1 **Volumetric Absorptive MicroSampling vs. Other Blood Sampling**

2 **Materials in LC-MS-based Protein Analysis – Preliminary**

3 **Investigations**

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5 Ida Kristine Lysgaard Andersen, Cecilie Rosting, Astrid Gjelstad, Trine Grønhaug Halvorsen

6 Department of Pharmaceutical Chemistry, School of Pharmacy, University of Oslo, Norway

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15 Corresponding author:

16 *Trine Grønhaug Halvorsen

17 Dept. Pharmaceutical chemistry

18 School of Pharmacy

19 P.O. Box 1068 Blindern

20 NO-0316 Oslo

21 Norway

22 Fax: +47 22854402 E-mail: t.g.halvorsen@farmasi.uio.no

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25 **Abstract**

26 The aim was to evaluate the performance of different microsampling materials in LC-MS-
27 based protein analysis. The evaluated materials were the Volumetric Absorptive
28 MicroSampling (VAMS) device, the pure cellulose sampling material (DMPK-C) and the
29 water-soluble material (carboxymethyl cellulose, CMC), with the main emphasis on VAMS.
30 Six proteins with different physicochemical properties were used as model proteins. A quick
31 and generic sample preparation consisting of extraction/dissolution of the blood spot, tryptic
32 digestion and subsequent matrix precipitation was applied prior to analysis. The recovery
33 from VAMS, compared to DMPK-C, was dependent on the protein analyte: Lower recovery
34 compared to DMPK-C was seen for β -lactoglobulin and myoglobin (74 % and 80 %, respectively)
35 while higher recovery compared to DMPK-C was seen for cytochrome c and
36 albumin (149 % and 197 %, respectively). The recovery from CMC was comparable to the
37 recovery from DMPK-C for all proteins except for cytochrome c (76 %). Hematocrit bias was
38 evaluated for DMPK-C and VAMS, and the blood hematocrit influenced the protein analysis
39 from both the materials. A preliminary evaluation was performed for VAMS: The correlation
40 coefficient ($R^2 \geq 0.983$), the accuracy (71-101 %) and the precision ($RSD \leq 20$ %) were
41 determined for blood spiked with the six model proteins.

42

43 **Abbreviation:**

44 ABC, ammonium bicarbonate; CMC, carboxymethyl cellulose; DBS, Dried Blood Spots; FA,
45 formic acid; hct, hematocrit; VAMS , volumetric absorptive microsampling

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47 **Keywords:** LC-MS; proteins; Dried Blood Spot; microsampling

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50 **1. Introduction**

51 Dried Blood Spots (DBS) has gained increased interest the latest years due to the many
52 advantages offered by this sampling technique. DBS was introduced already in the 1960s [1]
53 as an easy method for sampling of whole blood from newborns, and is today used to screen
54 newborns for several inborn disorders [2]. The DBS sampling technique is also of interest in
55 analysis of both small molecules and larger molecules such as proteins in various fields such
56 as drug discovery and development [3-6], therapeutic drug monitoring [7-11], doping analysis
57 [12, 13], toxicology [14], forensics [15] and in analysis of biobank material [16].

58 Immunoassay-based techniques have been the gold standard for protein analysis in the clinics,
59 but LC-MS has gained increased interest as a tool for protein analysis due to multiplexing
60 possibilities and the high specificity of this instrument [17, 18]. In recent years, several papers
61 describing DBS and LC-MS-based protein analysis have been published [12, 19-23].

62 Different materials and devices are available for DBS sampling. The most common sampling
63 material for DBS is pure cellulose-based materials (sampling cards). However, some
64 challenges are related to these DBS materials: One of the challenges is related to the influence
65 of hematocrit (the volume fraction of red blood cells in a blood sample) on the quantitative
66 assay. Hematocrit is important in determination of the blood viscosity, and hence the
67 spreading of the blood sample on a DBS sampling card. Several papers have demonstrated
68 that the hematocrit value of the blood influences the quantification of analyte from the DBS
69 samples [24-26], and alternative sampling materials or hematocrit markers have been
70 introduced to avoid the hematocrit bias [27-29]. The company Neoteryx launched in 2014 a
71 Volumetric Absorptive MicroSampling (VAMS) device, claimed to absorb a fixed volume of

whole blood independent of the hematocrit value [30]. Another challenge is related to the elution of sample from the microsampling materials (both VAMS and DBS cards), which can be both time-consuming and incomplete. Incomplete elution is especially a challenge for achieving low detection limits. Our group has worked with water-soluble materials to address these challenges [31-33].

