On-paper sample handling for bottom-up protein analysis

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Abstract

A concept integrating sampling and protein digestion is here introduced combining fast and simple fabrication by wax printing on filter paper with trypsin immobilized polymer beads. The paper reactors showed promising results with a high degree of protein digestion within 50 minutes. A six protein mixture consisting of bovine serum albumin, carbonic anhydrase 2, catalase, myoglobin, phosphorylase and β-lactoglobulin was used as model system. The performance was evaluated both with and without a reduction and alkylation step. The paper reactors *without* reduction and alkylation showed between 46 % and 75 % protein sequence coverage and between five and 20 high confidence peptides with one and five zero missed cleavage peptides, respectively. Compared to a conventional *in-solution* approach the paper reactor with reduction and alkylation before protein digest showed a slight reduction (10 %) in protein sequence coverage, 29 % less high confidence peptides and 19 % less high confidence peptides with zero missed cleavages. The placement of the protein reduction and alkylation step before or after protein digestion was shown to be of low importance. Alternatively, *only* a reduction step (no alkylation) could be performed after protein digestion. The stability of produced tryptic peptides in the reactor was satisfactory: Six weeks of storage showed a decrease of five percent in sequence coverage, 38% in number of high confidence peptides and 14% in number of high confidence peptides with zero missed cleavages. The performance of reactors stored *before use* was also satisfactory: After twelve weeks of storage prior to sample application, the reactors showed a decrease of only 11 % in sequence coverage, and 8 high confidence peptides and four high confidence peptides with zero missed cleavages was obtained. The ability of the paper reactors to digest complex biological samples like whole blood was evaluated by comparison with an established dried blood spot (DBS) procedure of human whole blood in a non-targeted analysis. The reactors showed comparable performance with 44 ± 10

unique proteins (high confidence) compared to 46 ± 7 for the DBS samples. Additionally, 151 and 140 unique peptides (high confidence) were identified for *on-paper* digestion and DBS, respectively.

Introduction

Bottom-up protein analysis has become a common approach during the last decade [1]. In a bottom-up approach peptides are separated post proteolysis most often with liquid chromatography (LC) and determined by mass spectrometry (MS). The bottom-up workflow is mainly comprised of in-solution protein digestion, sample clean up and enrichment (e.g. solid-phase extraction) which usually is a time consuming and complex process. Additionally, the sample amount is often limited making sample handling and treatment cumbersome. To simplify the required steps and time consumption new developments in protein sample handling are needed. In the recent years microfluidic devices have gained interest for diagnostic purposes e.g. glucose assays [2] and immunoassays [3,4]. These devices have been reported with low cost simple fabrication on non-intrusive paper [5]. However, protein analyses in combination with devices fabricated on-paper and MS are not widely reported. Nevertheless, a complimentary to the already established sampling techniques is dried blood spots (DBS). DBS has gained much interest and the main advantage with this way of collecting samples is that it can be done rapidly without extensive equipment. DBS has proven its applicability in combination with LC-MS most often for small molecules, either drugs or endogenous compounds in blood samples [6-8]. However, DBS has not been frequently used with proteolytic protein analyses, thus a limited number of publications are reported [9-11]. Nevertheless, there are some reports with promising results from our group on determination of proteins after storage on paper [3,12]. These reports focus on water soluble materials since it is assumed that these improve the accessibility of the analytes for extraction. Another way to improve analyte accessibility might be addition of proteolytic enzyme simultaneously with- or prior to extraction since smaller molecules are potentially easier to extract from DBS cards. Kehler et.al [13] demonstrated a concept of *on-card* enzymatic digestion with overnight digestion on commercial DBS cards, but with limited success due to poor signal intensities. Needless to say, there is still a contradiction between the easy on-card sampling and the time-consuming protein digestion prior to MS analysis hence, faster sampling techniques are called for. To accelerate protein digestion immobilization of enzymes (often referred to as enzyme reactors) has gradually gained more attention within protein analyses throughout the last decade. But, common for most enzyme reactors are that the processes are carried out static in liquid systems e.g. in-solution with polymer beads [14], on-line

LC columns [15] etc. We wanted to explore if paper sampling could be combined with immobilized trypsin to make an efficient *on-paper* enzyme reactor for protein samples. However, commercial sampling cards does not have volume restricting properties and this possesses a challenge since a liquid layer will be absorbed into the paper rather than creating a static system for prolonged enzyme-to-protein interaction. **Wax printing** could solve this problem. In wax printing, filter paper (most often fabricated by cellulose) is used as carrier to print on. The wax printer itself is a non-expensive printer where warm wax is used instead of regular toner. When the filter paper is heated (e.g. in a stove) after printing, the wax will melt and impregnate the paper creating hydrophobic barriers [16,17]. These barriers do not allow polar liquids to pass (**Fig. 1**) and therefore applied liquid will sit as a bubble within the wax barrier and maintain the structure until dry. By combining the static water droplet with immobilized polymer beads a proteolytic digest could be performed *on-paper* inside the sample droplet. Even though the basics of wax printing and paper substrates has been used for different immobilized applications (e.g. ELISA [18] and colorimetry [19]), it has not yet been used for protein samples in combination with *on-paper* protein digestion and LC-MS. Our goal was to investigate fabrication and use of a micro fluidic enzyme reactor as a concept for tryptic digestion of proteins and storage of tryptic peptides hence; study the usefulness of performing digestion *on-paper* to accelerate a protein analysis workflow. Moreover, to simplify the steps required to perform quantitative protein analyses in combination with sampling and preparation. The concept was evaluated based on number of unique peptides/total high confidence peptides, (high confidence) peptide sequences with zero missed cleavages, protein coverage and MS intensity of selected peptides. Finally the concept was applied in non-target analysis of whole blood samples and compared to a conventional dried blood spot (DBS) procedure to evaluate the ability of the paper reactors to digest complex biological samples.

Experimental

Chemicals and reagents

Acetonitrile (99.9%, (ACN)), sodium azide, sodium phosphate dodecahydrate (99-102%), sodium phosphate monobasic (99-102%) and hydrochloric acid (37%, (HCl)) were purchased from Merck (Darmstadt, Germany). Ammonium bicarbonate (99.5%, (ABC)) and ethanolamine (≥ 99%) was purchased from Fluka (Milwaukee, WI, USA). Benzamide (≥ 95%), calcium chloride dehydrate (≥ 99%), Tris(hydroxymethyl)aminomethane (trisma base), Tris(hydroxymethyl)aminomethane (HCL base)(≥ 99%) and sodium deoxycolate (≥ 98%) were

purchased from Sigma-Aldrich (St.Louis, MO, USA). Formic acid (≥ 98%, (FA)), iodoacetic acid (≥ 98%, (IAA)), DL-dithreitol (≥ 99.5%, (DTT)), tris(2-carboxyethyl)phosphine (≥ 98% (TCEP)), cytochrome C from equine heart (≥ 95%, (Cyt-C), catalase from bovine liver (≥ 10 000 units/mg protein), myoglobin from equine heart (≥ 90%), ß-lactoglobulin from bovine milk (≥ 90%), carbonic anhydrase isozyme 2 from bovine erythrocytes (≥ 3000.0 W-A units /mg protein), phosphorylase B from rabbit muscle (≥ 20 units/mg protein), bovine serum albumin (≥ 97%, (BSA)) and trypsin from bovine pancreas TPCK treated (≥ 10 000 BAEE units /mg protein) were purchased from Sigma-Aldrich. NHS activated Sepharose 4Fast Flow beads were purchased from GE Healthcare (Uppsala, Sweden). Water used in this experiment was filtrated through a Merck Millipore Milli-Q intergral 3 water dispenser (resistivity: 18.2 MΩ cm^{-1})

Immobilization of trypsin on polymer beads

The immobilized polymer beads were prepared in Eppendorf (Hamburg, Germany) Protein LoBind 1.5 mL tubes as previously described by Freije et al.[20] and Vukovic et al.[14]

Preparation of wax-printed filter paper

GE Healthcare life sciences (Buckinghamshire, UK) Whatman® grade 1 filter paper (pore size: ~10 μm) were cut to A4 sized sheets (compatible with the printer) and printed with 48 circles in Microsoft Power point 2010 (version 14.0.7177.5000) (internal diameter (ID): 6.0 mm, outer diameter (OD): 10 mm per circle). The printing was performed with a Xerox (Norwalk, CT, USA) Color Qube 8580 printer and Color Qube 8570 wax. The printed sheets were further baked at 110 ˚C for 15 minutes and subsequently cooled down prior to dry and dark storage at ambient temperature.

