ORIGINAL

Targeted Metabolomics of Dried Blood Spot Extracts

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Abstract Dried blood spot (DBS) samples are already successfully used in newborn screening and pharmacological analyses. The application of DBS matrix to further metabolomic methods will considerably extend the analytical options for the diagnostics of metabolic diseases. We present an MS/MS based method for the simultaneous extraction and quantification of 188 metabolites from dried blood spots. We provide a sensitive and reproducible method that adapts the Absolute*IDQ*^{TM} p180 kit of Biocrates to the DBS matrix for the quantification of metabolites of different substance classes including amino acids, biogenic amines, free carnitine, acylcarnitines, hexoses, glycerophospholipids, lysophosphatidylcholines, phosphatidylcholines, and sphingolipids.

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Introduction

The sample matrix dried blood spot (DBS) is commonly used for many years in the diagnosis of inborn errors of metabolism [1–3]. Recently, the analysis of DBS has been extended and become a practicable tool for many different applications ranging from clinical monitoring [4] over animal testing [5] to pharmacokinetics [6]. Studies analyse single substances [7] or perform complete metabonomic profiling [8] based on DBS as sample matrix.

DBS offers some essential advantages over fluid samples. (1) The sampling is minimally invasive and easy to perform (e.g. finger or heel prick, rather than conventional venous puncture) and can even be done by the patients or volunteers themselves [4]. (2) The required sample volume is significantly lower when compared to the venous collection. (3) DBS samples offer possibilities for easy shipment and storage (e.g. no requirement for dry ice). (4) Furthermore, DBS analysis could lead to the detection of whole blood markers that might not be present in plasma or serum samples. Clinical trials or cohort studies requiring collection, shipment, storage, and analysis of thousands of samples could benefit from these advantages. Several population-based studies have already employed the DBS sample matrix [9].

The Absolute*IDQ*^{TM} p150 kit from Biocrates is a frequently used assay kit for targeted metabolomics and several seminal discoveries in large-scale population profiling or mechanistic analyses have been published [10–14]. In 2010, the extended Absolute*IDQ*^{TM} p180 kit ("p180-kit") was released and first results have already

been published [15]. Whereas the p150-kit is applying FIA-MS/MS (flow injection analysis tandem mass spectrometry), the p180-kit includes LC-MS/MS (liquid chromatography tandem mass spectrometry) as well. Up to now, no study has applied these targeted metabolomics assays to dried blood spot samples. The p180-kit has been originally validated for the quantification of metabolites in human blood plasma sampled by conventional venous blood collection and centrifugation. The kit can be further adapted for the use with other sample matrices, e.g. cultured cells or tissues (Zukunft et al., manuscripts in preparation). We here describe the quantification of metabolites of DBS with the Absolute*IDQ*^{TM} p180 kit.

Experimental Section

Sample Preparation

Preparation of Dried Blood Spots

To assure homogenous sample properties during sample preparation like similar coagulation state and fluidity, we used EDTA-blood instead of native blood for dried blood spots (DBS) preparation. Venous blood from one male and one female volunteer was collected in 9 mL EDTA tubes (S-Monovette® K3E from Sarstedt, Nümbrecht, Germany) or in lithium-heparin tubes (Kabe Labortechnik, Nümbrecht-Elsenroth, Germany) according to the manufacturer's instructions. Aliquots (80 µL) of freshly taken blood were spotted onto the middle of Whatman 903^{TM} filter paper card circles (GE Healthcare, Westborough, MA, USA). It was ensured that the blood filled the circle uniformly. As described by the manufacturer, each circle has a diameter of 12.7 mm and holds 75–80 µL of blood. The samples were dried at room temperature (RT) for 4 h to receive DBS. The DBS were either processed directly on the day of preparation or stored until use as described below.

Preparation of Plasma and Blood Cells

In parallel to blood taking for the DBS sample preparation, venous blood from the male volunteer was collected in a second 9 mL EDTA tube to gain access to EDTA plasma and blood cells. The blood sample was centrifuged for 10 min at RT and $2,750 \times g$ and plasma was separated from the pelleted blood cells by pipetting.

Preparation of DBS with Defined Haematocrit

To investigate the influence of the haematocrit (HK) on the metabolite quantification results, blood cells and EDTA-plasma of the male volunteer were mixed in defined ratios [25, 33, 50, 66, 75 % blood cells/plasma (v/v)]. 80 µL of each blood cell/plasma mixture were spotted onto filter paper cards and further processed as described above for the regular DBS samples.

DBS Sample Disk Preparation

Sample Disk Preparation

Using a hand punch (GE Healthcare, Westborough, MA, USA), 3-mm diameter disks were punched out of the centre of the DBS ("sample disks") and immediately transferred to the wells of the p180-kit filter plate for further processing. Between each sample punch, two punches on clean, empty filter paper cards were performed (cleaning punches) to minimise cross contamination. From each DBS exactly one sample disk was prepared.

Cleaning Disk Preparation

For the examination of cross contamination, two 3-mm disks from clean and empty filter paper were successively punched out directly after a DBS punch without any cleaning punches in between (replicated three times).