The aim of the present paper was to evaluate some key parameters in targeted protein analysis using a simple sample preparation procedure and different microsampling materials/devices, with special focus on the VAMS device. Basis for the work was a paper previously published by our group [34] evaluating the method performance of proteins with different physicochemical properties from DBS using two different sampling materials (pure cellulose sampling material, DMPK-C and the water-soluble sampling material carboxymethyl cellulose, CMC) [34]. The main emphasis in the previously published paper was on method development and sample preparation from the water-soluble material. This method was subsequently applied to both water-soluble and pure cellulose material, demonstrating comparable recoveries for all monitored peptides except one. In addition, the storage stability of the proteins from both materials was evaluated.

In the present study five of the six model proteins (with different physicochemical properties (size, *pI* and hydrophobicity) applied in the previous paper were included together with the common model protein, bovine serum albumin. Isotopically labelled peptide internal standards were implemented (one per protein) to increase reproducibility of the assay. Focus was on quantitative method performance parameters such as effect of blood hematocrit, matrix effects, response curve, repeatability and accuracy using concentration levels corresponding to the levels of medium to high abundance proteins in blood (μM -range). These parameters were not evaluated in the previous study [34]. Three sampling materials/devices were evaluated: DMPK-C, VAMS and CMC. VAMS has mostly been

studied for small molecules [35-40] and to our knowledge this sampling device has only been evaluated for the small peptide, hepcidin [41] and for six mid- to high-abundance endogenous proteins [42]. The present paper will by such represent a valuable addition to the knowledge about VAMS. In addition, to our knowledge, this is also one of the first papers describing hematocrit effect of different materials/devices in LC-MS bottom-up protein analysis.

2. Materials and methods

2.1 Chemicals

The model proteins carbonic anhydrase lysozyme II from bovine erythrocyte, catalase from bovine liver, cytochrome C from bovine heart, β -lactoglobulin from bovine milk, myoglobin from equine heart and bovine serum albumin were all purchased from Sigma-Aldrich (St. Louis, MO, U.S.A). The chemicals 4-dithiothretiol (DTT), iodoacetic acid (IAA), tosyl phenylalanyl chloromethyl ketone (TPCK) treated trypsin from bovine pancreas, formic acid (FA) and ammonium bicarbonate (ABC) were also purchased from Sigma-Aldrich. LC-MS grade acetonitrile (MeCN) was obtained from Merck (Darmstadt, Germany). Internal standards (isotopically labelled peptides: QSPVDIDT[K- $^{13}\text{C}_6$ - $^{15}\text{N}_2$] (for bovine carbonic anhydrase II), NFSDVHPEYGS[R- $^{13}\text{C}_6$ - $^{15}\text{N}_4$] (for bovine catalase), TGPNLHGLFG[R- $^{13}\text{C}_6$ - $^{15}\text{N}_4$] (for bovine Cytochrome C), VYVEELKPTPEGDLEILLQ[K- $^{13}\text{C}_6$ - $^{15}\text{N}_2$] (for bovine beta-lactoglobulin), LFTGHPETLE[K- $^{13}\text{C}_6$ - $^{15}\text{N}_2$] (for equine myoglobin), DAFLGSFLYEYS[R- $^{13}\text{C}_6$ - $^{15}\text{N}_4$] (for bovine albumin)) were ordered from Innovagen AB (Lund, Sweden).

Whole blood was donated in BD vacutainer[®] K2 EDTA tubes by four voluntary persons. The initial experiments were performed using blood donated from one person, while the linearity, accuracy and precision experiments were performed by using pooled blood from four persons.

2.2 Preparation of stock solutions

Preparation and storage of the stock solutions of the model proteins have previously been described [34]. Stock solutions (1 mg/mL) of the internal standard were made in pure water for all peptides except for the internal standard for albumin (water:MeCN (80:20) was used). The stock solutions were aliquoted and stored in the freezer (-25°C) for maximum one month prior to use.

2.3 Preparation of DBS

All samples were freshly prepared on the day of the experiment. Whole blood was thawed on the day of the experiment (except for the hematocrit evaluation where freshly drawn whole blood was used). The whole blood was spiked (10 % spike volume) with the stock solution to a concentration of 30 µM for each protein (lower concentrations were prepared for the response curves). Three different sampling materials were used: The pure cellulose-based sampling card FTA[®] DMPK-C (GE Healthcare, Fairfield, CT, U.S.A), the water-soluble material, carboxymethyl cellulose (purchased as the wound dressing product Aquacel[®] Hydrofiber[™] (Convatec Skillman, NJ, U.S.A)), and VAMS (Mitra[®] from Neoteryx[®], Torrance, CA, U.S.A). Ten µL of blood were deposited on either a piece of Aquacel or a DMPK-C sampling card using a mLine 10 µL pipette (Biohit/Sartorius, Gröttingen, Germany), or absorbed onto the VAMS following the manufacturers guidelines by dipping the tip into a vial containing the blood (average blood wicking volume for VAMS calculated by producer and specified in the product sheet: 10.1 µL). The samples were always gently vortexed (500 rpm) for at least five minutes using a Thermomixer prior to spotting/absorbing the sample onto sampling cards or absorbing the samples with VAMS. Using all materials the samples were dried for two hours in room temperature (~25 °C) prior to further processing. For