Characterization

Characterization of the wax printed papers were carried out with a FEI (Hillsboro, OR, USA) Quanta 200 FEG E-SEM and a Leica (Wetzlar, Germany) Zoom 2000 optical microscope.

Protein solutions

Cytochrome-C solution

The cytochrome-C solution was freshly prepared by dissolving cyt-C in ammonium bicarbonate (ABC) buffer (4.0 mg/mL) to a final concentration of 20 µg/mL in a 1.5 mL Eppendorf LoBind tube.

Six protein mix

The protein mix was freshly prepared by dissolving catalase, myoglobin, ß-lactoglobulin, carbonic anhydrase isozyme 2 (CA2), phosphorylase B and bovine serum albumin (BSA) in ABC buffer (4.0 mg/mL) to a final concentration of 20 µg/mL (per protein) in a 1.5 mL Eppendorf protein LoBind tubes.

Whole blood

Whole blood was donated in BD (Franklin Lakes, NJ, USA) vacutainer® K2 EDTA tube by one voluntary person.

Protein digestion

Protein digestion was carried out *in-solution* and *on-paper*. The choice of peptide sequences were determined by analysis in Thermo Fischer Scientific (Waltham, MA, USA) Proteome Discover™ (version 1.4) after LC-MS analysis. The signature peptides (**Table 1**) were chosen for each protein with respect MS intensities. Depending on the nature of the experiments, the proteins were digested and analyzed without reduction and alkylation, only with reduction or with both reduction and alkylation (**Fig.2**).

In-solution digestion

For all *in-solution* digestions 80 µL ABC solution and 20 µL protein sample was pipetted to a 1.5 mL protein LoBind Eppendorf tube with a subsequent addition of five µL trypsin

immobilized beads. The sample was further mixed at 1100 rpm at room temperature for two hours. After the digestion the sample was added 95 µL water/ACN/FA (95/5/0.1, v/v/v) and centrifuged. Hundred µL supernatant was transferred to a new tube, centrifuged with a subsequent supernatant transfer to a HPLC vial.

On-paper digestion

- *General procedure without reduction and alkylation (see method A in **Fig.2**)*

Immobilized polymer beads washed three times with a tenfold volume of ABC buffer (4.0 mg/ml) was pipetted (5.0 µL) to the center of the wax printed circle and subsequently dried at room temperature for one hour. *On-paper* digestion was performed by adding 20.0 µL of protein sample. After drying, the wax printed circles where punched out along the inner wall of the wax print with a Philip Harris (Birmingham, UK) Uni-Core 6.0 mm puncher and extracted with 200 µL water/ACN/FA (95/5/0.1, v/v/v) for 15 minutes at 800 rpm with an Eppendorf comfort mixer. After centrifugation 140 µL of the supernatant was transferred and additionally centrifuged prior to a supernatant transfer of 100 µL to a HPLC vial for analysis.

Protein reduction and alkylation

- *Procedures using reduction and/or alkylating reagents (method B,C,D and E in **Fig.2**)*

The protein sample was reduced at 60 ˚C and 1100 rpm with 50 mM DTT for 15 minutes before a subsequent alkylation with 200 mM IAA at 4 ˚C (dark) for 15 minutes. This procedure was either done prior to protein digestion (Method **D** and **E, Fig. 2**) or post digestion (Method **B, Fig. 2**) after sample elution with 200 µL ABC buffer and subsequently reduced and alkylated prior to protein extraction. Additionally, only reduction was carried out post digestion (Method **C**). *On-paper* digestion was in all cases performed as described above.

Experiments to locate where digestion takes place

Wax printed circles (n=4) were spotted with five µL immobilized beads, dried and punched out before a subsequent transfer to an protein LoBind tube containing 180 µL ABC solution and 20 µL cyt-C solution (20 µg/mL). The sample was mixed for 15 minutes at 800 rpm and room temperature. The samples were further centrifuged twice prior to transferring the supernatant to HPLC vials

Experiments with whole blood samples

- *Dried blood spots*

The preparation and analysis of dried blood spot samples were carried out according to a previously described method by Chambers et.al [21]. In short; ten µL whole blood was pipetted onto FTA® DMPK-C cards from GE Healthcare (Fairfield, CT, U.S.A) (n=3), dried and subsequently extracted and reduced with 1.0 mL 50 mM ABC buffer with one percent deoxycolate and five mM TCEP at 60 ˚C and 1100 rpm. One hundred µL was transferred to new Eppendorf protein LoBind tubes and five µL 200 mM IAA was added and agitated at 37 ˚C and 1100 rpm. Excess IAA was removed by adding five µL 100 mM DTT. Ten µL trypsin (1.0 mg/mL dissolved in 50 mM ABC) was added for an overnight digest at 37 ˚C and 1000 rpm. Two µL concentrated FA was added to precipitate deoxycolat before subsequent centrifugation at 14 000 rpm for 10 minutes. Eighty µL of the supernatant were extracted with in-house made C8 solid phase extraction columns (C8 Empore® material from Teknolab AS, Kolbotn, Norway) before drying at 40 ˚C with a Thermo Scientific (Rockford, IL, USA) SpeedVac with a subsequent rehydrating in 500 µL 20 mM FA.

- *Paper reactors*

The preparation and analysis of *on-paper* digestion samples (n=3) were carried out as described for the dried blood spot analysis. However, digestion time was equivalent to drying of the sample instead of an overnight in-solution digest. The reduction and alkylation step were therefore performed after the tryptic digest.

Stability of the paper reactors

Paper reactors (n=16) were prepared as described above. Six paper reactors were tested immediately using cyt-C as model compound. Four paper reactors were dried and stored dark at ambient conditions for 12 weeks prior to addition of cyt-C and thus protein digestion. The reminding paper reactors (n=6) where applied cyt-C for immediate protein digestion before dark storage at ambient conditions for 6 weeks prior to extraction and analysis.

