
# Performance properties of filter-paper used in blood spot collection devices for quantitation of phenylalanine


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## Abstract

### Aims

Accurate and precise measurement of dried blood spot (DBS) phenylalanine (Phe) is vital for managing phenylketonuria (PKU). Standard DBS collection devices use grade-226 filter-paper, while the CapitainerB quantitative device utilizes grade-222 filter-paper. Although grade-226 filter-paper performance is well characterized, data on grade-222 filter-paper are sparse. This study aimed to investigate the analytical properties of grade-222 and grade-226 filter-papers.

### Materials and Methods

We compared grade-222 and grade-226 filter-papers for Phe measurement accuracy and imprecision in DBS generated using both filter-papers. Scanning electron microscopy (SEM) and slit lamp imaging was used to assess the physical properties of the filter-papers.

### Results

Using an aqueous calibrator as reference, grade-222 exhibited a mean bias of -1.1%, the mean bias for grade-226 was -7.3%. Intra-assay imprecision was 2.3% for grade-222, versus 4.2% for grade-226. SEM revealed that fibres in grade-226 filter-paper are bonded by an amorphous material, which is absent in grade-222 filter-paper. Total error analysis indicated grade-222 filter-paper reduced uncertainty of Phe measurement compared to grade-226 filter-paper.

### Conclusions

Grade-222 filter-paper was proven to have superior analytical performance for Phe quantification, providing improved differentiation between safe and harmful Phe concentrations and offering more reliable PKU monitoring compared to traditional grade-226 filter-paper.

## 1.0 Introduction

The analysis of phenylalanine (Phe) in dried blood spot (DBS) specimens is the universally accepted method for monitoring patients with phenylketonuria (PKU, OMIM 261600). DBS collection allows patients the freedom to manage their disorder from home, reducing the anxiety and resources associated with collecting plasma specimens. Clinical guidelines recommend the use of age-related Phe reference intervals to monitor patients' dietary restriction and cofactor therapy, to prevent adverse neurological outcomes [1]. Therefore, accurate and reproducible analysis of DBS Phe specimens is central to the management of PKU.

Several studies have shown that using conventional non-volumetric filter paper collection devices to reliably monitor Phe concentrations is challenging due to issues such as DBS size, quality and haematocrit [2,3,4]. Studies have shown that volumetric blood collection devices improve the accuracy and precision of analyte measurement compared to conventional filter paper through negating the effect of haematocrit [5,6,7,8]. Hence, the implementation of a volumetric blood collection device for Phe monitoring in PKU is highly attractive. The CapitainerB quantitative DBS collection device is one such device and displays favourable analytical performance [9]. The device accurately collects 10µL of blood, has a sample quality indicator and is unaffected by differences in haematocrit [7]. However, the CapitainerB device employs

a different grade of filter paper (Ahlstrom grade-222) compared to traditional DBS filter paper collection devices (Revvity grade-226).

Various grades of cotton cellulose filter paper are available commercially. The different grades of paper vary in pore size and thickness, these differences may affect the spreading and permeation of blood through the filter-paper matrix. The Newborn Screening Quality Assurance Program (NSQAP) at the Centers for Disease Control (CDC) and Prevention conducts the evaluation of all lots of Food and Drug Administration (FDA)-approved screening collection devices. NSQAP publishes the results of these evaluations annually [10]. Currently, the Revvity grade-226 and Cytiva Life Sciences grade-903 papers are the only commercial products approved by the FDA for Newborn Bloodspot Screening (NBS) device production. The filter-paper evaluation must comply with the Clinical & Laboratory Standards Institute (CLSI) standard NBS01-A6 [11]. Performance characteristics include; absorption serum volume, absorption time, physical appearance and homogeneity [12]. These international standardisation efforts ensure uniformity of manufactured calibrators, quality control (QC) and reference materials for NBS assays. The type of matrix used for calibration and QC materials will influence the analyte recovery. Therefore ideally, quantitative DBS methods for monitoring should also use DBS calibration and QC materials to correct for the filter-paper matrix.

Whilst several studies have assessed the performance properties of the Revvity grade-226 and Cytiva Life Sciences grade-903 papers, demonstrating that they had comparable performance when measuring a range of analytes including Phe [13], no previous studies have evaluated the performance of the Ahlstrom grade-222 filter-paper used in the CapitainerB quantitative device. The aim of this study was to

evaluate the performance of the Ahlstrom grade-222 paper and compare it to the existing filter-paper used to monitor Phe in PKU patients, Revvity grade-226 paper.

## 2.0 Materials and Methods

### 2.1 Ethical approval

This study was determined not to be research after discussion with the Cardiff and Vale University Health Board (CAVUHB) Research and Development department. A quality improvement project was submitted to the CAVUHB Patient Safety and Quality department for the work contained in this study.