DMPK-C and CMC the whole spot was cut out from the material and transferred to a 1.5 mL Eppendorf® protein LoBind tube (Eppendorf®, Hamburg, Germany). For preparation of VAMS the sampling tip of the VAMS was separated from the stick and transferred to an Eppendorf tube. The water-soluble DBS was dissolved within 30 minutes in 160 µL of 100 mM ABC buffer (pH ~ 8) using rigorous vortexing (1400 rpm) by Thermomixer (Eppendorf®, Hamburg, Germany). The commercial sampling card and the VAMS were extracted in 160 µL of 100 mM ABC for approximately 30 minutes using the same Thermomixer.

2.4 Tryptic digest

The proteins were reduced, alkylated and digested as previously described [34] by adding a volume of 4 µL of 500 mM DTT (reduction at 60 °C, 15 min), 6 µL 2000 mM IAA (alkylation at 25 °C in dark, 15 min) and 10 µL of 2.5 mg/mL trypsin. The samples were digested overnight at 37 °C (with the microsampling materials present in the buffer during the digest).

2.5 Matrix precipitation

The matrix precipitation was performed as previously described [34], the only difference was the inclusion of internal standard peptides. A volume of 20 µL of the internal standard (mixture consisting of 0.99 µM carbonic anhydrase II-IS, 0.71 µM catalase-IS, 1.7 µM cytochrome C-IS, 8.6 µM beta-lactoglobulin-IS, 1.6 µM myoglobin-IS and 0.63 µM albumin-IS) was added to the samples after the tryptic digestion. A volume of 1000 µL of icecold MeCN was then added to the samples prior to centrifugation at 14000 rpm (20817g) for 10 min using centrifuge 5804 from Eppendorf (Hamburg, Germany). Finally 500 µL of the supernatant was evaporated by a vacuum centrifuge (SpeedVac™) from Thermo Scientific (Rockford, IL, U.S.A) and the residue was redissolved in 50 µL of 20 mM FA.

2.6 Relative recovery

The relative recovery was evaluated by depositing 10 μ L of blood spiked with the six model analytes onto DMPK-C and CMC, while absorbing 10 μ L of the blood onto VAMS. The sample was eluted/dissolved overnight (during the tryptic digest) as described in [21] by using 180 μ L 100 mM ABC. Further clean-up of the samples was performed as described in the previous section (2.5 Matrix precipitation). The signal (peak area analyte/peak area IS) for all the six proteins from the three different sampling material was compared. The data is shown as percentage signal relative to the signal obtained from DMPK-C.

2.7 Hematocrit effect

Blood with different hematocrit was prepared by using the method proposed by Koster et al [43]: Fresh whole blood was drawn and kept in the fridge for maximum one week (within the recommendations described in [44, 45]. The blood was allowed to reach room temperature and gently mixed by rotating the vial to redistribute the blood cells after the storage. The initial hematocrit of the blood was measured by using the *Hematocrit 4* centrifuge from Meszansky (Oslo, Norway). The centrifugation of the blood (by the hematocrit 4 centrifuge) separated the blood into plasma and red blood cells. Cell lysis was therefore easy to detect by any change in color (pink/red) of the plasma (or unsuccessful separation of the red blood cells and plasma). Plasma was either removed (whole blood centrifuged for 5 minutes at 2000 rcf) or added to the whole blood to create blood with hematocrit of 20 %, 40 % and 60 % (percentage of red blood cells). The *Hematocrit 4* was used to confirm the hematocrit value after the adjustment. For the hematocrit experiments a larger volume of whole blood (30 μ L) was deposited onto the DMPK-C sampling card as only a part of the spot was punched out (3 mm diameter). Internal standard was added to the samples as previously described and the

signal ratio (signature peptide/internal standard) from the different hematocrit blood was compared.