LC-MS method

The chromatographic separation and detection was performed with a Dionex (Sunnyvale, CA,USA) ultimate 3000 pump module coupled to a Thermo Scientific Acclaim™ PepMap™100 C18 enrichment column (ID: 300 µm x 5.0 mm, D_p: 5.0 µm), a PepMap™100 C18 separation column (ID: 75.0 µm x 150 mm, D_p: 3.0 µm,) and a Thermo Scientific LTQ XL / LTQ Orbitrap Discovery. Elution was performed with a gradient where the mobile phases (MP) was water/ACN/FA (95/5/0.1, v/v/v) and ACN/water/FA (95/5/0.1: v/v) for MP A and MP B, respectively. The gradient ran from 0 to 50 % MP B over 18 minutes and further increased to 100 % MP B for two minutes. Loading was performed on five µL sample over four minutes. Total run time was set to 31 minutes. The system was controlled by Thermo Scientific Xcalibur (version: 3.0.63) and Dionex Chromeleon (version: 6.80 SR8) software. The MS analyses were performed with data dependent acquisition (full scan (300 – 2000 Da)) positive mode ESI. Spray voltage was set to 2.2 kV, heated capillary: 150 ˚C, capillary voltage: 45 V and tube lens offset: 100 V. The six most abundant peptides were fragmented with 35 % relative collision energy (helium) with 30 ms activation time and dynamically excluded for 15 seconds in order to fragment lower intensity *m/z* values.

Data interpretation

All samples with buffered proteins were processed with the Sequest algorithm in Thermo Fischer Proteome Discover™ (version 1.4) searches in FASTA libraries for each protein. For all whole blood samples data was interpreted with searches in the SwissProt human database (reviewed and canonical, downloaded March 2017) with exclusive reporting of high confidence proteins and peptides. All reports were generated with the following restrictions: collision induced dissociation activation, precursor tolerance of 10 ppm, fragment mass tolerance of 0.6 Da, maximum number of three missed cleavages, static modification at carboxymethyl (C) and dynamic modification at oxidation (M). A strict false discovery rate was used (0.01) for filtration of the data.

Results and discussion

Fabrication and test of paper reactors

Fabrication

Since the DBS workflow depend on punching out a circular sample, we decided to wax print a circular pattern compatible with a standard DBS puncher (6.0 mm inner diameter (ID)). In order to create the hydrophobic wax barrier (i.e. melt the wax into the paper structure) we found that 15 minutes at 110 ˚C was sufficient to keep the inner diameter of the wax circle constant (i.e. not decreasing/increasing) through the heating process. The baking step could be carried out at different temperatures however; lower temperatures needed longer time to obtain a complete cross sectional penetration. With temperatures higher than 110 ˚C the baking process was faster, however, the circle diameter shrank and increased in ID and OD, respectively. In order to perform a digest, an aliquot of five µL trypsin immobilized beads was pipetted into the middle of a wax printed circle and subsequently dried. Although digestion could be performed with fewer beads (data not shown), a minimum of five µL of liquid was needed to wet the whole circle. The filter papers were routinely examined in an optical microscope prior to sample application to confirm an evenly spread of beads. The beads were prone to clustering on the paper surface when dried (**Fig. 3**), but were more evenly distributed when an adequate volume of sample was used. Unevenly spread of beads would give less contact between protein and enzyme and thus, a less efficient digest. Protein digestion was started by pipetting 20 µL sample into the circle center creating a sample droplet within the wax barrier. The experiments were performed under ambient conditions and therefore drying time and therewith the reaction time was hard to control. Drying time proved to be prone to fluctuation in humidity and temperature, however, variations were small when the experiments were performed intraday. Nevertheless, fifty minutes was average with five minutes variation between circles prepared intraday. With regards to sample volume capacity the reactor had a tolerance beyond 20 µL, but due to drying time we chose to proceed with 20 µL. The work flow of *on-paper* digestion is presented in **Figure 3**.

Test of on-paper digestion reactors

As a preliminary experiment the performance of *on-paper* digestion was compared with *in-solution digestion* with trypsin immobilized beads (n= 3). Even though the reactor still is at a conceptual stage, we decided to compare the performance to a conventional procedure in such way we could determine whether or not the reactors gave adequate results for further evaluation. The performance was evaluated with respect to MS intensity, number of unique peptide sequences, peptide sequences with zero missed cleavages and protein coverage. For these initial experiments cyt-C was used as a model protein due to its simple composition, i.e. lack of disulfide bridges and relatively short sequence length (mass: 12 384 Da), avoiding the need of

treating the sample with reduction and alkylating reagents. To ensure a valid comparison between the digestion methods, the three most frequent peptides (from *in-solution* and *on-paper* digestion) were used as target peptide sequences (MIFAGIK, TGPNLHGLFGR and EDLIYLK). Both digestion methods were performed simultaneously and the dilution ratio of protein with respect to injected sample was kept constant (10 ng on column in case of maximal extraction recovery from paper). Regarding time consumption, both methods performed within approximately two hours (from digestion start to start of the LC-MS analysis). From the chromatograms (**Fig. 4**) it can be seen that *on-paper* digestion (**Fig. 4 *i***) exhibited less peaks than *in-solution* digestion (**Fig. 4 *ii***). However, the number of unique peptides was comparable with 13 and 15 for *on-paper* and *in-solution* digestion, respectively. Both methods gave 8 peptides with zero missed cleavages, and a total of 16 and 20 peptides (unique and non-unique) were observed for *on-paper* and *in-solution* digestion, respectively. Signal intensities for *on-paper* digestion (**Fig. 5**) showed no significant variation compared to *in-solution* digestion. Furthermore, signal intensities were in the same order of magnitude (~10^8) with adequate sequence coverage (~ 80%) for both methods. Thus, this experiment proved that *on-paper* digestion has a potential as a sample preparing method for simple protein samples.

Experiments to locate where digestion takes place

The *on-paper* digestion is achieved with beads on which trypsin is immobilized. These beads are not attached to the paper and are thus only laying on the paper surface (**Fig. 3)**. As the punched circle is shaken during extraction, the beads might loosen from the paper and start protein digestion *in-solution* during the extraction. To investigate whether the digestion is taking place during drying of the sample droplet or during extraction four unused wax printed filter paper reactors were extracted following the previously described procedure, however, with cyt-C added to the extraction solvent. From LC-MS analyses, no peaks from tryptic cyt-C peptides were present (**Fig. 4 *iii***) hence; protein digestion is occurring *on-paper* during the drying step.

On-paper digestion of protein mixture

After the preliminary experiments with cyt-C we wanted to explore the concept with a more complex sample. A buffered protein mix was prepared with six different proteins with different physicochemical properties (concentration of 20 µg/mL per protein); catalase, myoglobin, ß-lactoglobulin, CA-2, phosphorylase B and BSA. We included BSA and ß-lactoglobulin to investigate the necessity of reduction and/or alkylation of proteins after *on-paper* digestion

since these proteins contain cysteine and thus, disulfide bridges. The digestion *without* reduction and alkylation were evaluated with respect to: MS intensity, number of unique peptides, number of zero missed cleaved peptides and protein coverage. In compliance with our assumptions only those peptides without cysteins were identified. The average (n=6) number of total peptides (high confidence) ranged from five (myoglobin) to twenty (phosphorylase) with one and five peptides with zero missed cleavages, respectively. Additionally, the average sequence coverage was between 46 % and 75 % for the six proteins (**Table 2**). MS intensities (**Fig. 6**) showed large variations between the proteins with signal ranging from $1.82 \cdot 10^6$ (myoglobin, LFTGHPETLEK) to $2.14 \cdot 10^8$ (ß-lactoglobulin, VYVEELKPTPEGDLEILLQK), however, since the protein mix was prepared with respect to weight rather than molarity this was expected.

