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RESEARCH ARTICLE

Dried Blood Spot Proteomics: Surface Extraction of Endogenous Proteins Coupled with Automated Sample Preparation and Mass Spectrometry Analysis

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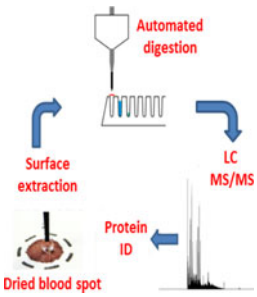

Abstract. Dried blood spots offer many advantages as a sample format including ease and safety of transport and handling. To date, the majority of mass spectrometry analyses of dried blood spots have focused on small molecules or hemoglobin. However, dried blood spots are a potentially rich source of protein biomarkers, an area that has been overlooked. To address this issue, we have applied an untargeted bottom-up proteomics approach to the analysis of dried blood spots. We present an automated and integrated method for extraction of endogenous proteins from the surface of dried blood spots and sample preparation via trypsin digestion by use of the Advion Biosciences Triversa Nanomate robotic platform. Liquid chromatography tandem mass spectrometry of the resulting digests enabled identification of 120 proteins from a single dried blood spot. The proteins identified cross a concentration range of four orders of magnitude. The method is evaluated and the results discussed in terms of the proteins identified and their potential use as biomarkers in screening programs.
Key words: Dried blood spots, Proteomics, Direct surface sampling, Liquid extraction surface analysis, Automated sample preparation

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Introduction

The analysis of dried blood spots (DBS) by mass spectrometry has become increasingly routine in clinical and pharmaceutical laboratories. There are many advantages to using DBS over whole blood samples. They are easier to store and transport than whole blood. Analytes preserved within a dried blood spot can be stable for years at ambient conditions [1] and can be eluted in solvents for later analysis. Many infectious agents such as blood borne viruses (e.g., HIV) are deactivated upon drying in the filter paper and so there are fewer biohazards associated with DBS than whole blood [2]. DBS also require very little sample (~20 μL of blood), which is advantageous when dealing with newborns or small mammals [1]. DBS can be collected via finger or heel prick, meaning there is no need for a specially trained phlebotomist or collection facility (i.e., samples can be taken by the patient in their own home [3]).

Mass spectrometry was first applied to the analysis of DBS samples in 1991 by Millington et al. [4] They used fast atomic bombardment (FAB) mass spectrometry to quantify derivitized esters of amino acids and acylcarnitines in DBS, with applications for PKU and medium chain acyl-coenzyme A dehydrogenase deficiency (MCAD) screening in newborns. The advent of electrospray ionisation led to multiple reaction monitoring (MRM) assays that detect underivatized metabolites, allowing for higher sample throughput. Many screening programs use a single-stage multi-analyte approach, where multiple markers of multiple diseases can be screened in a single analysis [5]. Mass spectrometry-based screening assays from DBS are now available for PKU, MCAD [6], maple syrup urine disease [7] , homocystinurea [8], and lysosomal storage disorders [9].

The pharmaceutical industry uses DBS in pharmacokinetic studies. Identification and quantitation has now been achieved for a range of drugs and their metabolites, as reviewed in [2]. More recently, DBS pharmaceutical analysis has coupled direct surface sampling with ambient ionisation. A variety of methods that have been trialled,

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including desorption electrospray ionization (DESI) [10], paperspray ionization [11], surface sealing sampling probe (SSSP) [12], and liquid extraction surface analysis (LESA) [13]. These methods have helped increase the throughput of DBS analysis, eliminating the need for any pretreatment or lengthy elution stages.

Despite the recent revolution in mass spectrometry-based proteomics, the DBS mass spectrometry field is almost entirely limited to the analysis of small molecule metabolites. Reports of protein analysis by mass spectrometry from DBS are scarce, and typically focus on the analysis of hemoglobin. MS/MS analysis of tryptic digests of samples eluted from DBS was demonstrated by Daniel et al. [14] and later by Boemer et al. [15] They performed both quantitative and qualitative analyses of hemoglobin by use of an MRM approach. Edwards et al. demonstrated direct surface sampling of DBS via liquid extraction surface analysis coupled with top-down mass spectrometry for the analysis of hemoglobin variants [16, 17]. Away from hemoglobin, the field is even more limited. In 2008, De Wilde et al. [18] demonstrated an MRM assay for the quantification of cerruloplasmin, a biomarker for Wilson's disease, from samples eluted from DBS. The assay gave comparable results to that of an immunoassay, but has not yet been taken to a population screening level. Kehler et al. reported MRM-based quantification of the 39-amino acid peptide exedin-4 that had been spiked into whole blood from monkeys and spotted onto a DBS card [19]. Sleczka et al. [20] used a similar MRM assay to quantify therapeutic proteins, administered intravenously, from DBS. Blood samples were pipetted onto filter card to form DBS and quantified against isotopically-labeled standards that had been spiked into the sample after the digestion. More recently, Chambers et al. [21] demonstrated a multiplexed MRM approach for the quantification of proteins eluted from DBS. From a panel of 60 human blood proteins, it was possible to quantify 37. While that study successfully analyzed multiple proteins, to date there has been no untargeted bottom-up proteomics analysis of DBS. Considering the wide body of research on the human plasma proteome [22, 23], DBS have been overlooked as a potential source of biomarkers [24]. Plasma is considered to be one of the most important proteomes from a clinical perspective [25]. DBS could offer a more convenient sampling method and could potentially be used in many different clinical assays where blood proteins act as biomarkers.

