, -37.5 and -25) also confirmed that the different CVs provided different information. In total was 173 proteins (*punch and elute* data) found from combining CV -55, -37.5 and -25, however only eight (≤ 5 %) of the proteins were found in all three CV steps. LC-FAIMS-MS/MS was also shown to work complementary with LC-MS/MS as 30 % of the proteins (found in total from *punch and elute* samples) was detected with both approaches. The same was also observed for the LESA samples. The drawback related to FAIMS was the increased instrument time required to run through the several CV steps. However, the time and labour spent on sample preparation were minimal. FAIMS was concluded to be beneficial in non-targeted analysis of DBS as FAIMS increased the proteome coverage.

4. CONCLUDING REMARKS

This thesis has demonstrated the potential of combining Dried Blood Spots (DBS) and both targeted and non-targeted Liquid Chromatography Mass Spectrometry-based (LC-MS-based) protein analysis. The key findings are discussed below:

Targeted LC-MS protein analysis of DBS was presented in *Paper I-V*. Different materials were demonstrated for sampling of microvolumes of biological matrices in the targeted approach. The water-soluble material (CMC) provided complete recovery of most of the protein analytes from the sampling material, and also showed comparable analyte recovery with the pure cellulose-based material (DMPK-C). The Volumetric Absorptive Microsampling (VAMS) device provided lower recovery for some proteins (compared to both DMPK-C and CMC) for the applied extraction time. Both CMC and DMPK-C was used as sampling material for stability studies and no major difference was observed regarding the stability in these two materials. Bias due to hematocrit was shown for both DMPK-C and VAMS device.

Model proteins with different physicochemical properties was also detected and quantified from DBS in targeted LC-MS-based protein analysis. Insulin was more prone to sticking to the DBS sampling materials, probably explaining the incomplete recovery of this protein from the sampling materials. Some proteins were also shown to have lower recovery from the VAMS compared to DMPK-C and CMC, without any clear relationship to the protein property. Mid- and high abundant proteins were in general shown to be stable at room temperature for at least one week in DBS samples, while the low abundant protein showed acceptable stability only when stored at reduced temperature.

The combination of immunoaffinity clean-up together with state-of-the-art nanoLC-MS enabled detection and quantification of the low abundant protein, human chorionic gonadotropin (hCG), in endogenous concentrations from dried urine, plasma, serum and blood spots. A brief evaluation of the quantitative performance was done by using Dried Serum Spots (DSS), and quantitative determination of the low abundant biomarker, hCG, from a real patient serum sample (spotted om sampling material, DSS) was demonstrated. These results showed the possibility of using DSS for quantification of low-endogenous biomarkers.

Non-targeted LC-MS-based protein analysis of DBS was also evaluated. Four different sampling DBS materials were initially tested for sampling in non-target protein analysis.

Only high abundant proteins were detected from DBS, and no differences were seen regarding the number or the type of proteins detected in the different materials. Further development of sample preparation or newer instrumentation is probably required to reveal differences between the four materials. The power of DBS for protein discovery studies was however demonstrated combined with High Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS). FAIMS doubled the total number of detected proteins compared to sample run without FAIMS and was also shown to work complementary to LC-MS analysis without FAIMS.

The work in this thesis has contributed to useful knowledge for future developments and application of microvolumes of dried biological samples. DBS has shown great potential for both targeted and non-targeted LC-MS-based protein analysis. However, advanced analytical tools such as nanoLC and FAIMS are necessary to achieve the required sensitivity for measuring low abundant proteins or the required fractionation for use in proteome discovery studies. In addition, there is still a need for optimization of sampling materials for DBS in order to achieve high and reproducible recoveries together with reduction of the hematocrit bias.

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ERRATA LIST

Name of candidate:

Cecilie Rosting

Title of thesis:

Dried Blood Spots in Liquid Chromatography Mass Spectrometry-based Protein Analysis

Errata:

Page	Figure	Original reference number	Corrected reference number
14	Figure 1.2	46	45
18	Figure 1.4	77	70
28	Figure 1.9	133	134