2.8 Matrix effects

Matrix effects were evaluated quantitatively as described by Matuszewski et al. [46] by preparation of non-spiked DBS samples on CMC, DMPK-C and VAMS. The samples were cleaned up as previously described and re-dispersed in 20 mM FA containing a digest of the protein analytes of interest and the internal standards and analysed (=matrix samples). Reference samples were prepared in 20 mM FA using the same digest as when preparing the matrix samples (=matrix-free samples). The level of the digested proteins and the internal standards were the same in both matrix samples and matrix-free samples. After analysis peak areas of the analytes in the matrix samples were compared to the peak areas of analytes the matrix free samples and presented as percentage relative to the reference sample to evaluate the effect of the matrix on the MS-signal. The comparison was performed both with and without internal standard correction of the analytes.

2.9 Response curve, precision and accuracy

The whole blood was spiked with the six proteins in a concentration range from 3 μ M to 30 μ M (3 μ M, 6 μ M, 9 μ M, 20 μ M and 30 μ M) before absorbing the blood onto the VAMS (n=5 for 3 μ M and 30 μ M, n=3 for the other levels). The precision (% RSD) was evaluated for the lowest and the highest concentration using all parallels (n=5). The accuracy was determined at low (3 μ M) and high (30 μ M) concentration level using two of the five parallels as unknown samples. A new response curve was made for the rest of the samples and used for calculating the concentration of the two left-out samples (i.e. not included in the response curve). In order to avoid bias when selecting the unknown samples it was decided which samples to use as unknowns prior to analysis of the samples. The accuracy was calculated based upon the average of two samples.

2.10 NanoLC-SRM-MS

The analytical system used for the analysis is described in the previous paper [34] and consisted of a UltiMate™ 3000 RSLCnano (including a trap system) coupled to TSQ Quantiva (both from Thermo Scientific, Bremen, Germany). The columns, mobile phases, flow (both loading and nano pump), injection volume and ESI parameters are also previously described [34]. The column temperature was set to 60 °C.

The heavy labelled peptides were incorporated in the existing SRM method and only peptides with corresponding internal standard were monitored. Fragment ions from the C-terminal (y-ions) of the internal standard were monitored as these fragments contained the labelled part and could therefore be distinguished from the corresponding non-labelled peptide. The SRM transitions for both the signature peptides and the internal standards are listed in **Table 1**.

2.11 Statistics

The significance was tested using Minitab 17 statistical software from (Coventry, UK). A two-sample T-test (two sided alternative) and a significance level (α) at 0.05 were applied.

3. Results and discussion

This work was a follow-up of a previously published paper from our group describing LC-MS analysis of six proteins of different physicochemical properties from DBS [34]. The six proteins used in the present project are listed in **Table 1** (overview of physicochemical properties see supplementary table S3). A few changes from the method used in the previous paper were introduced in the present study: A new protein analyte was included (bovine serum albumin) and isotopically labelled internal standards were available. The main change was the inclusion of a third microsampling material: Volumetric Absorptive Microsampling (VAMS) which sampled a fixed volume of ~10 μ L. The sampling volume was therefore

increased to 10 μ L whole blood for the two other sampling materials (CMC and DMPK-C). The elution (DMPK-C and VAMS) and the dissolution (CMC) were performed by using 100 mM ABC buffer as this buffer is directly compatible with both the following tryptic digestion step and the LC-MS analysis (volatile buffer). The buffer concentration was optimized previously [34]. Frozen blood was used for all experiments except for the hematocrit experiment as intact red blood cells were essential when investigating the effect of hematocrit.

3.1 Implementation of internal standards

Isotopically labelled internal standards were available for one of the signature peptides from each protein. The selection of signature peptides is described in supplementary material. The SRM transitions of the signature peptides and the internal standard are listed in **Table 1**.

Implementation of the internal standards improved the repeatability of the protein analysis of the DBS samples (using the DMPK-C sampling material). The repeatability (n=5) of the DBS analysis was improved from ≤ 30 % (RSD) to ≤ 13 % (RSD) for all proteins except for albumin (Table S2 in supplementary). The RSD of albumin (RSD 22 %, n=5) was high compared to the other proteins due to poor peak shape of the peptide and carry-over from previous samples. The carry-over was eliminated by increasing the column temperature from 35°C to 60°C as described by others [47, 48]. This also improved the peak shape of albumin (data not shown). **Figure 1A** shows chromatograms of the six signature peptides and their internal standard (all analysed from VAMS). The peaks from the signature peptides were always confirmed by comparing the retention time of the signature peptide with aqueous reference standards and with the internal standard. In addition, the presence of all fragments and the fragment ratio were confirmed for every chromatogram (Typical ratios are shown in Figure S1-6). Chromatograms after analysis of blank blood sampled with VAMS can be seen in **Figure 1B**.