Blank Disk Preparation

To determine the baseline effects of the filter paper matrix, three clean and empty 3-mm filter paper disks ("blank disks") were punched and processed in parallel to sample disks.

Direct Application of Blood to 3-mm Filter Paper Disks

3 µL of male EDTA-blood was pipetted directly onto clean and empty pre-punched 3-mm filter paper disks ("spotted disks") and dried at RT for 4 h. The spotted disks were immediately transferred to the wells of the p180-kit filter plate for further processing. The experiment was performed in triplicates.

DBS Storage

For the examination of the stability of metabolites over time at different temperatures, filter paper cards containing the DBS were placed in sealed bags (multi-barrier pouches, GE Healthcare, Westborough, MA, USA) together with two desiccant packages (1 g desiccant/package, GE Healthcare, Westborough, MA, USA). These bags and two additional desiccant packages were placed in zip-lock plastic bags. The storage conditions were −80, −20, 4 °C and RT each for 1, 2, and 4 weeks. Before disks were punched, the bags were equilibrated for 30 min at RT. For each temperature and each time point, triplicates were performed.

Reproducibility

The reproducibility of the method was determined by assaying six EDTA-DBS disks from a male and six from a female volunteer each. For inter-assay reproducibility, six additional replicates from the male volunteer were processed and analysed in parallel by a second experimenter.

Comparison of DBS with Plasma Samples

For comparison of DBS with plasma metabolite levels, 10 μL of EDTA plasma from the male volunteer were applied to the p180-kit filter plate in parallel to 3-mm EDTA-DBS disks from the same person and further processed. Six replicates per sample type were performed.

Linear Behaviour

To determine the relation between sample amount and metabolite signal, different sizes and numbers of disks were transferred to the p180-kit filter plate. From the male EDTA-DBS ¼, ½, one, two, or three 3-mm disks were applied per well and ½, one, two or three 3-mm disks from the female EDTA-DBS and further processed like regular DBS sample disks.

Metabolite Quantification

We used the AbsoluteIDQ^{TM} p180 kit of Biocrates (Biocrates Life Sciences AG, Innsbruck, Austria) for targeted metabolite quantification. The kit measurement consists of two parts: an HPLC separation step (LC) and a flow injection analysis (FIA) step, both followed by mass spectrometry analyses (LC-MS/MS and FIA-MS/MS, respectively). Mass detection and compound identification are performed by multiple reaction monitoring (MRM). The LC-MS/MS procedure enables the quantification of 42 metabolites, covering 21 amino acids (AA) and 21 biogenic amines (BA). The FIA-MS/MS procedure is used for the quantification of 146 additional metabolites, covering 40 acylcarnitines (AC) including free carnitine, 38 phosphatidylcholines with acyl/acyl side chains (PCaa), 38 phosphatidylcholines with acyl/alkyl side chains (PCae), 14 lysophosphatidylcholines (lysoPC), 15 sphingolipids (SM), and the sum of hexoses.

All liquid handling was performed by a Hamilton Microlab STAR^{TM} workstation (Hamilton Bonaduz AG, Bonaduz, Switzerland). The drying steps were performed by an Ultravap nitrogen evaporator (Porvair Sciences, Wrexham, UK). MS/MS measurements were performed on an API 4000 System (AB Sciex Deutschland GmbH, Darmstadt, Germany) coupled to an Agilent 1200 Series HPLC and an HTC PAL auto sampler (CTC Analytics, Zwingen, Switzerland), all controlled by the software Analyst 1.6 (AB Sciex Deutschland GmbH, Darmstadt, Germany).

Measurements were performed according to the manufacturer's manual with minor modifications in case of DBS disk measurements. Briefly, internal standards for the LC-MS/MS procedure were pipetted onto the filter inserts of the 96-well plate. This plate is pre-spiked with internal standards for the FIA-MS/MS procedure by the manufacturer. Either 10 μL plasma or DBS disks were placed into the cavities of the 96-well filter plate of the p180-kit. Plasma samples were dried in a nitrogen stream for 30 min. This initial drying step required with plasma was not performed for the DBS disks. Amino acids and biogenic amines in the samples were derivatized with an excess of 5 % phenylisothiocyanate (Sigma-Aldrich, Steinheim, Germany) for 20 min and dried under a nitrogen stream. Metabolites and internal standards were then extracted with 300 μL methanol (AppliChem, Darmstadt, Germany) containing 5 mM ammonium acetate (Sigma-Aldrich, Steinheim, Germany) by incubation for 30 min at RT with continuous shaking. Metabolites were eluted by a centrifugation step (5 min at $500 \times g$ at RT). One part of the eluate was diluted with Biocrates kit running solvent (1/5 (v/v)) for FIA-MS/MS analysis, the other part with water (50/50, (v/v)) for LC-MS/MS analysis. The LC run was performed using an XDB-C18 column (3×100 mm, 3.5 μm) (Agilent Technologies, Waldbronn, Germany).

Samples were always quantified without delay on the day of disk punching. Disks of different storage times were measured on different kit plates. To ensure comparability of the data, we normalised the results of each plate to the measurements of five parallel processed reference plasma samples which were included in every kit plate.