### 2.2 Chemicals and reagents

Liquid chromatography mass spectrometry (LC-MS) grade methanol (MeOH) and acetonitrile (MeCN) were obtained from ROMIL (Cambridge, UK). LC-MS grade formic acid was purchased from Greyhound (Birkenhead, UK) and concentrated hydrochloric acid (HCl) was purchased from VWR Chemicals (Leicestershire, UK). All solutions were made using deionised water (Milli-Q Advantage A10 water purification system, Merck, Darmstadt, Germany). L-phenylalanine (P2126, >98%) and bovine serum albumin (BSA) was obtained from Merck Sigma Aldrich (Darmstadt, Germany). Commutable amino acid certified reference material (CRM, TraceCert, 79248, 2.5 mmol/L in 0.1M HCl) was obtained from Merck Supelco (Darmstadt, Germany). Stable isotope L-phenylalanine (NSK-A, ring-$^{13}C_6$, 99%) was obtained from Cambridge Isotope Laboratories (Tewksbury, USA). CapitainerB devices were obtained from Capitainer (Solna, Sweden). Ahlstrom grade-222 filter-paper was obtained from

Ahlstrom-Munksjö (Helsinki, Finland) and conventional grade-226 filter-paper was obtained from Revvity (Turku, Finland).

### 2.3 Preparation of reagents

An aqueous stock solution of L-phenylalanine (200 mmol/L) was made by dissolving the powdered material under sonication at ambient temperature. Stock solution was stable for 6 months when stored at -20°C. Stable isotope labelled (SIL) internal standard working stock (50 mL, 10 µmol/L) was prepared by dissolving $^{13}C_6$- L-phenylalanine in 80% MeOH (1 mL) at 40°C under sonication. The volume was made up to 50 mL with MeOH to give the working standard which was stored at 4°C prior to use.

### 2.4 Preparation of enriched blood

Venous blood (20 mL) was collected from a healthy volunteer into lithium heparinised tubes (BD Vacutainer, UK). Informed consent was obtained from the volunteer in line with local protocols. Aliquots of whole blood was enriched with the stock solution of L-phenylalanine (200 mmol/L) to clinically relevant concentrations to generate calibrator and IQC material. The volume of aqueous L-phenylalanine added to each whole blood aliquot did not exceed 5% of the total volume. An aliquot of whole blood was centrifuged to assess for haemolysis prior to dried blood spot preparation. Following enrichment, the blood was gently mixed on a roller mixer for a minimum of 30 minutes before application to filter-paper.

### 2.5 Preparation of dried blood spots

Enriched blood (10 µL) was applied onto Revvity grade-226 and Ahlstrom grade-222 filter-papers using a calibrated positive displacement pipette (microman M25E volume range 3-25 µL, Gilson Inc., Middleton, USA). During the process of DBS preparation, the blood samples were continually manually mixed. Blood was applied to grade-226

and grade-222 filter-papers in an alternating method. After air-drying for 3 hours at ambient temperature, DBS were stored in sealed bags with a desiccant at -20°C.

**2.6 Extraction of dried blood spots**

DBS (10 µL) on grade-222 and 226 filter-paper were punched in their entirety with a 6 mm punch head on an automated punching device (1296-071 Wallac, Revvity, Turku, Finland), directly into 96-well plates (2mL 96-well deep square, Porvair, Wrexham, Wales). Working SIL (300 µL) was added to each well. Samples were extracted for 1 hour on the plate shaker (1296-003, Revvity, Turku, Finland) at ambient room temperature (25°C). The extracts were transferred to a new microtiter plate and dried under nitrogen at 40˚C (MiniVap Gemini Evaporator, Porvair, Wrexham, Wales). After evaporation the extracts were reconstituted in 80% MeCN (1.5 mL), the plate was sealed with easy pierce foil (AB-1720, ThermoScientific, Waltham, USA) using a manual heat sealer (ALPS30, ThermoScientific, Waltham, USA) then mixed for 5 min on the plate shaker. Prior to analysis the plate was centrifuged for 5 min at 3000 rpm (Heraeus Megafuge 16, ThermoScientific, Waltham, USA).

**2.7 Analysis of samples by Flow Injection Analysis Tandem Mass Spectrometry (FIA-MS/MS)**

Samples (5 µL) were injected into a Waters Xevo TQ mass spectrometer with an electrospray ionisation source. Sample temperature was controlled at 10°C. The mass spectrometer settings were: cone voltage 30 V, capillary voltage 3.2 kV, source block temperature 150 °C, desolvation temperature 350°C, desolvation gas flow 800 L/h. The mobile phase was 80% MeCN with 0.1% formic acid. The flow rate varied over the course of an injection as follows: 0 minutes, 0.18 mL/min; 0.28 m, 0.018 mL/min; 1.21 m, 0.6 mL/min; 1.50 m, 0.18 mL/min. Data was acquired by multiple reaction

monitoring (MRM) using positive ionisation mode (Phe 166>120 m/z, $^{13}C_6$ Phe 172>126 m/z).