3.2 Relative recovery from the sampling materials

The recovery from CMC and DMPK-C was evaluated in the previously published paper (without internal standards) [34]: High analyte recovery ($\geq 80\%$, although high standard deviations) was seen from the water-soluble sampling material for all proteins. Comparable protein recovery from CMC and DMPK-C was also demonstrated in the previous study. The relative recovery from the different sampling materials was also evaluated in the present study, and both CMC and DMPK-C were re-evaluated together with VAMS as improved repeatability was expected due to inclusion of internal standards. This might also reveal some differences not seen in the previous study.

The protein recovery from CMC and VAMS were calculated relative to DMPK-C and the results are shown in **Figure 2**. The recovery from CMC and DMPK-C was comparable for all the model proteins except cytochrome c (76 % compared to DMPK-C), while only two of the proteins analysed from VAMS had comparable recoveries to DMPK-C. The other proteins showed either increased recoveries (cytochrome c and albumin) or decreased recoveries (β -lactoglobulin and myoglobin) from VAMS compared to the other materials. The difference observed in recovery may be due to both physiochemical properties of the protein analytes and the properties of the sampling material. Different degree of matrix effects from the different sampling materials may also explain the differences in recovery; however evaluation of matrix effects (discussed in section 3.5) did not reveal any change in matrix effects for the different materials. The number of model proteins included in this study is not sufficient to find a clear relationship between analyte recovery and the protein properties. The following observations were still made: The two proteins (carbonic anhydrase II and catalase) which showed comparable recovery from all three sampling materials have similar isoelectric point. Cytochrome c and albumin showed increased recovery from VAMS (149 % and 197 %, respectively) compared to DMPK-C. No common properties were observed between these

two proteins. The recovery of albumin was not evaluated in the previous study, while the recovery of cytochrome c (signature peptide: TGP_NLHGLFGR) was ~ 80 % from CMC [34]. The increased recovery of these two proteins from VAMS showed that this sampling material can be beneficial to increase the recovery for some proteins. β -lactoglobulin and myoglobin showed lower signal intensity (74 % and 80 % respectively) from VAMS compared to DMPK-C and CMC. These two proteins have the highest hydrophobicity of the six model proteins. Reduced recovery from VAMS has previously been seen for hydrophobic analytes (small molecules) [49], and may therefore also explain the reduced recoveries for β -lactoglobulin and myoglobin in the present study.

Optimization of the elution buffer may increase the recovery, as demonstrated for small molecular analytes, of the proteins which showed relatively low recovery from VAMS (β -lactoglobulin and myoglobin) [35, 37]. However, a buffer directly compatible with the following tryptic digest step was required and hence ABC buffer was chosen. The relative recovery for these two proteins was nevertheless high compared to DMPK-C (≥ 74 %) and optimization of the buffer was therefore not crucial. In development of a method for clinical use the robustness of the recovery towards the age of the blood sample and the concentration of the analyte should also be evaluated.

3.4 Influence of hematocrit

The influence of hematocrit (hct) on the analyte recovery was evaluated for the DMPK-C cards and the VAMS device. The water-soluble material (CMC) was not included in this experiment as diffusion of the blood in this material was irreproducible especially for the low hematocrit sample (see Supplementary Figure S7). This was probably due to inhomogeneous composition of the fibers in the material. Fresh blood was used to prepare blood with high,

316 mid and low blood hct: containing 60 % (n=4), 40 % (n=4) and 20 % (n=4) red blood cells.
317 The levels of hematocrit was chosen based on the average hematocrit level in adult humans
318 (female: 36-44 %, male: 41-50 %) [26], and the recommendation of Jager et al of choosing
319 minimum two levels of hematocrit [50]. The results from the evaluation of the hematocrit
320 effect are shown as signal relative to the mid hct value (40 %) in **Table 2**. RSD values of the
321 individual hct values can be found in supplementary Table **S4**.

322 An hct effect was observed for both materials in the present study. DMPK-C showed in
323 general lower signal intensities (23-72 %) from the low hct samples compared to the mid hct
324 samples (except for myoglobin, 134 %). Higher signal intensities were also observed for some
325 of the proteins at high hct level, but only the signal from β -lactoglobulin was significantly
326 different from the signal obtained from the mid hct samples. The reason for the high signal
327 from myoglobin and the exceptional low signal from albumin at low hct is unknown, but may
328 be due to different degree of matrix effects at different hct levels [51], and that the internal
329 standards are not able to correct for this difference as described in [31, 52]

330 For the VAMS samples, no clear trend between the hct level and the signal from the model
331 proteins was seen. Carbonic anhydrase, catalase and myoglobin showed higher signal from
332 low hct compared to mid hct (120-203 %), while albumin showed lower signal (59 %)
333 intensities at this hct level. All the proteins showed higher signal intensities (104-133 %) at
334 high hct level compared to mid hct level, however the signal was significantly different for
335 four of the six proteins (carbonic anhydrase II, catalase, cytochrome c and β -lactoglobulin).