Data evaluation for quantification of metabolites and quality assessment were performed with the MetIDQ^{TM} software package, which is an integral part of the p180-kit. Internal standards served as references for the calculation of metabolite concentrations. Statistical analysis was performed in R [16] and with the metaP-server [17].

For metabolite specification and the detailed protocol of metabolite quantification we refer to [14, 15].

Results and Discussion

Rationale for the Experiments

The AbsoluteIDQ^{TM} p180 kit of Biocrates (p180-kit) has been already validated for human blood plasma following FDA criteria [18] but up to now for no other biological matrix. In this study, we applied the kit to dried blood spots (DBS) and analysed the concentrations of 188 metabolites in this matrix. Based on internationally accepted criteria

[19] we also provide a partial validation for the use of the p180-kit with the new matrix. Here, it should be noted, that we aimed to show the feasibility of applying the sample matrix DBS to the already validated p180-kit and that the accuracy of the quantification in DBS samples was not part of the validation.

To enable method validation with homogenous samples, we used EDTA-blood for the preparation of DBS instead of native blood. Native blood samples would have coagulated during DBS preparation and probably lead to inhomogeneities of the DBS. However, for amino acids or acylcarnitines, the use of EDTA-blood does not affect the absolute measured values as shown in [20].

As outlined in the following sections, we looked at the comparability of DBS to plasma samples, tested the reproducibility and linearity of the method, analysed matrix effects, the influence of haematocrit and anticoagulants, checked for cross contamination, and analysed metabolite stability under different storage conditions.

Comparison of DBS and Plasma Metabolite Concentrations

Metabolite quantification from DBS might be favourable over the respective analyses from plasma because the sample source DBS has many advantages: it is easy to obtain, process, and store, and can be generated even from low sample amounts. However, the number of detectable metabolites might be different to that of plasma and the absolute concentration levels might vary significantly.

Thus, to compare the metabolite levels between DBS and plasma, sample disks from the male DBS were quantified in parallel to 10 μL male EDTA plasma samples (10 μL are the standard sample volume recommended for the p180-kit). After data evaluation, the concentrations and the absolute number of metabolites that exceeded the limit of

detection (LOD), as defined for the p180-kit, were used for the further analyses.

We were able to detect nearly the same number of metabolites in one 3-mm DBS sample disk as in 10 μL plasma. In DBS disks, 137 metabolites were found in levels above LOD (29 of 42 metabolites in LC-MS/MS and 108 of 146 metabolites in FIA-MS/MS) compared to 155 in plasma (35 of 42 metabolites in LC-MS/MS, 120 of 146 in FIA-MS/MS) (Supplementary Table 1).

We directly compared the absolute concentrations of the two blood derived sample matrices, the 10 μl plasma samples and the 3-mm DBS sample disks (the latter containing estimated volumes of 3 μL blood each). We found a high level of accordance between DBS and plasma metabolite concentrations.

For metabolites measured by LC-MS/MS, the absolute concentrations found in DBS were mostly within the range of 0.5–1.5-fold from that observed in plasma. Only a few metabolites showed considerably lower (one metabolite) or higher (seven metabolites) levels in DBS. For biogenic amines, metabolites were mostly equal or higher concentrated in DBS compared to plasma samples (Fig. 1a).

Looking at the FIA-MS/MS results, most of the metabolite concentrations in DBS were found to be in the range of 0.5–1.5-fold of that in plasma. Only acylcarnitines (with one exception) and long-chain sphingomyelins were concentrated considerably higher in the DBS samples (Fig. 1b). Absolute concentrations for the DBS and the plasma samples are listed in Supplementary Table 1.

Our data indicate that metabolites can be quantified from DBS with the Absolute*IDQ*^{TM} p180-kit. In DBS and plasma, nearly the same number and types of metabolites were accessible for quantification. However, the DBS provided slightly different metabolite levels than plasma. Such differences were expected due to the fact that DBS contains blood cells beside plasma. Considering the above
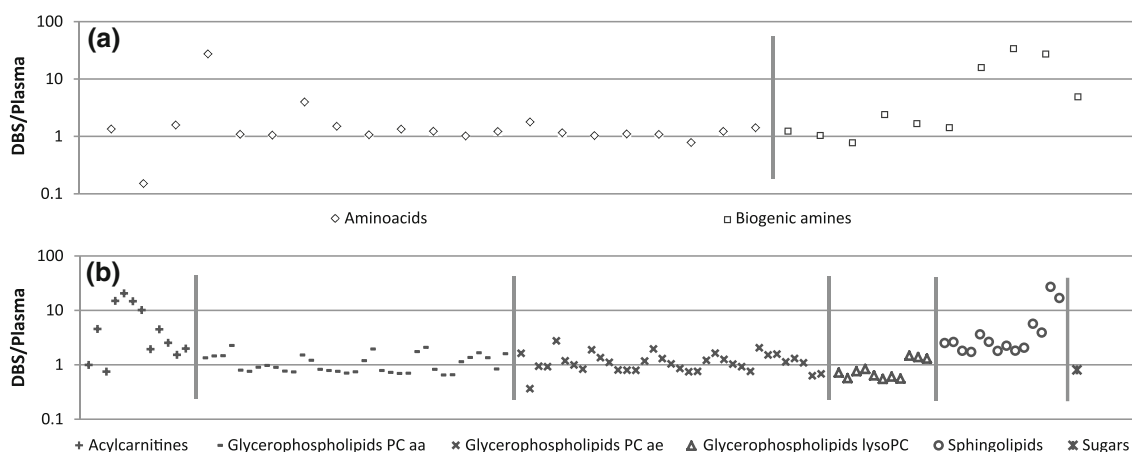
Fig. 1 DBS/plasma ratios of the mean metabolite concentrations for all metabolites above LOD on a log scale. **a** metabolite ratios of LC-MS/MS data, **b** metabolite ratios of FIA-MS/MS data