Quantification of Phe was made by stable isotope internal calibration (SIIC). In SIIC, the response of the measurand is compared to the response of the measurand internal standard to give the response ratio (RR). The concentration of the measurand can therefore be calculated using the relationship between the RR, the internal standard concentration and the volume of sample and internal standard. Thus, the concentration of Phe in the samples in this study was calculated using the formula:

Sample Phe concentration = RR x ((SIL concentration/sample volume) x SIL volume)

### 2.8 Evaluation of bias

Bias was assessed by analysis of CRM. Aqueous and DBS standards on grade-226 and grade-222 filter-papers were generated at the following concentrations: 25, 75, 200, 500, 1000 and 2000 µmol/L (n=3 preparations). The mean percentage difference of DBS standards prepared on grade-226 and grade-222 filter-papers compared to CRM and aqueous standards was calculated using the slope of the curve over the concentration range 25-2000 µmol/L. Acceptable test bias for Phe is deemed to be ±10.4% [14].

### 2.9 Evaluation of extraction recovery

The recovery of Phe from DBS specimens prepared on grade-222 and 226 filter-paper was investigated by comparing the DBS extract with the aqueous Phe stock used to enrich the whole blood before application onto the filter-paper (n=40 each IQC and grade). Aqueous Phe stock aliquots (10 µL) were added to working SIL (300 µL) in the 96-well plate at the same time as the DBS and followed the same extraction and analysis protocol as DBS.

### 2.10 Evaluation of imprecision

Intra-assay imprecision was determined by replicate measurements of three concentrations of in-house IQC applied to the different filter-papers (n=20 per IQC per grade). The coefficient of variation (CV) was calculated for both types of filter-paper. Inter-assay imprecision was determined by replicate measurements (n=5) of three concentrations of in-house IQC over 5 different days (n=25 per IQC per grade). Intra-well imprecision was used to validate the impact of the tandem mass spectrometer injection profile. This was calculated by repeat injections (n=12 per IQC) of combined (i.e both grade-222 and grade-226) pooled IQC extracts. The IQC material was generated at concentrations near treatment window cut-offs for PKU patients, using the European treatment guidelines [1]. Acceptable test imprecision for Phe was deemed to be <4.7% [14].

Total error (TE) was calculated using the following equation to give a function of systemic and random error for the analysis of Phe on each type of filter-paper:

TE = bias + (1.96 x maximum inter-assay imprecision)

Total allowable error ($TE_a$) for Phe is 11.1% [15].

### 2.11 Evaluation of filter-paper characteristics using electron microscopy and slit lamp analysis

The physical appearance of the grade-222 and 226 filter-papers was assessed using scanning electron microscopy (SEM). Blank filter-paper sub-punches (6 mm discs) were attached to 12 mm SEM stubs with conductive carbon adhesive discs, sputter-coated with gold in an EMscope vacuum coater (EMScope, Ashford, Kent, UK) and viewed at 10 kV in a JEOL 840A scanning electron microscopy fitted with a Point control and imaging system (Point Electronic GmbH, Erich-Neuß-Weg 15 D-06120 Halle (Saale), Germany).

Slit lamp analysis was also used to assess the filter-papers before and after solvent extraction. Blank 6 mm filter-paper sub-punches were placed in Eppendorf tubes containing MeOH (450 µL), dry control discs were placed in empty Eppendorf tubes. After 1 hour the samples were gently removed from the Eppendorf tubes and imaged at 25x magnification on a Topcon SL-D7 Photo slit lamp (Tokyo, Japan) with a Nikon D700 (Tokyo, Japan) body attached.

### 2.12 Data analysis

NeoLynx software on the Waters Xevo TQ automatically processed raw data and performed SIIC calculations, data was exported to Microsoft Excel 2016 to perform statistical analysis. Graph-Pad prism v6.0b was used to generate figures and tables. The Wald-Wolfowitz runs test was used to assess deviation of data from a curve fit by linear regression. The normality of data distributions was assessed using the Shapiro-Wilk test for normality (suitable for sample sizes <5000) at the 5% significance level. If data was Gaussian distributed, the paired or unpaired t-test with unequal variances was used to test statistical significance. Whereas paired non-Gaussian distributions were assessed using the Wilcoxon Rank sum test.