Here, we present an untargeted bottom-up proteomic analysis of DBS. Our method involves extraction of intact proteins via automated surface sampling of the DBS coupled with automated sample preparation (trypsin digestion), both performed by use of the Advion Biosciences Triversa Nanomate. The resulting peptide digests were analyzed by LC MS/MS. Over 120 proteins were identified, covering known concentration ranges of over four orders of magnitude.

Materials and Methods

The work was approved by the University of Birmingham STEM Ethical Review Committee (ERN_12-0782A). Normal adult DBS specimens were collected via finger prick onto standard NHS blood spot (Guthrie) cards, Ahlstrom grade 226 filter paper (ID Biological Systems, Greenville, SC, USA) and dried overnight. Conditions in the laboratory remained within a temperature 23–24 °C and a humidity of 21 %–26 %. Three DBS were analyzed.

Automated Trypsin Digestion

Samples were mounted onto a 96-well microtitre plate (Abgene PCR plate; Thermo Scientific, Loughborough, UK) and placed in the Triversa Nanomate chip-based electrospray device (Advion Biosciences, Ithaca, NY, USA). One of the 96 wells contained 50 mM NH_4HCO_3 (Fisher Scientific, Loughborough, UK), and a second contained 0.1 μg/μL trypsin (Trypsin Gold; Promega, Southampton, UK). The three DBS were sampled and digested in parallel to demonstrate the capability for multiplexing the method.

Surface sampling of the DBS and trypsin digestion was performed by use of the advanced user interface (AUI) feature of the ChipSoft Manager software, which controls the Triversa Nanomate. The sampling/digestion routine was based on robotic arm movements (X, Y, Z) of the Nanomate probe, and is illustrated in Figure 1. Liquid extraction surface analysis (LESA): seven μL of 50 mM NH_4HCO_3 was aspirated from the solvent well. The robotic arm relocated to a position above the DBS and descended approximately 0.8 mm above the sample. Six μL of the solution was dispensed onto the DBS to form a liquid microjunction. The liquid microjunction was maintained between the probe tip and the DBS surface for 4 s to allow diffusion of analytes into the solvent. Five μL were re-aspirated and dispensed into a clean well in the microtitre plate. The conductive pipette tip was then ejected, and a clean tip selected. Four and a half μL of trypsin solution was aspirated from the trypsin-containing well and dispensed into the sample well. (The 0.45 μg aliquot of trypsin used in this digestion equates to a 1/20 ratio of enzyme to protein. Protein concentration was calculated via a Bradford assay from a separate surface extraction). After the enzyme was added, the sample was mixed by one aspiration and dispense cycle of 4 μL and the second tip was ejected. The sample was incubated at 40 °C for 1 h by use of the temperature control unit of the Triversa Nanomate. At 30 min and 1 h of incubation, a further 7.5 μL of 50 mM NH_4HCO_3 was aspirated from the solvent well and added to the sample well, in order to account for evaporation of the solvent.

LC MS/MS

Once digestion was complete, samples were analyzed by LC MS/MS. The microtitre plate was removed from the Triversa Nanomate and placed in the autosampler of the HPLC