Reduction and alkylation

Even though our experiments have showed that it is possible to perform protein analysis without reduction and alkylation for certain proteins, we wanted to investigate the possibility to reduce and alkylate *after* protein digestion. Protein reduction and alkylation to disrupt the disulfide bridges are in most protocols accomplished prior to digestion and may therefore be a limiting factor for *on-paper* digestion. Therefore, in order to use the reactors as a likely sampling technique for DBS reduction and alkylation is only applicable after sampling. For this investigation, post proteolysis reduction and alkylation (n = 6) (Method **B, Fig. 2**) was compared with the conventional pre proteolysis reduction and alkylation (n = 6) (Method **D, Fig. 2**). Mainly the same peptides were produced for both methods. However, it was difficult to state which method gave the best results since the data variation through each data set as whole were similar. However, certain proteins (e.g. phorphorylase and BSA) favored either pre- or post- reduction and alkylation. But given that BSA contain disulfide bridges and phosphorylase not, this could indicate that the sample preparation is more protein dependent (e.g. stickiness of proteins to the paper) rather than exclusively when the reduction and alkylation is performed (**Table 3**). The MS intensities for the peptides (**Fig. 8, Supplementary**) were comparable for the two methods, although, for BSA, the methods showed some differences. Overall, it is hard to state which method yielded the best results. Thus we showed that it is possible to reverse the normal work flow for protein sample preparation and therewith the application of the paper reactors as a likely sampling technique.

Reduction without alkylation

With respect to the latter experiments we wanted to evaluate the need of alkylation. Limiting the use of modifying reagents is desirable since reduction and alkylation involves EHS hazardous reagents, and in addition is time-consuming. We therefore explored the possibility of only reducing the proteins after digestion (Method **C, Fig. 2**). The digestion was carried out as previously described (n = 6), but the extraction (from the paper reactor) was performed in ABC buffer containing DTT. Different peptides were generated when *only* the reducing reagent was used rather than reducing *and* alkylation reagents. Nevertheless, it was not possible to distinguish whether method **C** outperformed method **B** significantly or vice versa with respect to protein sequence coverage, number of high confidence peptides and number of high confidence peptides with zero missed cleavages (**Table 3**).

Can on-paper digestion be compared to in-solution digestion?

Our experiments have shown that wax printed filter papers with trypsin immobilized beads can readily be used for protein sample preparation. In addition, the experiments demonstrated that reduction and alkylation could be performed either *before* or *after* protein digestion. Even though protein modification prior to sample application is not desirable for the paper reactors, we wanted to investigate sample loss and digestion efficiency of *on-paper* digestion compared to the more common *in-solution* approach. Reduction and alkylation was carried out before protein digestion for both *in-solution* (Method **E, Fig. 2**) and *on-paper* (Method **D, Fig. 2**) digestion. *On-paper* digestion gave similar chromatograms (**Fig. 7**) to the *in-solution* digestion, but with a few more intense peaks. Therefore, it was no distinction in complexity. The signal intensities were, for both methods in the order of magnitude ~ 10^9. However, with respect to quantitative determinations the *in-solution* digestion proved higher signal intensities for most of the tryptic peptides. The *in-solution* approach showed 10 % (average from all proteins) higher sequence coverage, 29% more high confidence peptides (average from all proteins) and 19 % more peptides with zero missed cleavages (average from all peptides) compared to *on-paper* digestion (**Table 3**). These differences might not be of significance with exception of the number of high confidence peptides given that the sample only contained six proteins. However, it must be stressed that this concept is not intended for proteomic investigations and thus, the

results were adequate. Nevertheless, the *on-paper* digestion proved promising results as a concept sampling technique (with integrated sample preparation) for rapid protein analyses.

Stability and storage at ambient conditions

For biological analyses, DBS has many advantages over the conventional sampling methods. With the exception of storage, the biggest advantage is that biological matrices are stable and in-active when dried. Therefore, it was important to determine if the paper reactors could be stored both with beads *and* protein, but also *only* with beads to prove the reactors as a complimentary DBS technique. Functionality of the paper reactors after long-term storage was important to determine since this sampling format could have potential for easy "at home" sampling. Many of the already existing kits for personal diagnostics are carried out by the postal service [22,23], and therefore we tried to mimic a scenario of everyday transport. The paper reactors were stored in a box frequently moved without special consideration. To determine the storage's impact on digestion efficiency six reactors were fabricated and used for immediate cyt-C digestion and analysis. The immediate analysis gave average protein sequence coverage of 76 % with 13 high confidence peptides and seven peptides with zero missed cleavages (**Table 4**). To test the stability, six reactors were fabricated and applied with cyt-C before drying and subsequent storage. After six weeks these reactors showed a drop of five percent in protein sequence coverage with 33 % fewer high confidence peptides was observed compared to the reactors prepared with immediate analysis. However, with only a loss of one high confidence peptide with zero missed cleavage (**Table 4**). To investigate if the reactor could be stored over a longer period of time; four reactors were applied with beads, stored for 12 weeks before an addition of cyt-C with subsequent analysis. For these reactors protein sequence coverage was 11 % lower than the freshly prepared reactors with only a decrease of four high confidence peptides and two high confidence peptides with zero missed cleavages (**Table 4**). Thus, lower functionality was expected since the polymeric beads as previously elaborated were not attached to a solid support. Thus, risking loss of beads (and hence lowering of the digestion efficiency) if the reactors were carelessly handled during storage. The stored paper reactors (both with and without digested protein) showed average intensities in the same order of magnitude compared to the freshly prepared reactors (data not shown). All in all the paper reactors were functional after storage and as a tentative conclusion the concept were successfully proven from a qualitative perspective.

Analysis of whole blood samples

Our experiments have shown that *on-paper* digestion is readily used for protein digestion in buffered samples. However, since we believe this concept could be used complimentary to DBS, we sought to prove the concept with human whole blood. To determine if the paper reactors could be used for whole blood sampling "as is" we compared the performance with DBS samples according to a described sample preparation [21] with non-targeted analysis. The procedure was duplicated for the DBS samples as well as for the paper reactors, but with variation in the digestion step. In contrast to overnight *in-solution* digest of DBS samples the paper reactors performed the digest within an hour, similar to our previous buffered samples. The paper reactors showed comparable performance with 44 ± 10 unique proteins (high confidence) compared to 46 ± 7 for the DBS samples (average). Roughly less than half of the proteins found in the DBS method were also found in the paper reactor method. For both the DBS- and paper reactors more than half of the found proteins were exclusive for either the DBS-method or the paper reactor method. Most of these proteins were only identified with one unique peptide. The same trend was observed regarding number of unique peptide sequences with 151 and 140 for *on-paper* digestion and DBS, respectively. Compared to results by Chambers et.al [24] (following the same procedure) we were not able to obtain as many identified proteins with neither *on-paper* digestion nor DBS digestion. However, it should be noted that a much shorter digestion was carried out. Additionally instrumental limitations could be a significant factor for the low protein count. Nevertheless, with these considerations taken into account DBS did not outperform the paper reactors. On the contrary our paper reactors were able to perform adequate protein digestion within an hour and thus this concept holds potential for faster sample preparation with marginal loss in quality compared to the established methods for whole blood sampling on paper. Furthermore, by using the paper reactors the most frequent trypsin autolysis products (in both methods) were significantly lower (**Fig. 8**) and this could contribute to a more sensitive platform for paper sampling of proteins if further optimization is carried out. All in all the paper reactors showed promising results for sampling and digestion of proteins in whole blood samples.