## 3.0 Results

### 3.1 Effect of filter paper type on analytical bias

The bias of 10 µL DBS standards generated using grade-222 and 226 filter-papers, and 10 µL aqueous in-house standards were assessed by comparison to 10 µL aqueous CRM. The mean RR for each standard was plotted against the nominal concentration. Linear regression analysis was used to produce curves for each set of standards, shown in Figure 1a. Figure 1a indicates that the quantified concentrations of Phe in the aqueous standards generated in-house are concordant with the CRM.

10 µL DBS standards generated using grade-222 filter-paper also indicate concordance with the CRM. However, 10 µL DBS standards generated using grade-226 filter-paper have the lowest slope in comparison to the CRM. The linear curve characteristics for each standard curve displayed in Figure 1a are represented in Table 1 for comparison. The 95% CI for the Y-intercept for all sets of standards encompass zero, enabling the slope of the curve to be used to determine bias. The mean percentage difference of each set of standards was calculated against the CRM and the aqueous standard using the slope of the curve, shown in Table 1.

### 3.2 Effect of filter paper type on phenylalanine recovery

An assessment of recovery was performed on n=40 replicates of three concentrations of in-house IQC material (10 µL DBS on grade 222 or 226 paper), analysed on discontinuous days (n=5). Extraction recovery of the DBS was calculated against the aqueous IQC material used to enrich the whole blood [16]. Percentage recovery results are shown in Figure 1b.

The mean percentage recovery for Phe across all IQC was 91% and 84% for grade-222 and 226 filter-paper respectively. The maximum imprecision of recoveries for grade-222 and 226 filter-paper was 3.3% and 4.7% respectively. The data were normally distributed and an unpaired t-test with unequal variance indicated a significant difference ($p<0.001$) between the percentage recoveries of the two filter-paper grades.

### 3.3 Effect of filter paper type on analytical imprecision

The intra-assay and inter-assay imprecision of both filter-paper grades is presented in Tables 2a and 2b respectively. The maximum intra-assay imprecision of IQC was 2.9% on grade-222 filter-paper, and 4.4% on grade 226 paper. The maximum

inter-assay imprecision of IQC rises to 3.3% on grade 222 paper and 4.6% on grade 226 paper. This demonstrates that the imprecision of 10 µL DBS generated using grade 226 paper is overall increased in comparison to the imprecision of 10 µL DBS generated using 222 filter-paper. Between-injection imprecision was assessed using pooled extracts of low, medium and high DBS IQC. The imprecision between-injections was ≤1.0% across three concentrations.

### 3.4 Imaging studies of the filter papers

SEM images were taken on blank 6 mm punches of grade-222 and 226 filter-paper. Figure 2 reveals the differences in the internal structure of the two grades of filter-paper. An amorphous material is present in the grade-226 filter-paper.

Slit lamp imaging was performed to visualise the difference in thickness of both grades of filter-paper post-extraction. The imaging was performed at 25x magnification against a scale, shown in figure 3. After 1 hour in MeOH, the estimated percentage increase in thickness of the grade-222 and 226 filter-papers post extraction were 84% and 52% respectively.

## 4.0 Discussion

It has previously been demonstrated that the use of volumetric blood collection devices improves accuracy and precision in the measurement of DBS Phe and Tyr concentrations, compared to conventional filter-paper cards [9]. In this study the performance properties of Ahlstrom grade-222 filter-paper, used in CapitainerB volumetric blood collection devices, and conventional Revvity grade-226 filter-paper were compared for the purpose of Phe measurement.

The linearity of Phe in the DBS calibrators produced on both grades of filter-paper was excellent ($r^2$ ≥0.995) over the measured range of 25-2000 µmol/L, which encompasses patient results routinely observed in the laboratory. The 95% CI of the slope of the CRM and the in-house aqueous standards did not overlap, indicating a significant difference. This expected difference is secondary to the purity of the reagent grade material. However, the concordance between the CRM and the in-house aqueous standards (-4.0%) was acceptable, inferring its suitability for use as a routine laboratory calibrant. The bias between the two different grades of filter-paper was 6.2%. Whilst both grades of filter paper meet the biological variation acceptance criteria (±10.4%) [14], the recovery of phenylalanine from the 222 filter-paper was superior to grade-226.

The same enriched whole blood was used to generate the grade-222 and 226 DBS standards, and the entire DBS (10 µL) was punched out during analysis of both papers. Consequently, the observed difference in bias must be secondary to the extraction process. This observation was confirmed in the recovery experiments. Recovery of Phe in DBS IQC material was calculated against the result of the in-house aqueous IQC material (as 100%) which was generated using the same volumes of stock solution. Therefore, although not matrix-matched the aqueous IQC underwent identical post-extraction analysis as the DBS, consistent with that reported previously [17]. Mean proportional error of recovery was 9% for grade-222 paper and 16% for grade-226 paper, no concentration dependant change in recovery was indicated. A significant difference between recovery in grade-222 and 226 paper was identified at each IQC concentration.