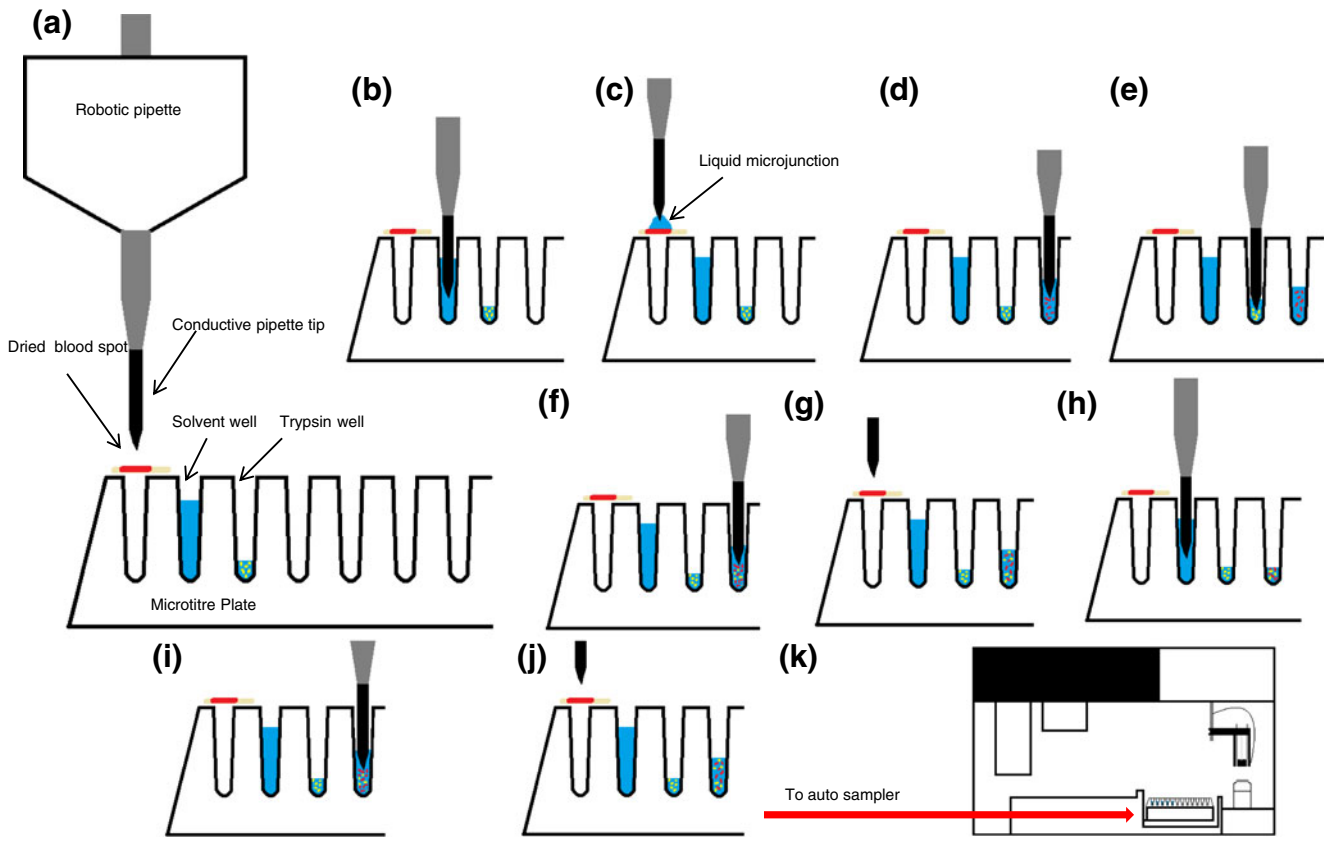

Figure 1. Summary of robotic workflow. **(a)** Starting position: DBS is mounted on the microtitre plate. One well contains extraction solvent and a second well contains trypsin solution; **(b)** 7 µL of 50 mMol NH_4HCO_3 is aspirated from solvent well; **(c)** 6 µL is dispensed onto DBS surface. Liquid microjunction is maintained between the pipette tip and the DBS surface (for 4 s) allowing intact proteins to dissolve into solvent; **(d)** solution of intact proteins (5 µL) is re-aspirated and dispensed into clean sample well; **(e)** 4.5 µL of 0.1 µg/µL trypsin solution is aspirated from trypsin well; **(f)** trypsin solution is added to sample well; **(g)** sample is incubated at 40 °C for 1 h. Enzyme digests intact proteins into peptides; **(h)** and **(i)** as solvent begins to evaporate from sample well, additional solvent (7.5 µL) is aspirated from solvent well and added to sample well [**(h)** and **(i)** are performed at 30 min and 1 h]. **(j)** Proteins are digested into peptides after 1 h. **(k)** Plate is transferred to HPLC autosampler and peptides are analyzed by LC MS/MS