Conclusion

We have demonstrated a concept for integrated sampling and sample preparation *on-paper* for proteins. With fast and simple fabrication on nonintrusive filter paper hydrophobic wax barriers was printed and baked into the paper structure within 15 minutes. The wax printed papers

showed promising results for digestion and storage of tryptic peptides after addition of trypsin immobilized polymer beads. A high degree of protein digestion was accomplished within 50 minutes. The performance of the paper reactors was comparable to a conventional *in-solution* approach with respect to protein sequence coverage, number of high confidence peptides and number of high confidence peptides with zero missed cleavages. It was also demonstrated that the reduction and alkylation step could be performed after protein digestion. Furthermore, adequate results were obtained when only performing reduction after protein digestion. Compared to freshly prepared and analyzed reactors, storage stability of the paper reactors both *with* (six weeks) and *without* (twelve weeks) tryptic peptides was sufficient showing only a small decrease in performance. The performance of the paper reactors were also compared with an established DBS procedure of human whole blood with non-targeted analysis. The reactors showed similar performance regarding number of identified proteins and peptides. The paper reactors showed much less autolysis products which potentially contribute to cleaner chromatograms and more robust quantification. Since sampling and preparation are integrated, the potential of this approach lies with a minimal amount of laboratory work. All in all, the concept of sampling and storage of tryptic peptides on wax printed filter paper could readily be used for qualitative analysis of protein samples and showed promising results for at-home sampling for protein samples.

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Table 1 Peptides chosen from each protein in a six-protein mix to evaluate protein digestion performance.

Protein	**Peptide 1 (*m/z*)**	**Peptide 2 (*m/z*)**	**Peptide 3 (*m/z*)**
Bovine serum albumin	LGEYGFQNALIVR (*740.39*)	GLVLIAFSQYLQQc PFDEHVK (*624.06*)	RPcFSALTPD ETYVPK (*941.45*)
Carbonic anhydrase isozyme 2	MVNNGHSFNVEYDDSQDK (*700.29*)	AVVQDPALKPLAL VYGEATSR (*733.40*)	VGDANPALQ K (*506.77*)

Catalase	DGPMcMMDNQGGAPNYYP NSFSAPEHQPSALEHR (*952.39*)	FNSANDDNVTQV R (*740.34*)	LVNANGEAV YcK (*669.81*)
Myoglobin	VEADIAGHGQEVLIR (*536.28*)	LFTGHPETLEK (*636.33*)	ELGFQG (*650.31*)
Phosphorylase	QIIEQLSSGFFSPK (*791.42*)	LITAIGDVVNHDP VVGDR (*630.67*)	VFADYEEYV K (*632.30*)
ß-Lactoglobulin	YLLFcMENSAEPEQSLAcQcL VR (*941.07*)	VYVEELKPTPEGD LEILLQK (*771.75*)	LSFNPTQLEE QcHI (*858.89*)

Table 2 Average (n=6) protein coverage, numbers of high confidence sequences and number off sequences with zero missed cleavages of the six-protein mixture digested on paper. All data points are presented with standard deviation.

	Bovine serum albumin	**ß-Lactoglobulin**	**Catalase**	**Phosphorylase**	**Carbonic anhydrase isozyme 2**	**Myoglobin**
Mass (Da)*	69 293	19 883	59 915	97 289	29 114	17 083
Number of disulfide bridges*	17	3	0	0	0	0
Protein sequence coverage (%)	56 ± 6	75 ± 8	50 ± 4	46 ± 9	58 ± 5	64 ± 13
Total number of peptides (high confidence)	8 ± 1	11 ± 2	17 ± 2	20 ± 5	12 ± 1	5 ± 1
Number of peptide sequences with zero missed cleavages	6 ± 1	8 ± 1	14 ± 2	17 ± 2	10 ± 1	4 ± 1

* Theoretical values (molecular weight and number of disulfide bridges) are obtained from Uniprot [25]

Table 3 Average (n=6) protein coverage, numbers of high confidence sequences and number off sequences with zero missed cleavages the six-protein mixture digested *on-paper* with protein modification (DTT/IAA) before (B) and after (D) digestion. C was carried out only with reduction post protein digestion and E was carried out *in-solution* with reduction and alkylation before protein digestion. All data points are presented with standard deviation.

Preparing method	Digest parameter	**Bovine serum albumin**	**ß-Lactoglobulin**	**Carbonic anhydrase isozyme 2**	**Myoglobin**	**Phosphorylase**	**Catalase**
B	Protein sequence coverage (%)	51 ± 8	85 ± 7	57 ± 12	57 ± 18	40 ± 11	52 ± 12
	Total number of peptides (high confidence)	16 ± 4	16 ± 2	11 ± 2	6 ± 3	16 ± 6	5 ± 3
	Number of peptide sequences with zero missed cleavages	12 ± 4	12 ± 2	9 ± 1	4 ± 2	15 ± 5	14 ± 3
D	Protein sequence coverage (%)	75 ± 7	84 ± 7	58 ± 5	61 ± 9	25 ± 6	43 ± 4
	Total number of peptides (high confidence)	30 ± 3	12 ± 1	10 ± 1	4 ± 1	5 ± 1	13 ± 1
	Number of peptide sequences with zero missed cleavages	26 ± 2	8 ± 1	9 ± 2	3 ± 1	5 ± 1	11 ± 1
C	Protein sequence coverage (%)	82 ± 8	87 ± 6	48 ± 20	42 ± 12	48 ± 12	56 ± 6
	Total number of peptides (high confidence)	29 ± 9	18 ± 2	8 ± 3	4 ± 2	12 ± 8	14 ± 2
	Number of peptide sequences with zero missed cleavages	14 ± 2	12 ± 1	7 ± 3	2 ± 2	11 ± 7	10 ± 1
E	Protein sequence coverage (%)	79 ± 3	83 ± 6	68 ± 8	74 ± 3	24 ± 7	52 ± 4
	Total number of peptides (high confidence)	45 ± 3	16 ± 3	12 ± 2	9 ± 1	4 ± 2	18 ± 1
	Number of peptide sequences with zero missed cleavages	30 ± 2	10 ± 1	11 ± 1	7 ± 1	4 ± 2	15 ± 1

Table 4 Protein sequence coverage, total number of high confidence peptides and number of high confidence peptides with zero missed cleavages for digestion of cytochrome-C with immediate analysis, analysis after six weeks of digest storage on paper and protein digestion after 12 weeks of stored paper reactors.

	Protein digestion with immediate analysis	Six weeks storage after protein digestion	Stored beads on paper with protein digest after 12 weeks
Protein sequence coverage (%)	76 ± 5	72 ± 8	68 ± 2
Total number of peptides (high confidence)	12 ± 2	8 ± 0	8 ± 2
Number of peptide sequences with zero missed cleavages	7 ± 0	6 ± 0	5 ± 1