mentioned advantages and results, DBS is an alternative sample material for the p180-kit.

Reproducibility

DBS preparation and processing are already applied in a standardised way worldwide [21]. However, DBS as a sample source for the AbsoluteIDQ^{TM} technology is not established yet and reproducibility has to be ascertained.

To test for intra-experiment precision, the coefficients of correlation (CVs) of six DBS sample disk replicates each, of a male and a female sample were determined. Additionally, we verified the inter-experiment performance. For this, we compared the CVs of data produced by two different experimenters, both preparing and processing six DBS sample replicates of the male sample.

For analysis of reproducibility, only metabolites with concentrations above the limit of detection were considered. These covered 32 metabolites from the LC-MS/MS and 117 metabolites from the FIA-MS/MS step and were the same in the male and female samples. Intra- and inter-experiment precision were within the internationally applied acceptance criteria (CV < 15 %) for nearly all of these metabolites (Fig. 2) with some exceptions as outlined below.

In the LC-MS/MS step, the intra-experiment performance in both male and female samples showed CVs < 15 % for all quantified metabolites. In the inter-experimental comparison all except two outliers within the class of amino acids (Arg: CV 19 %, Asp: CV 22 %) did match the acceptance criteria.

For FIA-MS/MS measurements, the intra-experimental CVs of most of the 117 metabolites matched the 15 % acceptance criteria. Only five metabolites showed higher CVs of up to 28 %, four of them with the male samples (PCaa C38:1, lysoPC a C26:0, lysoPC a C28:1, SM C20:2) and one with the female samples (PCaa C38:1). In the inter-experiment performance, we observed seven out of 117 metabolites with CVs higher than 15 %, but not higher than 27 % (PCaa C24:0, PCaa C38:1, PCae C30:1, lysoPC a C26:0, lysoPC a C28:0, lysoPC a C28:1, SM C20:2). Detailed results for absolute concentrations and CVs are listed in Supplementary Table 2. In our experiments, the reproducibility was satisfactory for DBS derived from blood samples collected by venous puncture.

While the sample material used here was very homogeneous, blood samples collected in real experiments are usually more heterogeneous leading to higher variances. In theory, each sample disk should represent a homogenous blood volume, but this can vary from DBS to DBS after spotting and drying. Furthermore, native blood samples can coagulate during DBS preparation leading to inhomogeneities of the DBS. Indeed, it was reported that analytes of interest were not equally distributed on the filter paper after spotting [20, 22]. However, the standardisation of clinical or biological sampling procedures to obtain homogeneous DBS samples goes beyond the focus of this feasibility study and was not addressed here.

Linearity

Depending on experimental setup, sample size might vary for real samples. It is therefore important to know the relation between sample amount and signal output. A linear correlation would be the preferred relation.

To test for linearity, we assayed different sizes and numbers of DBS sample disks representing the different sample amounts (¼ up to 3 disks for male EDTA-DBS and ½ up to 3 disks for female EDTA-DBS) and calculated the coefficient of correlation as well as the Pearson correlation coefficient r for each metabolite. To assign a single metabolite concentration to be linear with sample amount, an r above 0.99 together with a CV above 50 % is requested.

For the analysis of linear behaviour, only metabolites with concentrations above the limit of detection were considered (33 metabolites from the LC-MS/MS and 108 metabolites from the FIA-MS/MS step). For all of the assessed metabolites in the LC-MS/MS step, linear