system (Ultimate 3000; Dionex, Thermo Fisher Scientific, Loughborough, UK), which is coupled to a Thermo Fisher Orbitrap Velos ETD mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) via the Triversa Nanomate. Five µL aliquots were injected onto a Pepmap 100, C_{18} 100 µm nanoviper trap (Thermo Fisher Scientific, Loughborough UK) for online desalting. The trap was treated to a 5 min wash cycle with 0.1 % formic acid prior to injection onto the analytical column. This online desalting stage was performed with the aim of eliminating any off-line purification prior to LC MS/MS analysis, thus reducing the need for manual intervention. Peptides were separated on a Pepmap 100 reversed phase C_{18} 75 µm, 3 µm, 100 Å (Thermo Fisher Scientific, Loughborough, UK) by use of a 30 min 3.2 %–44 % ACN (J. T. Baker, The Netherlands) gradient at a flow rate of 0.35 µL/min. Samples eluted into the mass spectrometer via the Triversa Nanomate chip-based nanoelectrospray device. Ionization voltage was 1.4 kV, gas pressure was 0.3 PSI, and capillary temperature was 250 °C. Mass spectrometry analysis was performed via a 'top 7' CID method, in which a survey scan was followed by CID fragmentation of the seven most abundant precursor ions. Survey scans were acquired in the Orbitrap with a *m/z* range of 350–2000, a resolution of 60,000 at *m/z* 400, and an automatic gain control (AGC) target of 1×10^6 charges. CID was performed in the ion trap (AGC target: 30,000 charges) and a collision energy of 35 %. Dynamic exclusion was applied ensuring that no peptide of the same precursor *m/z* was selected within 60 s. All singly-charged peaks and those multiply-charged peaks with an intensity of <5000 charges were rejected for fragmentation.

Data Analysis

MS/MS data were searched against the SwissProt human database (downloaded in December 2012), composed of 20,233 sequences, using the Mascot and Sequest algorithms in Proteome Discoverer 1.3. For both algorithms, the following

parameters were applied: precursor ion mass accuracy 10 ppm, fragment mass tolerance 0.8 Da, methionine oxidation was allowed as a dynamic modification, up to two missed cleavages in the digestion. Peptide false discovery rates were calculated from a decoy database using the percolator function of Proteome Discoverer. Data were filtered to a false discovery rate of 1 %. The protein grouping algorithm was applied, which grouped all non-unique peptides to the highest scoring protein. All proteins identified by only one unique peptide were confirmed manually.

Results and Discussion

LESA extraction of proteins from DBS followed by automated trypsin digestion and LC MS/MS was performed in triplicate. The workflow employed is illustrated in Figure 1. The number of proteins identified in the three replicates was 120, 115, and 107 proteins. (Preliminary experiments [data not shown] in which the LESA extraction and trypsin digestion were performed manually and the digest analyzed by direct infusion MS/MS resulted in identification of hemoglobin α- and β-chains only, highlighting the need for LC separation). Across the replicates, 68 proteins were common to all three, with ~80 common between pairs of replicates, see Figure 2. Figure 3a–d show the results obtained from one of the DBS samples. The total ion chromatogram, shown in Supplemental Figure 1, demonstrates that samples prepared via LESA sampling of DBS and automated trypsin digestion contain peptides of suitable abundance, but without leading to any overloading of the HPLC column.

In the example shown in Figure 3, 120 proteins were identified, and these are summarized in Table 1 and detailed in Supplemental Table 1. Proteins identified on the basis of single peptides were checked manually and the CID MS/MS spectra are shown in Supplemental Figure 2. The proteins identified include many of the classic plasma proteins, along with hemoglobin chains and other proteins derived from erythrocytes. The proteins identified cross concentration ranges of over four orders of magnitude in human blood. The most abundant proteins identified were hemoglobin and albumin, and the least abundant was protein S100-A9. Hemoglobin makes up 97 % of the protein in human red blood cells [26]. Albumin has a plasma concentration of 500–800 μmol/L, whereas protein S-100 A9 has a concentration of 0.05–0.014 μmol/L [27]. The CID MS/MS spectrum obtained from $[M + 2H]^{2+}$ ions of peptide VGAHAGEYGAEALER from hemoglobin α-chain is shown in Figure 3a. Full peptide sequence coverage is obtained. Figure 3b and c show MS/MS spectra obtained from $[M + 2H]^{2+}$ ions of peptides from proteins of intermediate concentration in blood: LGVYELLLK from inter 1 alpha trypsin inhibitor H4 and ALYLQYTDETFR from cerruloplasmin. Inter alpha trypsin inhibitor H4 has an plasma concentration of 2–4 μmol/L and cerruloplasmin has a plasma concentration of 2–5 μmol/[27]. Figure 3d shows the CID MS/MS spectrum obtained from $[M + 2H]^{2+}$ ions of peptide NIETIINTFHQYSVK from protein S100-A9, which has a low plasma concentration of 0.05–0.014 μmol/L [27].