Figures

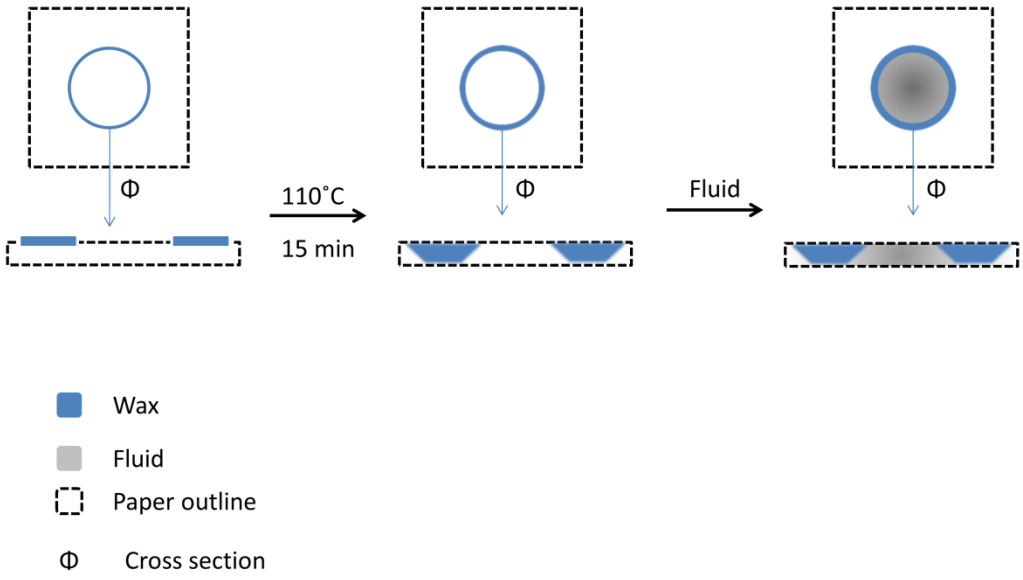

Figure 1 Schematic illustration of a typical wax printing work flow on fibrous paper.

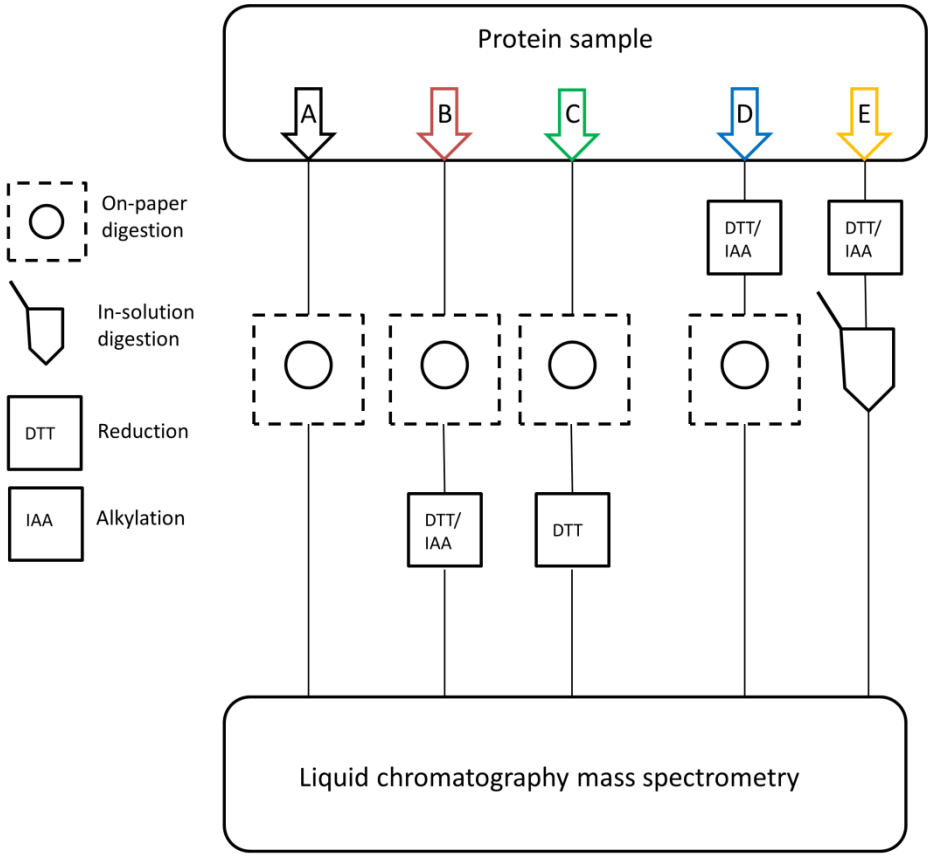

Figure 2 Schematic illustration of the protein digestion workflow

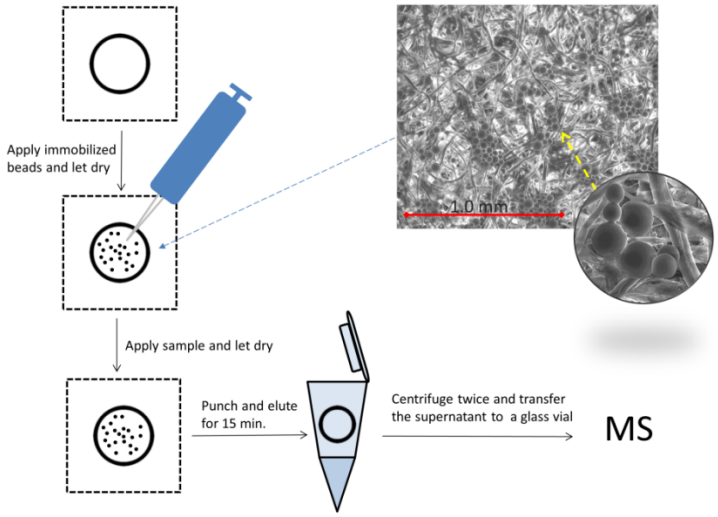

Figure 3 Schematic illustration the paper based enzyme reactor work flow. The micrograph was captured in high vacuum mode, 30 kV and 16.6 mm working distance with a FEI Quanta 200 FEG SEM. Detailed description of the procedure is given in the experimental section