Fig. 2 Distribution of CVs for intra-experiment reproducibility (male and female samples), and the inter-experimental reproducibility (male samples). The median values of the CVs per metabolite class with the 20 and 80 % quintiles are shown. The number of metabolites per class is given in parentheses (only those with concentrations >LOD were considered)
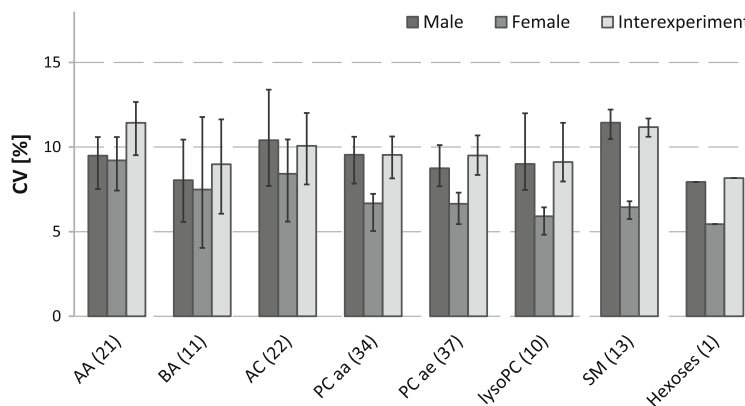
responses over the whole concentration range were observed except for two in the male (Arg, putrescine) and two in the female sample (Arg, Glu), which have r-values below 0.99. Of the metabolites detected by FIA-MS/MS, nearly all reached r-values above 0.99 and CVs above 50 %, except six metabolites in the male (C3-OH, PC aa C42:1, PC ae C44:4, PC ae C44:5, lysoPC a C28:1, SM C20:2) and 12 in the female sample (C3-OH, PC aa C24:0, PC aa C42:1, PC aa C42:6, PC ae C30:1, PC ae C36:0, PC ae C40:1, PC ae C42:0, lysoPC a C26:0, lysoPC a C28:0, lysoPC a C28:1, SM C20:2). A complete list of the metabolite concentrations, r values and CVs are given in Supplementary Table 3.

For nearly all investigated metabolites, the concentrations correlated in a linear manner with the sample amount (disk size and number). In LC-MS/MS, only 6.0 % of the quantified metabolites were not linearly correlating with sample amount both in male and female samples. For FIA-MS/MS, these included 5.6 and 11 % of metabolites, respectively.

The use of one disk per measurement is the standard procedure for most described applications of DBS but in some cases it might be necessary to apply more or less sample material (e.g. at to low or to high concentrations of metabolites). As depict with our results, the use of different DBS disk sizes or numbers in those cases would be possible with the exception of only a few metabolites of the AbsoluteIDQ™ p180-kit panel.

Ion Suppression

Matrix effects originate from endogenous compounds that are either in the biological sample or introduced during sample preparation and analyses [23]. These endogenous compounds may interfere with the analyte (metabolite or feature of the metabolite) of interest and can suppress or enhance the ionisation efficiency of the analyte and consequently alter the analyte MS/MS signal intensity.

We evaluated the suppression effects by analysing the signal intensities of all 40 internal standards of the p180-kit in three phosphate buffered saline (PBS) samples, three blank filter disks, six plasma samples, and six EDTA-DBS disks. As illustrated in Fig. 3a, LC-MS/MS analysis of blank filter paper did not result in additional suppression effects compared to PBS (a common blank sample for plasma analyses and used here as well as reference). The application of blood onto filter disks adds further compounds to the analysis. However, the internal standards in DBS samples had the same intensities as in blank filter paper or PBS. In comparison to plasma (the validated sample matrix for the p180-kit) DBS samples showed slightly lesser intensities of the internal standards, i.e. slightly higher suppression. However, this did not affect the
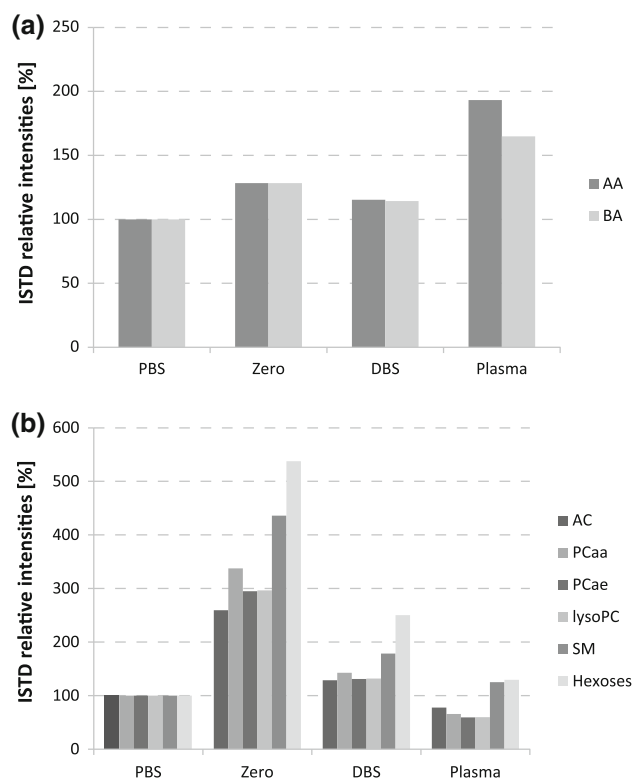
Fig. 3 Suppression effects of different sample matrices. The sums of relative intensities of internal standards per metabolite class in different matrices (PBS, blank filter paper, DBS and plasma) are shown. Intensities of internal standards in the reference matrix PBS ("zero sample" in the p180-kit) were set to 100 % **a** data from LC-MS/MS measurements, **b** data from FIA-MS/MS analysis

linear correlation of concentration to signal intensity of the metabolites from DBS samples.