The categories of proteins identified from the DBS are illustrated in Figure 4, and detailed in Supplemental Figure 3. The proteins identified include four hemoglobins, 61 known plasma proteins [22, 27, 28], 14 structural proteins such as keratins, 19 proteins of known erythrocyte or platelet origin [29–31], and 22 proteins of an unknown cellular origin, eight of which were enzymes. Of the 61 plasma proteins, there were eight complement proteins such as C3 and C9, 15 transport proteins (e.g., albumin and apolipoproteins), and

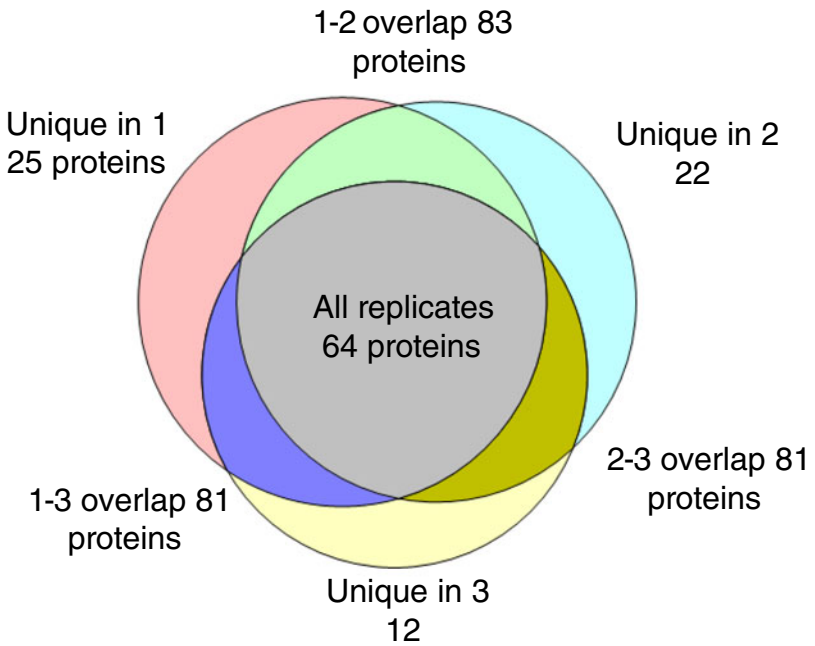

Figure 2. Reproducibility of identifications. Protein identifications from dried blood spots across the three replicates