336

337 A positive correlation between the hematocrit and the signal intensities has also been seen in
338 other papers evaluating pure cellulose sampling cards (such as DMPK-C) [53-55]. The data
339 obtained for DMPK-C using blood with different hct was therefore as expected. VAMS has

previously shown to be unaffected by volume bias caused by the blood hematocrit, both for small molecules and proteins [28, 42]. A wider hct range was tested in the present study compared to the paper by Van den Broek et al. [42], which also examined the hct effect for analysis of proteins from blood sampled with VAMS. The use of a wider hct range may explain why an hct effect was shown for VAMS in the present paper. Hct can also influence the recovery of analyte from the sampling material [49, 56], give different degree of matrix effects [51] which might result in both increased ion enhancement and ion suppression. In addition, hct may influence the digest efficiency. Differences in digest efficiency were however unlikely as 10 μ L of blood (eluted from the material) was diluted in a total volume of 200 μ L of buffer prior to digest. Optimization of the elution buffer may eliminate the hematocrit effects, as shown by others [49, 56], but ABC buffer was required in the present project as described in the previous section. To sum up, removal of hematocrit bias will not solely be solved by changing the sampling material and further optimization of elution process or the following sample preparation steps is required to avoid hematocrit bias for the volumetric sampling material.

3.5 Evaluation

A brief evaluation was performed using the six model proteins with different physicochemical properties. Matrix effects were evaluated for all three materials while linearity, precision and accuracy were evaluated for VAMS. Due to limited availability of fresh whole blood the blood used in this brief evaluation had been frozen prior to preparation of the standards/application to the sampling materials. By introducing a freeze-thaw cycle to the blood hemolysis of blood cells is expected. There are some reports on the effect of hemolysis on analytical parameters mainly on small molecules [44, 57] but also some reports on proteins [57, 58]. Hemolysis alters the composition of the blood and might hence influence the parameters evaluated in the present paper due to i.e. increased matrix effects, effects on

recovery and precision. However, we believe that despite this valuable information regarding the applicability of dried micro sampling in LC-MS/MS based protein analysis can be derived from the following experiments, as a method developed using fresh blood is likely to be more robust. The described method was never intended for use in a clinical laboratory as six non-human model proteins were used, the main goal was to gather information about parameters important in quantitative analysis such as hematocrit and the parameters described below. This means that if the quantitative performance of the method is satisfactory using a less ideal matrix (freeze-thawed whole blood) the method most likely will be robust when developing methods for the clinic using fresh blood. Frozen blood was therefore considered satisfactory in order to gather preliminary information about matrix effects in dried microsampling using various sampling materials and for quantitative assessment of VAMS in protein analysis.

Matrix effects

Matrix effects were evaluated quantitatively by preparation of non-spiked blood samples on DMPK-C, VAMS and CMC as described in *Experimental*.

The signal (relative to reference) for each peptide and all three sampling materials/devices is shown in **Table 3**, both without internal standard (A) and with internal standard compensation (B). Both ion suppression and ion enhancement (not always significantly different from reference due to high standard deviations) were observed for most of the signature peptides (relative signal ranging from 33 to 464 % compared to aqueous reference samples). However, the internal standard corrected for these matrix effects (relative signal of 86-103% compared to aqueous reference samples) for most of the proteins, except for β -lactoglobulin (relative signal of 55-74 % compared to aqueous reference samples) and albumin (relative signal of 136-142 % compared to aqueous reference samples). Carbonic anhydrase was for some

reasons not detected from DMPK-C sampling cards in this experiment, but was expected to behave similar as in samples from CMC and VAMS.

Matrix effects have previously been shown by others [52], despite the use of isotopically labelled internal standard. Our group has also previously described ion enhancement during LC-MS-based protein analysis of human chorionic gonadotropin from DBS, after using a highly specific sample preparation method (immunoaffinity clean-up) [31]. The reason for the unsuccessful correction of the matrix effects by the internal standards for β -lactoglobulin and albumin is unknown, but the internal standard enabled at least acceptable repeatability ($< 6\%$ RSD) for the two proteins. It is also important to keep in mind that the use of frozen blood as sample matrix (as in the present study) might influence the analytes differently than the use of fresh blood. Matrix effect should therefore always be determined for all relevant analytes and sample matrices during method validation.