In a FIA-MS/MS run, all compounds of a sample are applied at the same time to the mass spectrometer. Thus, analytes of interest are not separated from endogenous compounds what can lead to stronger interferences. As expected, ionization efficiency for metabolites comprised by FIA-MS/MS varied from those detected by LC-MS/MS. As illustrated in Fig. 3b, suppression effects in DBS samples analysed by FIA-MS/MS were clearly lower than in plasma and slightly fewer than in PBS samples. Of all the samples the blank filter paper had the highest intensities for internal standards, i.e. the lowest suppression effects.

Our experiments showed that suppression effects were present with the DBS matrix. The observed matrix effects of DBS samples on the internal standards were comparable to those in plasma or even lower. As expected, suppression effects were lower in the LC-MS/MS than in the FIA-MS/MS runs because a chromatographic separation step like LC reduces interfering compounds from the analysis [24]. Matrix suppression effect analysis is usually not requested for LC-MS/MS method validation. Nevertheless, matrix suppression effects should be evaluated for every new

developed method as they can significantly reduce sensitivity and reproducibility and also have a negative impact on the quality of the data [25]. Generally in DBS, adverse matrix effects were found to be smaller than in the original zero sample (PBS) of the p180-kit. Thus, our FIA- and LC-MS/MS measurements are considered trustworthy in regard to ion suppression effects.

Effects of Filter Paper Matrix

Dried blood spots are generated on filter paper. Beside the loaded biological sample, the filter paper adds an artificial matrix to the analysis. This matrix might influence the measurement and change the baseline levels. We therefore analysed clean and empty Whatman 903™ filter paper disks ("blank disks") to estimate this effect.

We indeed observed that the filter paper matrix had an impact on the concentration baseline since some metabolites were visible in the blank filter paper in amounts higher than their LODs.

In LC-MS/MS, all metabolites were below the lower limit of quantification (LLOQ) as defined for the p180-kit. While no LC-MS/MS peaks at all could be found for the class of biogenic amines, 10 of 21 amino acids showed levels at or above the LOD. Four amino acid concentrations were slightly and six were more than two fold above the LOD (Table 1).

In FIA-MS/MS measurements, 131 out of 146 metabolite signals were in the range of the background noise (<LOD) and two metabolites showed no MS/MS signals. The concentrations of 13 metabolites were above LOD, 9 of them slightly above the LOD and 4 at levels of more than two times of the LOD (Table 1).

The blank Whatman 903™ filter paper which is routinely used for DBS sampling was found to contain metabolites that were detected by the p180-kit, although in fairly low amounts. Those blank values were 5 % at most of the respective metabolite concentrations seen in DBS. Especially when the metabolites are expected in lower concentrations, we recommend a correction of the quantitation results by subtracting the blank values to improve study power and avoid false absolute concentrations.

Influence of Haematocrit

Haematocrit levels (HK) are known to vary between individuals and with pathological state. The HK was shown to affect the accurate quantification of small molecules in DBS samples from EDTA blood [20]. To investigate if the metabolite quantification from DBS by the p180-kit is also dependent on HK, we prepared a set of samples with defined blood cell/plasma ratios (25, 33, 50, 66, and 75 % blood cells) by mixing male blood cells with EDTA-plasma of the male volunteer accordingly. The mixtures were processed like the regular DBS samples.

To test for linear response to haematocrit, we calculated the Pearson correlation coefficient r and the coefficient of correlation over the five tested haematocrit levels. To assign a single metabolite concentration to respond linear to haematocrit, an r above 0.8 together with a CV above 20 % is requested.

We observed that higher HKs led to higher metabolite concentrations mainly in the metabolite classes of acylcarnitines and sphingolipids, whereas the metabolites of other metabolite classes showed less or no change. Amino acids and biogenic amines seemed scarcely affected by the HK as the concentrations of most metabolites of these classes were not significantly influenced. Only two amino acids and two biogenic amines were positively correlated with the haematocrit level, with $r > 0.9$ and CV > 25 %. The concentrations of free carnitine, PCs, lysoPCs, and hexose were mostly not affected by HK, except of three PC metabolites. The concentrations of most long chain, as well as some medium and short chain acylcarnitines increased with haematocrit. Only four acylcarnitines C3-OH, C4, C5, and C14:1 seemed to be independent of the haematocrit level. The here observed correlation of AC and haematocrit corresponds in part with other published results [22]. The concentrations of the other AC were below LOD and they are not considered here. Nine of 15 SM were correlated with haematocrit, for one (SM C22:3) concentration was below LOD (Table 2).