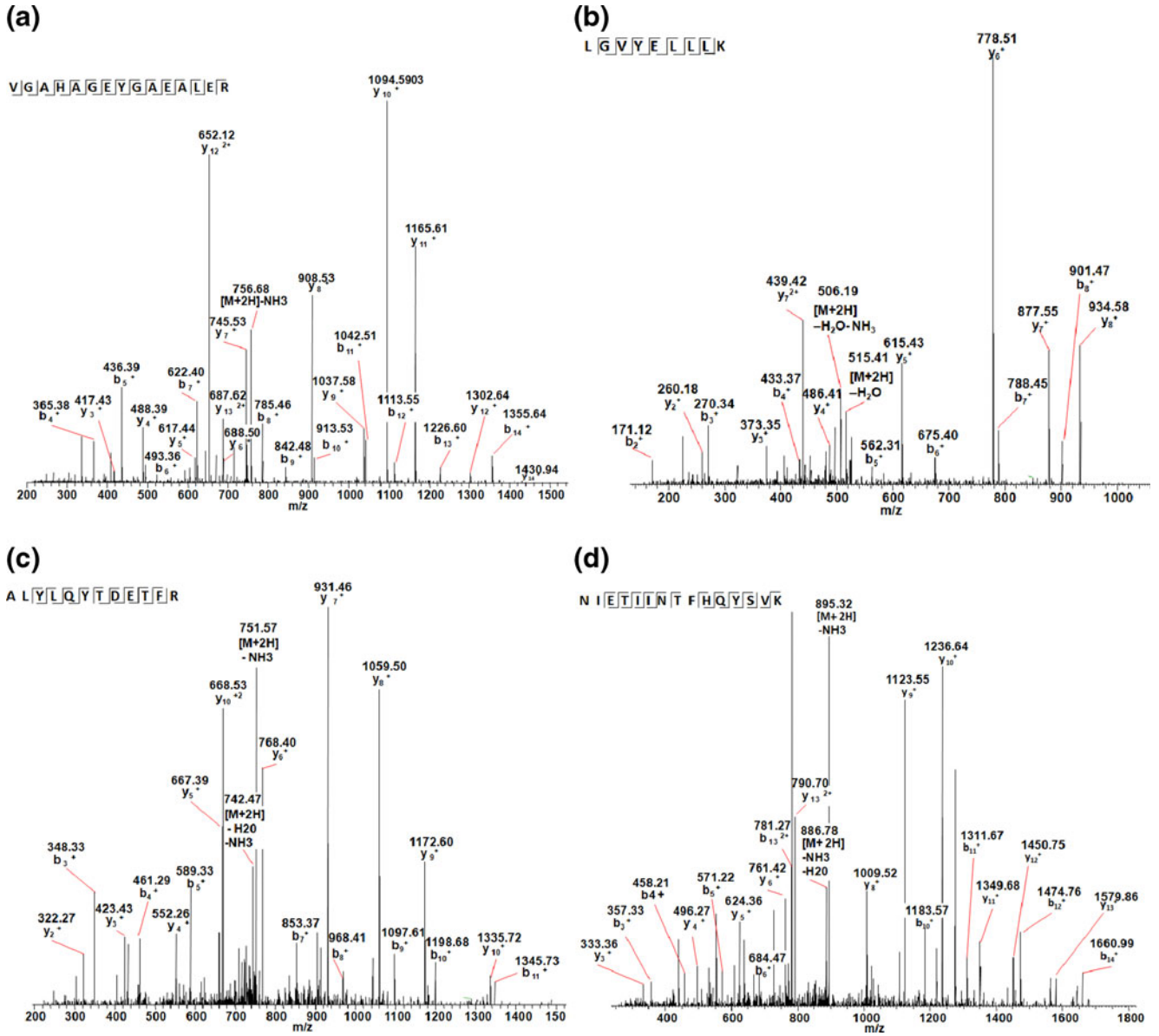

Figure 3. **(a)** CID MS/MS spectrum of peptide VGAHAGEYGAEALER in the +2 charge state, from alpha chain of hemoglobin, a very high abundance protein. **(b)** CID MS/MS spectrum of peptide LGVYELLLK in the +2 charge state, from inter alpha trypsin inhibitor H4, a medium abundance protein. **(c)** CID MS/MS spectrum of peptide ALYLQYTDETFR in the +2 charge state from cerruloplasmin, a medium abundance protein and biomarker of Wilson's disease. **(d)** CID MS/MS spectrum of peptide NIETIINTFHQYSVK in the +2 charge state from protein S100 A9, a very low abundance protein

five coagulation factors, including fibrinogen, prothrombin, and kininogen 1. The remaining plasma proteins were composed of 10 immunoglobulins and 23 other plasma proteins, including glycoproteins and protease inhibitors. Twenty-nine of the 37 proteins quantified by Chambers et al. [21] in their targeted analysis were identified here. In addition, two of the proteins targeted but not detected in that study were identified here.

A large number of the structural proteins identified were keratinous. Keratin is a common contaminant in proteomics experiments, originating from dust in the laboratory or the users themselves contaminating the sample. In this case, the keratin is likely to originate from the blood collection method: the blood was acquired via a finger prick, releasing a skin plug into the blood and onto the card.

Many of the proteins identified are known to be found in erythrocytes or platelets [29–31]. These include the hemoglobin proteins and several others, of which many were enzymes. Examples include carbonic anhydrase, catalase, and peroxiredoxin 2. There were some proteins whose exact cellular origin could not be classified. These include histone H2A and mucin-like protein. Although not classic plasma proteins or associated with erythrocytes, these could originate from other types of blood

Table 1. Proteins identified from LC MS/MS run of trypsin digestion of endogenous proteins sampled from surface of DBS