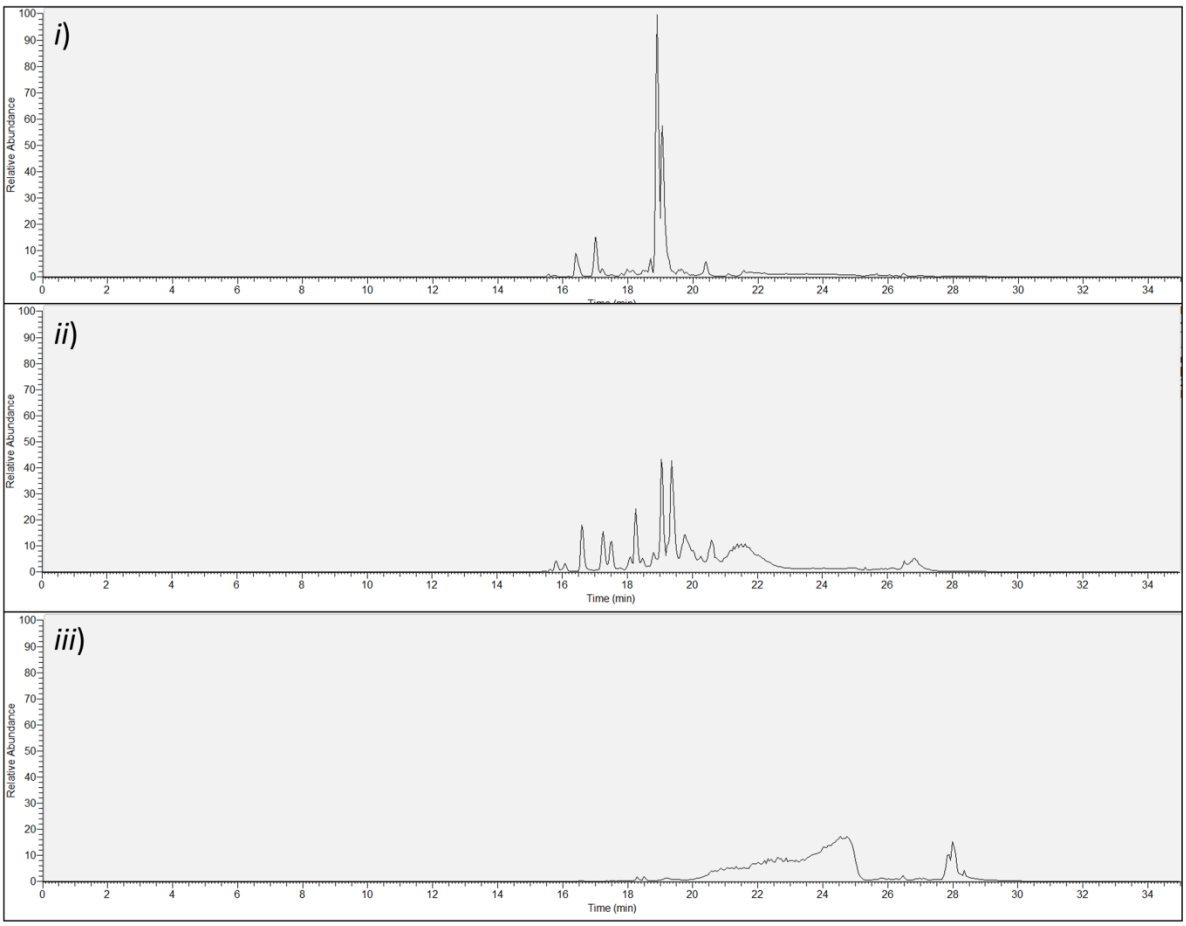

Figure 4 LC-MS Chromatograms from *on-paper* digestion (*i*), *in-solution* digestion (*ii*) and analysis of supernatant when trypsin beads were added during extraction of wax printed filter paper with cyt-C (*iii*). All chromatograms are normalized with a fixed MS intensity of $4.8 \cdot 10^9$.

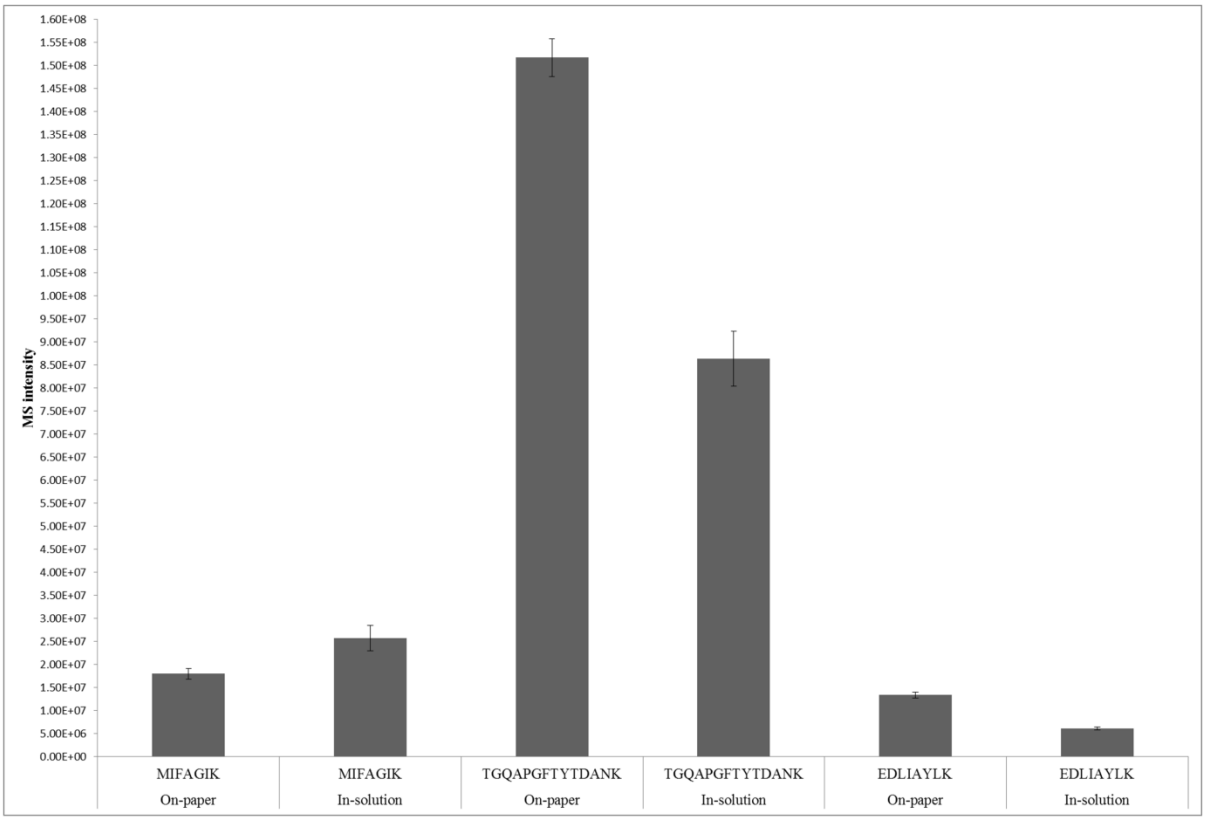

Figure 5 MS intensities of the three target peptides after *on-paper* digestion and *in-solution* digestion of cytochrome-C. The data set is presented with relative standard deviation error bars.