The observed influence of the haematocrit on metabolite concentrations might limit the use of dried blood spots for studies especially when the study population shows varying haematocrit levels. For such cases, a parallel measurement

Table 1 Metabolites found in concentrations above LOD in blank Whatman 903™ filter paper disks

Metabolite (by LC-MS/MS)	Amount (fold above LOD)	Metabolite (by FIA-MS/MS)	Amount (fold above LOD)
Gly	8.8	PCae C44:4	3.7
Met	8.4	SM C16:0	2.6
Ala	4.3	PCae C38:0	2.3
Val	3.1	PCae C44:3	2.1
Ile	2.3	PCae C38:5	1.7
Gln	2.2	SM C24:1	1.7
His	1.8	PCaa C34:1	1.4
Ser	1.4	PCae C38:4	1.3
Trp	1.4	SM C20:2	1.3
Phe	1.0	PCaa C34:2	1.1
		C3-OH	1.0
		PCaa C36:4	1.0
		PCaa C36:5	1.0

Table 2 Metabolites in DBS affected by the haematocrit

Metabolite	Blood cell content of DBS				
	25 (%)	33 (%)	50 (%)	66 (%)	75 (%)
Asp	66.00	73.00	96.67	122.00	152.67
Glu	63.33	67.33	72.00	97.67	132.33
Spermidine	2.20	2.07	2.90	5.30	8.13
Spermine	2.47	2.50	3.17	4.90	6.27
C2	5.00	4.67	6.00	9.27	11.40
C3	0.53	0.50	0.70	1.37	1.87
C5-OH (C3-DC-M)	0.10	0.10	0.20	0.23	0.27
C14	0.10	0.10	0.10	0.17	0.20
C16	0.37	0.33	0.73	1.30	1.87
C16:1-OH	0.03	0.03	0.03	0.07	0.07
C18	0.27	0.27	0.57	0.97	1.03
C18:1	0.50	0.50	0.93	1.77	2.23
C18:2	0.17	0.13	0.27	0.50	0.60
PCaa C30:0	5.13	4.83	6.87	8.03	8.03
PCaa C32:0	18.27	16.43	27.20	35.67	35.00
PCae C34:0	2.23	2.00	3.60	4.70	4.27
SM(OH) C14:1	9.03	8.87	12.33	15.83	15.87
SM(OH) C16:1	4.03	4.30	5.87	7.10	7.23
SM(OH) C24:1	2.23	2.33	3.03	3.77	4.10
SM C16:0	155.00	150.33	216.33	264.00	269.33
SM C18:0	24.67	23.30	32.37	39.00	38.00
SM C24:0	59.67	61.00	94.00	125.67	129.33
SM C24:1	94.00	98.00	149.33	183.00	196.67
SM C26:0	2.00	2.10	4.27	5.10	5.33
SM C26:1	2.53	2.57	4.70	5.87	6.33

Concentration values (μM) of those metabolites correlating with haematocrit levels. Parameters indicating a response to haematocrit were defined as $R^2 > 0.8$ together with a CV > 20 % over the five haematocrit levels

of the HK should be performed to be able to perform data normalisation.

Influence of Anticoagulant

The p180-kit is validated for the analysis of human EDTA plasma. While heparin plasma can be used without loss of performance, plasma with citrate as anticoagulant is not recommended.

To check if the choice of the anticoagulant for DBS preparation has an effect on metabolite quantification, DBS disks of male EDTA and heparin blood were analysed. As observed for plasma, both anticoagulants seemed to have no different influence on the DBS measurements with the p180-kit method. Metabolite concentrations of EDTA and heparin samples varied only within the acceptance criteria of the kit (CV < 15 %). Only a single metabolite, SM C20:2, showed significant differences in concentrations

depending on the anticoagulant (CV 56.3 %). But this might have been an outlier, which depended more on the poor reproducibility of this analyte (CV 28 %) than on the influence of the anticoagulant.

The testing of citrate blood was not included in this study because Biocrates disadvises to use citrate with the Absolute*IDQ*™ kits. Additionally, we cannot speculate on the influence of the coagulants on the measurements. For this, native blood would have had to be tested in direct comparison but this would have gone beyond the focus of this feasibility study.

Cross Contamination

Cross contamination can occur while using the same punching device without cleaning between punches. A transfer of sample matrix is possible which can affect the analysis of the following samples. Cross contamination can be avoided by performing cleaning punches between the sample disk punches. Here, we investigated the possible carry-over of sample matrix from a sample disk onto two following cleaning disks.

While performing p180-kit measurement on the two cleaning punch disks, we found no signs for carry-over of metabolites in the second cleaning disk (Supplementary Table 4). In LC-MS/MS, 32 out of 42 metabolites had concentration values below the LOD or even did not show up at all and 10 metabolites were concentrated below LLOQ. Using FIA-MS/MS, 122 out of 146 metabolites had levels below the LOD or were not measurable. The other 24 metabolites were concentrated in the range of the LOD.

The metabolite concentration patterns observed in this experiment corresponded with that of the blank filter paper matrix. Only the same metabolites, which were detectable in the blank filter matrix showed up in similar concentrations in the second cleaning disk. This means there was no carry-over of metabolites onto the second cleaning disk. Since we also found only vestigial concentrations for some of the metabolites in the first cleaning disk (Supplementary Table 4), two cleaning punches between the sample punches should be sufficient to avoid cross contamination.

Direct Application of Blood to Pre-Cut Disks

DBS needs only a small amount of sample volume. However, 80 μL may sometimes be a very large volume, for example when only limited blood amounts are available as from small mammalians like mice. The application of too small volumes to the 12.7 mm circles of the sampling filter paper leads to inhomogeneities and the method becomes irreproducible. This can be avoided by the application of small, defined blood volumes to pre-cut filter paper disks. It

is assumed that a 3-mm Whatman 903™ filter paper disk is soaking with about 3 μL blood [26].