Protein Description	Concentration in Plasma/μmol/L*	Protein Description	Concentration in Plasma/μmol/L *
Hemoglobin subunit beta		Ras-related protein Rab-14	
Hemoglobin subunit delta		Glutathione S-transferase A1	
Hemoglobin subunit gamma-1		Ig alpha-1 chain C region	8–50
Hemoglobin subunit alpha		Polyubiquitin-C	
Protein S100-A9	0.05–0.14	Serum amyloid A-4 protein	4
Apolipoprotein A-I	30–70	Alpha-synuclein	
Glyceraldehyde-3-phosphate-dehydrogenase		Thioredoxin	
Ig kappa chain C region	68–150	Cofilin-1	
Carbonic anhydrase 1		Protein DJ-1	
Carbonic anhydrase 2		Mucin-like protein 1	
Peroxiredoxin-6		Complement C4-B	0.5–2
Protein S100-A6		Ig gamma-3 chain C region	2–16
Keratin, type I cytoskeletal 10		Alpha-1B-glycoprotein	3–5
Complement C3	5–10	Vitronectin	1–3
Serum paraoxonase/arylesterase 1		Gelsolin	3–5
Keratin, type II cytoskeletal 1		Haptoglobin	0–40
Apolipoprotein A-II	30–60	Keratin, type II cytoskeletal 5	
Serum albumin	500–800	Lactotransferrin	
Ig gamma-1 chain C region	68–150	Semenogelin-2	
Flavin reductase		Ig kappa chain V-I region Lay	68–150
Keratin, type I cytoskeletal 9		Trypsin-1	
Keratin, type II cytoskeletal 2 epidermal		Angiotensinogen	1
Alpha-1-antitrypsin	18–40	Keratin, type II cytoskeletal 6A	
Keratin, type I cytoskeletal 13		Heat shock protein beta-1	
Apolipoprotein C-III	6–20	Prothrombin	1.5
Dermcidin		Histidine-rich glycoprotein	1-3
Protein S100-A8	0.05–0.14	Apolipoprotein E	0.6–2
Inter-alpha-trypsin inhibitor heavy chain H4	2–4	Ig lambda chain V-III region LOI	36–48
Bisphosphoglycerate mutase		Alpha-enolase	
Peroxiredoxin-2		Zinc-alpha-2-glycoprotein	0.8–1.6
Actin, cytoplasmic 1		Alpha-1-antichymotrypsin	4–9
Histone H2A type 1-H		Apolipoprotein-L1	0.2
Inter-alpha-trypsin inhibitor heavy chain H2	2–3	Kininogen-1	3
Ceruloplasmin	2–5	Adenylate kinase isoenzyme 1	
Apolipoprotein C-II	2–7	Fibrinogen alpha chain	10–27
Vitamin D-binding protein		Delta-aminolevulinic acid dehydratase	
Nucleoside diphosphate kinase A		Alpha-2-HS-glycoprotein	9-30
Serotransferrin	25–45	Plasma protease C1 inhibitor	
Complement factor B		Ig gamma-2 chain C region	20–90
Ig kappa chain V-III region SIE	68–150	Arginase-1	
Alpha-1-acid glycoprotein 1	9–20	Zymogen granule protein 16 homolog B	
Keratin, type II cytoskeletal 2 oral		Complement factor H	2–5
GTP-binding nuclear protein Ran		Rab GDP dissociation inhibitor beta	
Hemopexin	9–20	Heat shock-related 70 kDa protein 2	
Catalase		14-3-3 protein theta	
Apolipoprotein A-IV	3–6	Plasminogen	2–4
Clusterin N]	1–2	Fibroblast growth factor 2	
Alpha-2-macroglobulin	7–17	Liver carboxylesterase 1	
Fibrinogen beta chain	10–27	Heparin cofactor 2	1.5
Lysozyme C	0.01–1	Serpin B3	
Ig lambda-7 chain C region	68–150	Protein disulfide-isomerase A2	
Peroxiredoxin-1		Zinc finger protein 611	
Fibrinogen gamma chain	9–24	Apolipoprotein B-100	1–3
Semenogelin-1		Phosphoglycerate kinase 1	
Peptidyl-prolyl cis-trans isomerase A		Retinal dehydrogenase 1	
Apolipoprotein C-I	6–12	Lumican	
Purine nucleoside phosphorylase		Complement C1s subcomponent	1
Keratin, type I cytoskeletal 14		Plasma kallikrein	
Inter-alpha-trypsin inhibitor heavy chain H1	2–4	Ig mu heavy chain disease protein	4–25
Fructose-bisphosphate aldolase A		Complement component C9	0.4–1

*Reference concentrations of named protein in healthy human plasma [27]

cells or merely be a result of tissue leakage into the blood stream, which is known to account for some of the lesser abundant proteins in human plasma [25].

Several proteins were identified that could act as biomarkers in newborn screening programs or other clinical assays [18, 32, 33]. In addition to hemoglobin, suitable