Response curve, precision and accuracy using VAMS

A response curve of the six model proteins was made in the concentration range from $3\ \mu\text{M}$ to $30\ \mu\text{M}$ by using VAMS as sampling material ($n=5$ levels). The correlation coefficient (R^2) of the signature peptides from carbonic anhydrase II ($R^2=0.983$), catalase ($R^2=0.999$), cytochrome c ($R^2=0.999$) and albumin ($R^2=0.985$) was acceptable as shown in **Table 4**. β -lactoglobulin was not detected at the two lowest concentration levels, while signal from myoglobin was only present in three out of five parallels for the lowest level ($3\ \mu\text{M}$). A response curve was therefore made in the range from $9\ \mu\text{M}$ to $30\ \mu\text{M}$ for β -lactoglobulin ($R^2=0.998$) and in the range from $6\ \mu\text{M}$ to $30\ \mu\text{M}$ for myoglobin ($R^2=0.999$). The quantitative data for these two proteins was therefore obtained by using a shorter range and fewer levels

(3-point calibration line for β -lactoglobulin and 4-point calibration line for myoglobin)

compared to the other proteins, and these data are hence less robust.

The accuracy and precision were also evaluated for all proteins, and the results are presented in Table 4. Precision was $\leq 20\%$ (RSD) for all proteins at their lowest concentration level and $\leq 16\%$ (RSD) at the highest level ($n=5$, both levels), and was within the acceptance criteria (except for carbonic anhydrase at the high concentration level) as defined by FDA (bioanalytical validation guidelines) [59]. The accuracy for the lowest level was $\leq 29\%$ and $\leq 13\%$ for the highest level ($n=2$, both levels). The accuracy, although calculated based on only two parallels, was acceptable for all proteins except for carbonic anhydrase II and albumin (-29% and -21% respectively) at their lowest concentration level.

Our evaluation, although preliminary, suggests that it is possible to obtain acceptable quantitative data for proteins with different physicochemical properties analysed from blood sampled with VAMS, despite the rapid and non-specific sample preparation method used for clean-up of the samples and the use of frozen blood as sample matrix.

4. Conclusions

This is, to our knowledge, one of the first papers demonstrating VAMS in analysis of proteins. VAMS was evaluated for use in targeted protein analysis by the bottom-up principle together with two other sampling materials, DMPK-C and CMC. Key parameters for DBS were evaluated for six model proteins with different physicochemical properties. A quick and generic sample preparation method was applied and analyte recovery was in general comparable for the sampling materials DMPK-C and CMC, while VAMS showed more variations in the recovery. Hematocrit was shown to influence both the pure cellulose-based material and the VAMS. The latter material was probably influenced by hematocrit due to differences in analyte recovery or matrix effects rather than volume bias. In a preliminary

evaluation of the quantitative performance using frozen blood acceptable quantitative data was obtained for four of the six model proteins in a concentration range of one order of magnitude. This demonstrated the possibility of using VAMS for quantification of mid- and high-abundance proteins with different physicochemical properties using a non-specific and simple sample preparation method. Further studies will be needed to confirm the quantitative performance of VAMS using fresh whole blood as sample matrix and a more rigorous experimental design.

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Figure captions:

Figure 1: Chromatograms of the signature peptides and their internal standard (IS, showed below its signature peptide) analysed from VAMS: carbonic anhydrase (30 μ M) and IS, catalase (30 μ M) and IS, cytochrome c (30 μ M) and IS, β -lactoglobulin (90 μ M) and IS, myoglobin (60 μ M) and IS and albumin (30 μ) and IS (**A**). The retention times of the peptides are shown above each peak. Chromatograms after analysis of blank blood sampled with VAMS (**B**). The shaded areas indicate the expected retention window of the signature peptides.

Figure 2: Relative recovery of the model proteins from the three different sampling materials: Recovery (n=5) from DMPK-C (white), VAMS (light grey) and CMC (dark grey) shown as percentage (%) relative to recovery from DMPK-C (100 %).

Tables

Table 1: The monitored signature peptides and the internal standards (IS) are listed together with the corresponding protein, the peptide sequence, the precursor m/z and the fragment m/z .