Using the bias against the in-house aqueous standard and the maximum inter-assay imprecision, our experiments indicated that the total error for the grade-222 and

226 papers was 7.6% and 16.3% respectively. Fonnesbeck *et al* [18] described that paediatric patients <6 years old with a Phe concentration >400 µmol/L over their lifetime were linked to poorer IQ scores. Similarly, Leuzzi *et al* [19] demonstrated that in older paediatric patients aged 8-13 years a lifetime Phe concentration of >400 µmol/L resulted in a poorer ability to perform executive function tests, in comparison to PKU patients of the same age with a lifetime Phe <400 µmol/L. Therefore, there is a significant clinical requirement for an analytical test to be able to distinguish between a Phe concentration of >400 µmol/L and the top of the paediatric target treatment window, 360 µmol/L [1]. Based on this study, the total error at 360 µmol/L would be ± 27 µmol/L using grade-222 paper, and ± 59 µmol/L using grade-226 paper. This suggests that grade-222 paper would reduce the uncertainty of measurement of Phe in DBS, thus enabling safe and detrimental (>400 µM) concentrations of Phe to be confidently distinguished.

The extraction of analytes from the solid to the liquid phase is a critical step in DBS specimen analysis and is achieved by the addition of a specific volume of an extraction solvent containing the appropriate internal standards. It is essential that the extraction solvent can re-solubilize the analytes of interest but also disrupt the hydrogen bonding between the analyte and the cellulose filter-paper matrix. The physical properties of the grade-222 and 226 filter-papers are different; the basis weight and thickness of the grade-222 paper (291.3 g/m$^2$ and 0.83 mm respectively [20]) is greater than that of the grade-226 paper (179 g/m$^2$ and 0.52 mm [21]). It can be theorised that due to its increased thickness there is more space for erythrocytes and serum to spread among the fibres in grade-222 filter paper. Slit-lamp imaging was performed on dry and extracted (1 hour in MeOH) sub-punches of both grades of filter-paper. The increase in depth of the swollen filter-paper sub-punches was estimated

315 using a scale imaged at the same magnification. The limitation of the slit lamp images
316 is that only a single image could be taken at one time, and identifying the edge of the
317 filter-paper using the curvature of the light is subjective. The depth estimates suggest
318 that grade-222 filter-paper expanded more than grade-226 filter-paper during
319 extraction, which can be visualised by eye. Therefore, the extraction of Phe may be
320 improved by the increase in space provided by grade-222 paper before and after
321 addition of solvent.

322 The use of SEM enabled characterisation of the physical appearance of the
323 filter-papers, to help explain the differences in performance between the two grades
324 of filter-paper. SEM revealed that the grade-226 filter-paper is bonded together with
325 an amorphous material which is not present in the grade-222 filter-paper. The
326 amorphous material visible in the grade-226 filter-paper may be the cause of the
327 reduced extraction efficiency if it disrupts the hydrogen bonding between analyte and
328 fibres.

329 Interestingly, the physical appearance of the Ahlstrom-226 paper is comparable
330 to that of electron micrographs of the Whatman-903 grade paper [22]. SEM imaging
331 of both filter-papers reveals long interwoven cellulose fibres with similar morphology.
332 There is no significant difference in fibre orientation, giving a randomly distributed
333 fibrous matrix in both filter-papers. The SEM images indicate similar pore sizes and
334 fibre packing density..This explains the comparable performance of the 226 and the
335 Whatman-903 grade papers used in NBS filter-paper collection devices [13].
336 Therefore, the composition and physical properties of blood collection devices appear
337 to be an important characteristic of their performance. Additional investigations are
338 required to assess the surface porosity and distribution of fibres in both 222 and 226

filter-papers to explain differences in the chromatography of whole blood through the paper, which may affect extraction of other analytes.

In a previous study several volumetric collection devices were assessed for quantification of Phe, in comparison to the conventional grade-226 collection card [9]. Of the volumetric devices assessed, the Mitra (Neoteryx) device demonstrated the greatest imprecision and bias relative to liquid whole blood. To investigate whether the composition of the Mitra may have affected its performance characteristics, SEM images of this device were obtained (Figure 4). The appearance of the Mitra device is not uniform, this observation may impact the ability of analytes to desorb from the tip. A possible explanation is at higher concentrations of haematocrit there is a higher relative number of erythrocytes trapped in the hydrophilic matrix, impacting extraction [9].