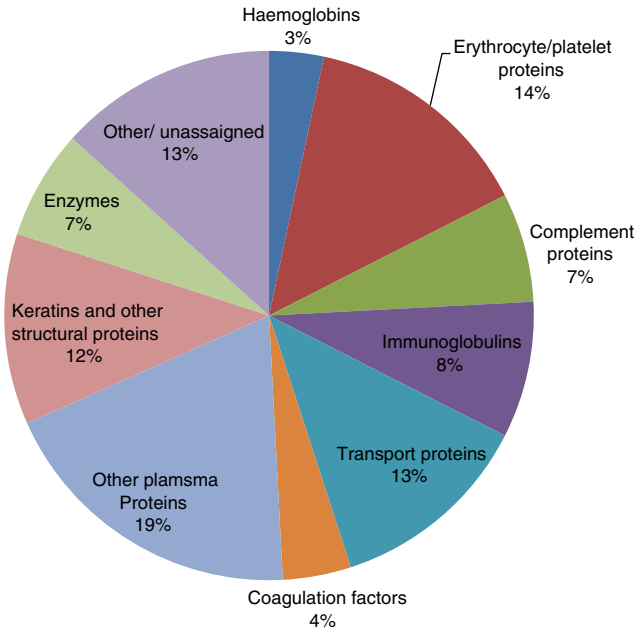

Figure 4. Categories of proteins identified

candidates include cerruloplasmin, which can act as a biomarker for Wilson's disease, a copper transport deficiency [18], and alpha-1-antitrypsin, a biomarker for alpha-1-antitrypsin deficiency. Alpha-1-antitrypsin deficiency can result in chronic obstructive pulmonary disease (COPD), emphysema, and liver failure, and often goes undiagnosed. It has been suggested that screening of individuals suffering from COPD for alpha-1-antitrypsin deficiency could be beneficial [33]. Protein S100 A9 and S100 A8 were the least abundant proteins identified in this study and have been shown to be up-regulated in blood plasma in several inflammatory diseases, such as rheumatoid arthritis, cystic fibrosis, and bronchitis [34]. As more plasma proteins are beginning to become associated with more disease states, it is expected that the number of biomarkers that can be identified from DBS will increase. For example, pancreatic cancer has been associated with increased levels of alpha-1B-glycoprotein, alpha-2 macroglobulin, complement C3, complement C9, complement factor H, and hemopexin, all of which were identified in this study.

As described above, multiple proteins can be successfully identified from a single dried blood spot via this simple automated tryptic digestion. The DBS require no pretreatment, no separate elution from the spot, and no lengthy sample preparation or cleanup stages. A range of digestion times (30 min, 1, 2, 4, and 8 h) were investigated. Supplemental Figure 4 shows representative total ion chromatograms from each time point. As expected, the amount of undigested protein observed decreased with digestion time; however, that did not correlate with increased numbers of protein identified (see Supplemental Figure 5). The optimum digestion time was 1 h, which correlates well with the timescale of LC MS/MS analysis and offers the potential for automated parallel analysis on a large scale. It is possible that use of proteases with broader specificity, such as pepsin or proteinase K, might result in greater numbers of protein identifications on this timescale, and work in this area is ongoing. It is also worth noting that the short digestion time may lead to larger proteolytic fragments for which separation by C18 liquid chromatography is not ideal. Use of C4 or C8 stationary phase may further increase the number of protein identifications.

The method also offers advantages in terms of ease-of-use: the digestion is fully automated and multiple samples can be digested in parallel, as performed here. Whilst the LC analysis is not fully integrated with the sample preparation, the only manual intervention required is to take the microtitre plate out of the Triversa Nanomate and place it into the HPLC autosampler. Online desalting was performed to eliminate the need for any off-line purification and any manual intervention.

A particular advantage of the DBS LESA extraction method presented here is that it requires very small amounts of trypsin. The results of a Bradford assay (data not shown) revealed that 8.7 µg of protein is extracted from the surface of the DBS using LESA. With only 8.7 µg of protein extracted from the spot, very little enzyme is required. In previous studies, Chambers et al. used 3.5 µg of trypsin per microliter of blood applied to the DBS, totaling 52.5 µg of enzyme per blood spot [21], Daniel et al. used 25 µg [14] , De Wilde et al. used 27.2 µg [18], and Sleczka et al. used 200 µg [20]. The method presented here requires just 0.45 µg of trypsin per sample.

The Triversa Nanomate used for DBS sampling and sample preparation can be interfaced with most mass spectrometers. Although this study used the high resolution Orbitrap mass spectrometer, the instrument of choice for bottom-up proteomics, it is entirely feasible that the DBS sampling and sample preparation methods could be employed in conjunction with other mass spectrometers. For example, it is possible that MRM assays on triple quadrupole mass spectrometers could be developed using this approach.

Conclusion

We have demonstrated an automated method incorporating liquid extraction surface analysis of DBS and trypsin digestion coupled with LC MS/MS. The results show that DBS are a suitable sample format for proteomics analyses. Over 100 proteins were identified in each analysis, crossing a concentration range of four orders of magnitude. The lowest known concentration protein identified was S100 A9 (0.05–0.014 µmol/L in plasma). A number of the proteins identified are potential biomarkers for disease.

The DBS sampling and digestion procedure is quick, automated, reproducible, requires minimal reagents, and can be multiplexed. The robotic platform is commercially available, does not require any specialist programming skills or any software development, and can be interfaced with most mass spectrometers. The method could be of particular use for high throughput analysis and screening

programmes. To achieve this goal, full integration of the automated sample preparation and LC MS/MS is required and work in that area is ongoing.

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