To test if direct sample application to disks leads to comparable results to the above-described standard DBS sampling procedure, we spotted 3 μL of male EDTA blood directly onto blank, pre-punched disks each, measured the disks after drying and compared the results with those of the standardised produced sample disks.

Using LC-MS/MS, the observed concentrations for the direct application were around 15–25 % lower than for standard sampling. CVs were generally lower for the direct application and matched the acceptance criteria for the method. Using FIA-MS/MS, the concentrations for most metabolite classes were generally 5–15 % lower for the direct application than for the standard sampling procedure. For lysoPCs, the direct application showed the same or higher concentrations compared to regular DBS disks. Both procedures resulted in comparable CVs mostly below 15 %, except for two metabolites (lysoPC a C26:0 (CV 16.2 %), SM C20:2 (CV 32.0 %)).

In our experiments, the majority of metabolites were lower concentrated in direct spotting than in regular DBS disks. We assumed to have the same blood volume applied in both procedures. The blood volume in a 3-mm DBS sample disk is estimated to be about 3 μL and we used this volume for direct spotting. However, the disks punched from DBS might have carried more than 3 μL blood, due to, e.g. incorrect volume assumption, blood inhomogeneity, or variances in DBS preparation. The different behaviour of lysoPCs cannot be explained yet and would need further investigation.

Anyhow, the direct application of blood on pre-cut disks is also a suitable method for the reproducible quantification of metabolites with the Absolute*IDQ*™ p180 kit.

Metabolite Stability During DBS Storage

Depending on the experimental setup, sample measurement might follow immediately after sampling or might be postponed to a time point long after sample collection. Safe storage conditions are required which keep metabolites stable preferable over extended time periods. Here, we tested metabolite stability in DBS, which were stored in bags with desiccants according to published standard procedures at different temperatures (−80, −20, 4 °C, and RT) for 1, 2 and 4 weeks. We compared the outcome with the results from immediate measurement at the day of DBS sampling.

Overall, the metabolites were relatively stable over the 4 weeks storage period and at different storage temperatures. We observed some alterations in the metabolite profile, but more with tendencies than significances since the performance of Kruskal-Wallis tests with Bonferroni

corrections did not find significant changes in metabolite concentrations. Detailed results for single absolute metabolite concentrations together with the uncorrected Kruskal-Wallis p values are given in Supplementary Table 5. The impact of storage to the different metabolite classes is outlined below.

Amino acids, biogenic amines, and lysoPCs were mostly stable over 4 weeks at all temperatures. Most of the ACs were stable as well but some ACs presented first instabilities after 4 weeks. PCaa metabolites were stable for up to 4 weeks at −20 and −80 °C, while slight changes in metabolite concentrations appeared at 4 °C after 4 weeks. For most of the PCaa metabolites, the concentration altered strongly at RT. PCae concentrations were fairly stable over the 4 week storage time at −20 and −80 °C. But most of the PCae metabolites had an overall tendency to increase with time with slight alterations at 4 °C and strong at RT. Stability of SM was observed to be independent of temperature, but not of storage time. After the first week, nearly all SM had lower concentrations at all temperatures while no further changes were seen during the following weeks. Hexose concentrations were stable at −80, −20 and 4 °C for 4 weeks, but diminished at RT after two weeks.

Based on our results, metabolites are stable for up to 4 weeks at −20 or −80 °C without significant alteration in metabolite concentrations. This corresponds with prior published results for the storage stability of amino acids and acylcarnitines [20] as well as fatty acids [27] in DBS samples. In addition, the observed alterations during storage at 4 °C and RT were also not significant. Under all storage conditions, the metabolite concentrations remain measurable above LOD. However, other long term storage experiments investigated the stability of amino acids and acylcarnitines under different storage conditions. They observed an appreciable breakdown for carnitine, and several acylcarnitines and amino acids over several years [28–30]. We did not test long-term storage as this would be beyond the scope of this study. Based on our and the published results, we recommend to store DBS at lowest temperatures possible (preferably at −80 °C) if the storage time exceeds several weeks.

Summary and Outlook

We successfully applied and partially validated the metabolite quantification from DBS by the Absolute*IDQ*™ p180 kit. Factors that could potentially affect the quantification method (haematocrit, anticoagulant, ion suppression, sample handling, storage condition) were investigated. Despite a much smaller analysis volume of DBS compared to plasma, those factors had no significant influence on the ability of the method to quantify most of the metabolites in DBS, and did not restrict the usability of

the method. Even the direct application of low amounts of blood to pre-cut filter paper disks was shown to be a feasible and reproducible method which can be very useful when only small sample volumes are available. Furthermore, the observed matrix effects do not influence the reproducibility or the assay linearity. Long-term storage of DBS turned out to be possible with best overall metabolite stability when samples are kept at $-80\,^{\circ}\text{C}$ under dry conditions. The here presented method application now awaits full validation, especially for accuracy of quantification. However, our results already show the huge potential of the new DBS matrix application for further development of metabolomics technologies and biological testing.

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