## 5.0 Conclusions

In conclusion, the grade-222 filter-paper used in the CapitainerB volumetric blood collection devices offers superior analytical performance for the measurement of Phe in DBS specimens. By design, the CapitainerB device also mitigates the analytical bias seen in poor quality DBS, DBS of different sizes (variable blood volume) and differences in haematocrit. These are all inherent limitations to DBS collected using grade-226 filter-paper, which is routinely used in NBS programmes worldwide. With the positive characteristics described, the CapitainerB device provides a promising development in the blood spot collection device market and should initiate further discussion around filter-paper performance.

There are no published studies directly assessing the performance or physical characteristics of grade-222 filter-paper. We conclude that it is important to assess the characteristics of a new grade of filter-paper when validating a DBS assay. The performance characteristics may affect the utility of the device in question and the clinical impact on patients. There has been much interest in blood collection devices for the monitoring or diagnosis of other inherited metabolic disorders, endocrine disorders, toxicology and therapeutic drug monitoring [23,24,25,26], we hope that this work will complement the research in these important areas.

## 6.0 Article Highlights

- This article presents the results of novel work assessing the performance characteristics of Phe measurement in DBS generated on grade-222 filter-paper.
- Grade-222 filter-paper is used in the CapitainerB blood collection device. This device is advantageous due to its superior analytical resilience to poor quality blood spots, variable blood volume and the impact of haematocrit compared to traditional grade-226 filter-paper.
- Analytical bias, imprecision and recovery was assessed in both filter-paper grades. Grade-222 filter-paper was found to improve the total error of Phe measurement, therefore reducing the harm associated with inaccurate at-home Phe monitoring in PKU patients.
- Physical characteristics of both grades of filter-paper and the Mitra blood collection device were assessed with scanning electron microscopy. These

images illuminated differences in the micro-structure of the devices and help to improve the understanding of their performance.

## Disclosure Statement

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties

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**Figure and Table Legends**

Figure. 1a. Standard curves for 10 µL certified reference material (CRM), 10 µL aqueous stock solution, 10 µL dried blood spots (DBS) on 222 filter-paper and 10 µL DBS on 226 filter-paper. The nominal concentration is plotted against the response ratio (RR) for the six standards (25, 75, 200, 500, 1000 and 2000 µmol/L). Mean ±SD. (n=3 preparations)

Figure. 1b. Percentage recovery of phenylalanine (Phe) in a 10 µL DBS on 222 and 226 paper at three concentrations. Calculated via stable isotope internal calibration (SIIC). Mean±SD. *p<0.05 compared to 226-grade paper; unpaired t-test with unequal variance. (n=40 each concentration and grade)

Figure. 2. Scanning electron microscopy (SEM) images of 6 mm punches of grade-222 (left-hand) and 226 (right-hand) paper. (A) dry 30x magnification, (B) dry 100x magnification, (C) dry 250x magnification.

Figure. 3. Slit lamp imaging; (A) grade-226 filter-paper at 25x magnification, (B) grade-222 filter-paper at 25x magnification. In both panels, the left-hand filter-paper punch is dry (pre-extraction) and the right-hand filter-paper punch is post-extraction (1 hour in methanol, MeOH). The solid red lines denote 1 mm as determined by the photographed scale, the dashed red lines indicate the top edge of the filter-paper, determined by the curvature of the slit lamp.

Figure.4. SEM images of the Mitra (Neoteryx) volumetric blood collection device at 30x, 100x and 250x magnification.

Table 1. Linear regression analysis of Phe standard curves; mean slope and Y-intercept with 95% confidence intervals (CI), coefficient of determination ($R^2$) and calculated mean percentage difference of each slope against the CRM and the in-house aqueous calibrator. (n=3)

Table 2a. Intra-assay imprecision. Means, standard deviations (SD) and percentage coefficient of variation (CV) of repeat 10 µL DBS samples on 222 and 226 filter-paper. (n=20 each IQC)

517 Table 2b. Inter-assay imprecision. Means, SD and percentage CV of 10 µL DBS on
518 grade-222 and 226 filter-papers. ≥5 replicates of IQC material were analysed over 5
519 discontinuous days. (n=5 days).