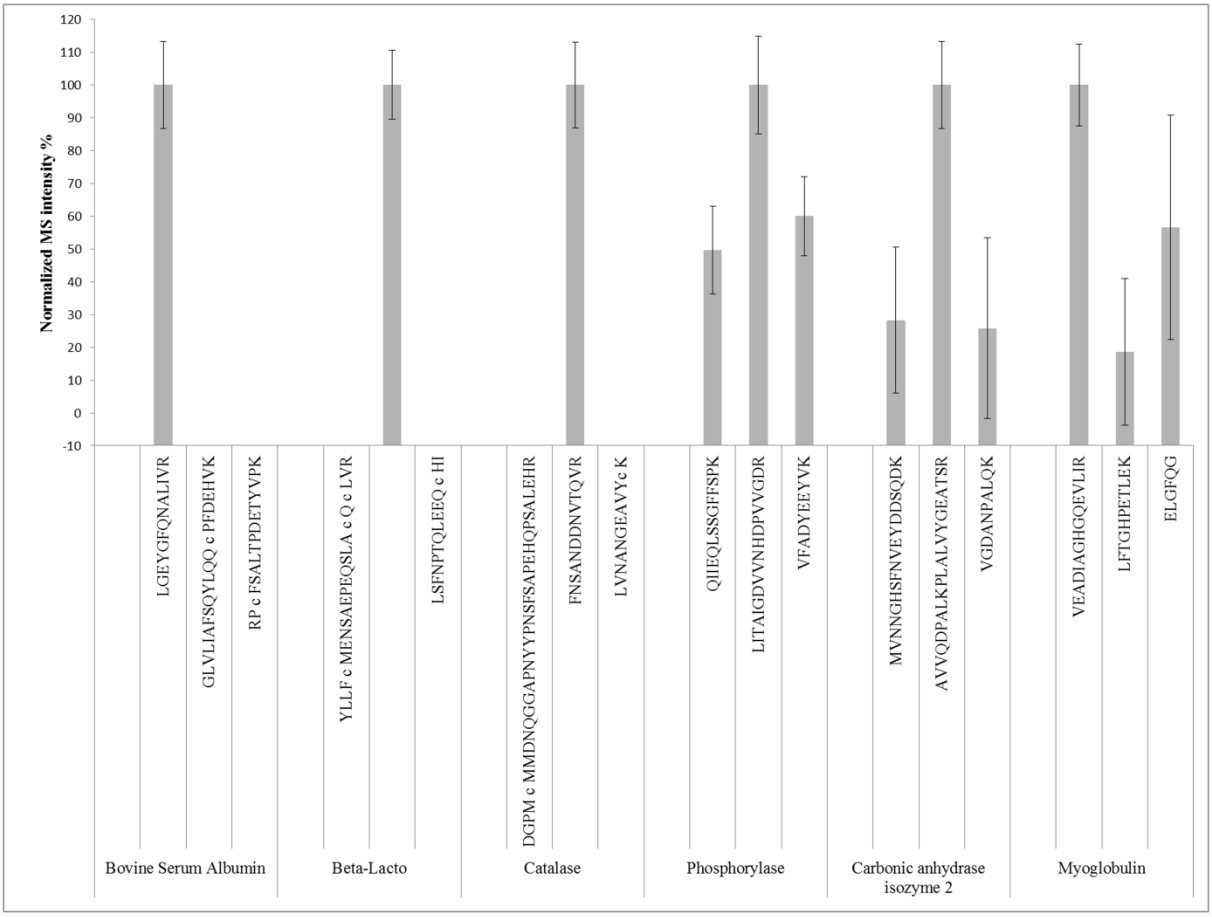

Figure 6 Normalized MS intensities (average for each protein with respect to the highest abundant peptide, n = 6). The dataset is presented with relative standard deviation error bars.

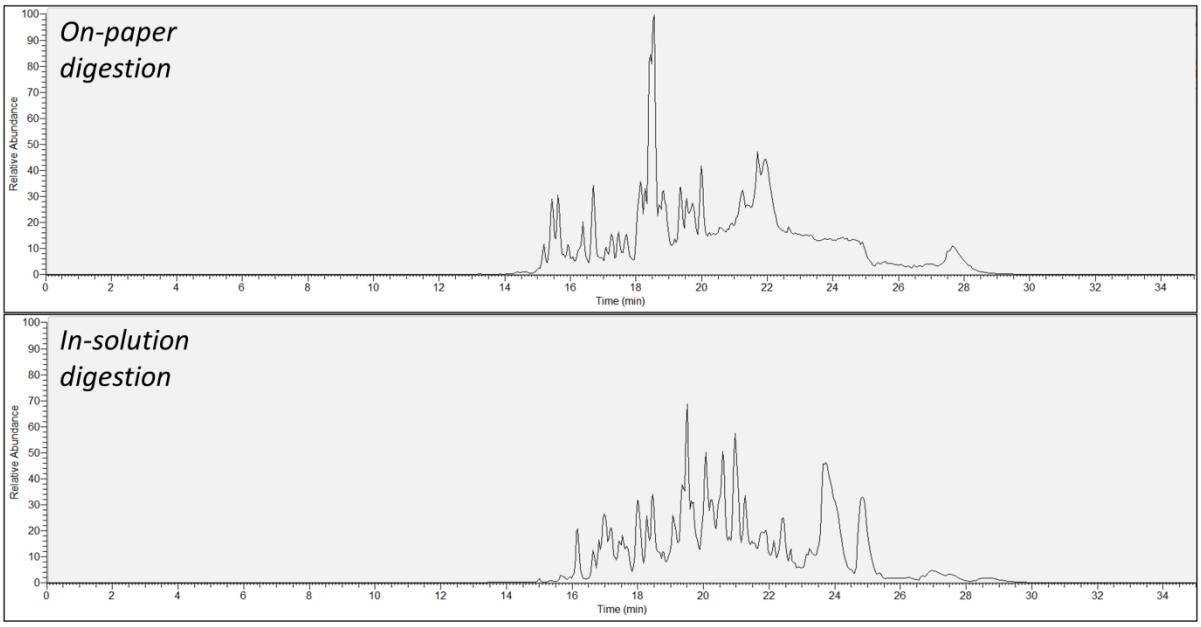

Figure 7 LC-MS Chromatograms from *on-paper* digestion and *in-solution* digestion of a six protein mixture with reduction and alkylation after protein digestion. The intensities are normalized to $6.70 \cdot 10^9$

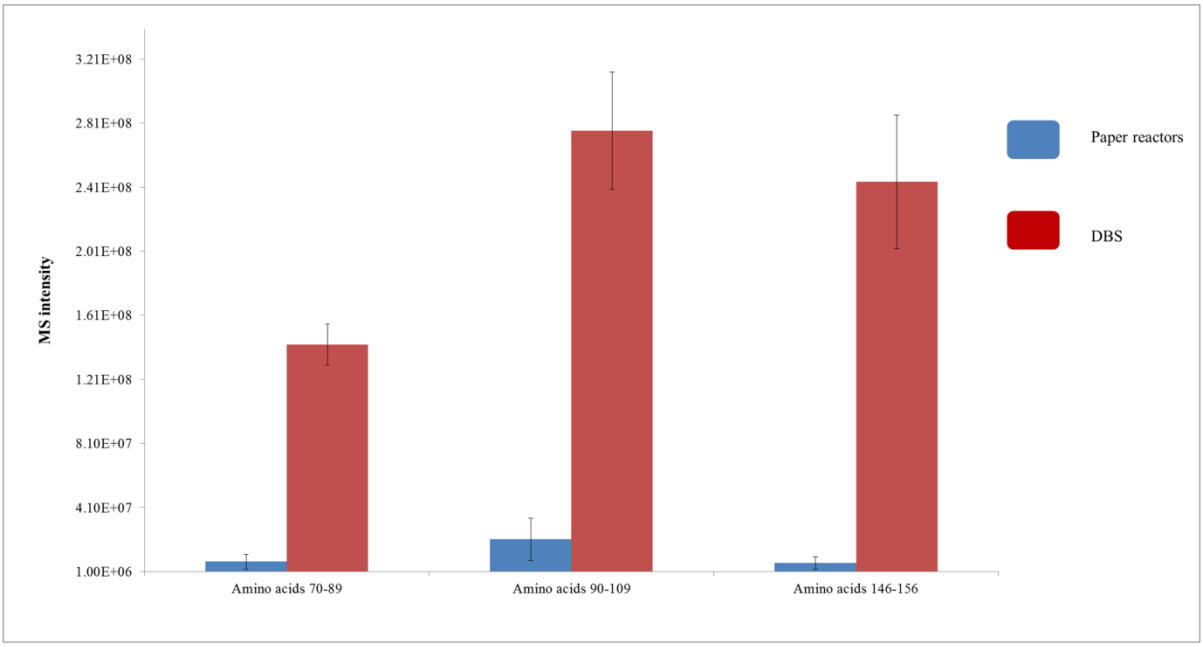

Figure 8 MS intensity of the three most frequent trypsin autolysis products found in pooled human whole blood with *on-paper* digestion and dried blood spot analyses. The data is presented with average values (n=3) and standard deviation

Tables

Table 5 Peptides chosen from each protein in a six-protein mix to evaluate protein digestion performance.

Protein	**Peptide 1 (*m/z*)**	**Peptide 2 (*m/z*)**	**Peptide 3 (*m/z*)**
Bovine serum albumin	LGEYGFQNALIVR (*740.39*)	GLVLIAFSQYLQQcPFDEHVK (*624.06*)	RPcFSALTPDETYVPK (*941.45*)
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