Organism	Protein	Peptide	Precursor m/z	Fragments m/z
Bovine	Carbonic anhydrase II	QSPVDIDTK	501.8	394.4; 591.3; 787.4
	IS	QSPVDIDT[K- ¹³ C ₆ - ¹⁵ N ₂]	505.8	398.4; 599.3; 795.4
Bovine	Catalase	NFSDVHPEYGSR	704.3	708.4; 845.5; 944.5
	IS	NFSDVHPEYGS[R- ¹³ C ₆ - ¹⁵ N ₄]	709.3	718.4; 855.4; 954.5
Bovine	Cytochrome c	TGPNLHGLFGR	584.8	505.9; 549.3; 686.4
	IS	TGPNLHGLFG[R- ¹³ C ₆ - ¹⁵ N ₄]	589.8	510.9; 559.3; 696.4
Bovine	B-lactoglobulin	VYVEELKPTPEGDLEILLQK	771.8	912.3; 976.6; 1026.5
	IS	VYVEELKPTPEGDLEILLQ[K- ¹³ C ₆ - ¹⁵ N ₂]	774.5	916.3; 980.6; 1030.5
Horse	Myoglobin	LFTGHPETLEK	636.3	716.4; 910.5; 1011.5
	IS	LFTGHPETLE[K- ¹³ C ₆ - ¹⁵ N ₂]	640.3	724.4; 918.5; 1019.5
Bovine	albumin	DAFLGSFLYEYSR	784.4	334.3; 717.3; 1121.5
	IS	DAFLGSFLYEYS[R- ¹³ C ₆ - ¹⁵ N ₄]	789.4	727.6; 1131.6

Table 2: Relative recoveries ($\pm$ standard deviation) of the proteins shown as percentage (%) relative to mid hematocrit (hct) for DMPK-C (n=4) and for VAMS (n=4).

Protein	DMPK-C		VAMS	
	<i>Low hct^a</i>	<i>High hct^b</i>	<i>Low hct^a</i>	<i>High hct^b</i>
<i>Carbonic anhydrase II</i>	72 $\pm$ 5 %*	100 $\pm$ 5 %	125 $\pm$ 5 %*	115 $\pm$ 4 %*
<i>Catalase</i>	63 $\pm$ 3 %*	105 $\pm$ 12 %	120 $\pm$ 6 %*	123 $\pm$ 14 %*
<i>Cytochrome c</i>	47 $\pm$ 5 %*	127 $\pm$ 18 %	94 $\pm$ 8 %	123 $\pm$ 10 %*
<i>β-Lactoglobulin</i>	62 $\pm$ 5 %*	132 $\pm$ 18 %*	116 $\pm$ 15 %	133 $\pm$ 7 %*
<i>Myoglobin</i>	134 $\pm$ 8 %*	106 $\pm$ 46 %	203 $\pm$ 23 %*	126 $\pm$ 12 %
<i>Albumin</i>	23 $\pm$ 4 %*	125 $\pm$ 28 %	59 $\pm$ 3 %*	104 $\pm$ 7 %

*significantly different from mid hct (40% hct).

^a20 % hct

^b60 % hct

Table 3: Matrix effects for blood sampled on CMC (n=4), DMPK-C (n=4) and VAMS (n=4) shown as percentage relative to reference sample (aqueous digest, n=4). A) Data obtained without internal standard and B) data obtained by internal standard compensation.

	A: Without IS (%)			B: With IS (%)		
	CMC	DMPK-C	VAMS	CMC	DMPK-C	VAMS
Carbonic anhydrase II	61±21	ND ^a	33±15	91±9	ND ^a	86±15
Catalase	98±18	112±5	112±20	102±10	103±7	97±11
Cytochrome c	384±81*	464±46*	464±94*	93±4	98±2	98±5
β-Lactoglobulin	83±14	48±15*	57±15	74±4*	56±5*	55±6*
Myoglobin	144±8*	106±23	131±49	102±1	97±7	97±7
Albumin	105±8	64±16*	75±29	137±3*	142±4*	136±3*

^aNot detected

*significantly different from reference

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663 **Table 4:** Coefficient of determination (R^2), precision (% RSD, n=5) and accuracy (%)664 deviation from theoretical concentration, n=2) for the six model proteins (range 3 μ M-30 μ M).

Protein	R^2	Precision (%)		Accuracy (% deviation)	
		<i>Low level</i>	<i>High level</i>	<i>Low level</i>	<i>High level</i>
Carbonic anhydrase II	0.983	14	16	- 29	- 4
Catalase	0.999	18	4	1	- 2
Cytochrome c	0.999	3	6	- 9	- 3
β -lactoglobulin	0.998 ^a	20 ^c	7	*ND	- 11
Myoglobin	0.999 ^b	7 ^c	15	*ND	- 13
Albumin	0.985	19	13	- 21	- 2

665 *ND: Not determined

666 ^aRange 9 μ M-30 μ M667 ^bRange 6 μ M-30 μ M668 ^cn=3

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