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Fig. 1a

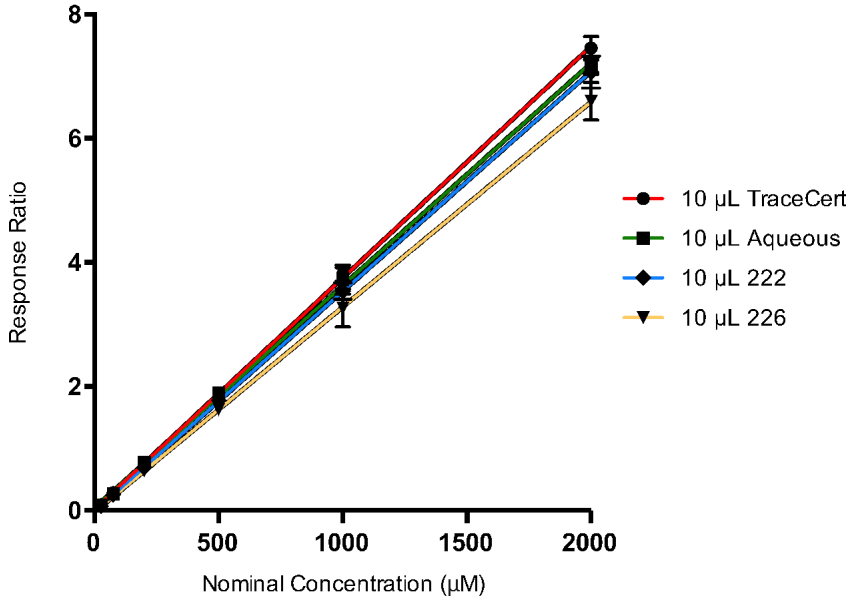
Response Ratio
0
2
4
6
8
0
500
1000
1500
2000
Nominal Concentration (µM)
10 µL TraceCert
10 µL Aqueous
10 µL 222
10 µL 226

Fig. 1b.

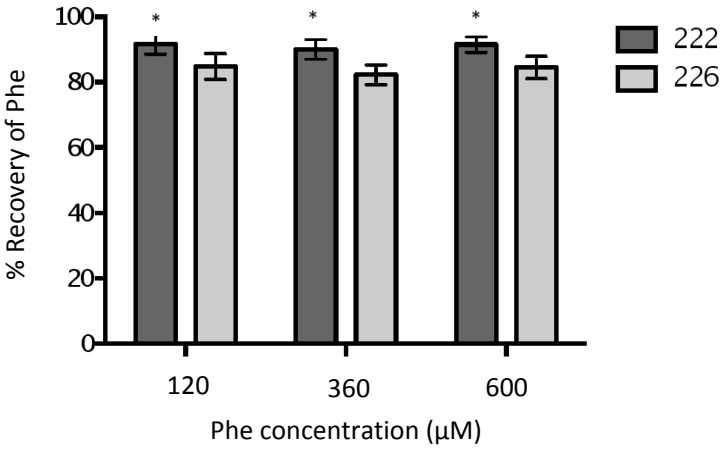
% Recovery of Phe
0
20
40
60
80
100
*
*
*
120
360
600
Phe concentration (µM)
222
226

Fig. 2.

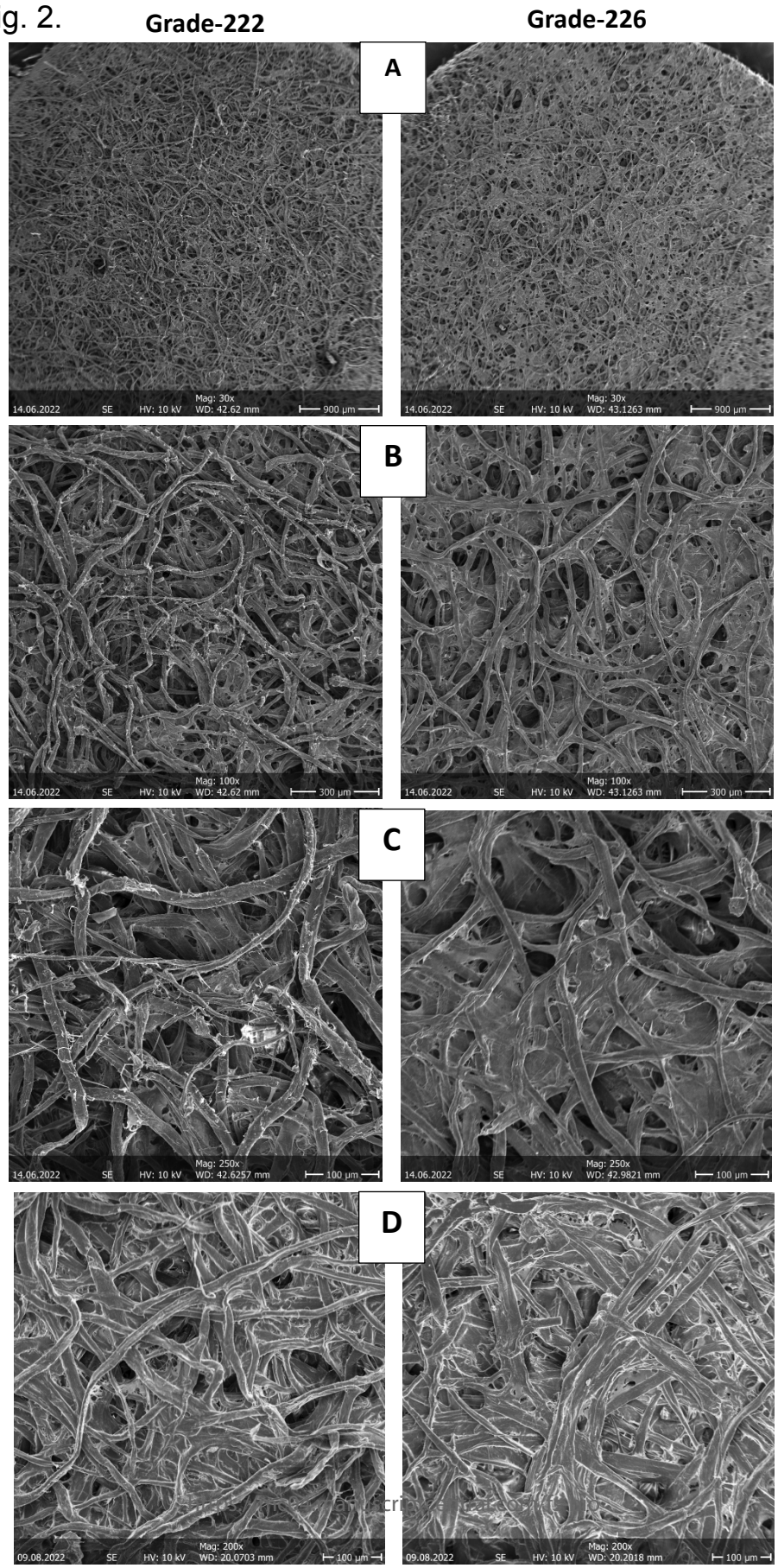
Grade-222
Grade-226
A
14.06.2022 SE HV: 10 kV Mag: 30x WD: 42.62 mm 900 µm
14.06.2022 SE HV: 10 kV Mag: 30x WD: 43.1263 mm 900 µm
B
14.06.2022 SE HV: 10 kV Mag: 100x WD: 42.62 mm 300 µm
14.06.2022 SE HV: 10 kV Mag: 100x WD: 43.1263 mm 300 µm
C
14.06.2022 SE HV: 10 kV Mag: 250x WD: 42.6257 mm 100 µm
14.06.2022 SE HV: 10 kV Mag: 250x WD: 42.9821 mm 100 µm
D
09.08.2022 SE HV: 10 kV Mag: 200x WD: 20.0703 mm 100 µm
09.08.2022 SE HV: 10 kV Mag: 200x WD: 20.2018 mm 100 µm

Fig. 3.

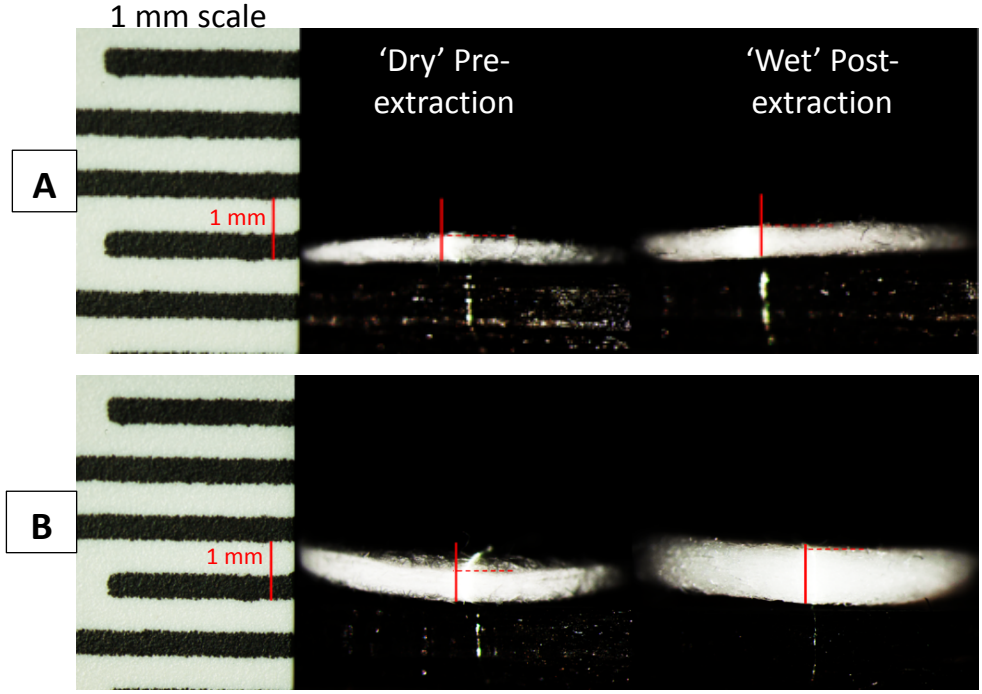

Fig. 4.

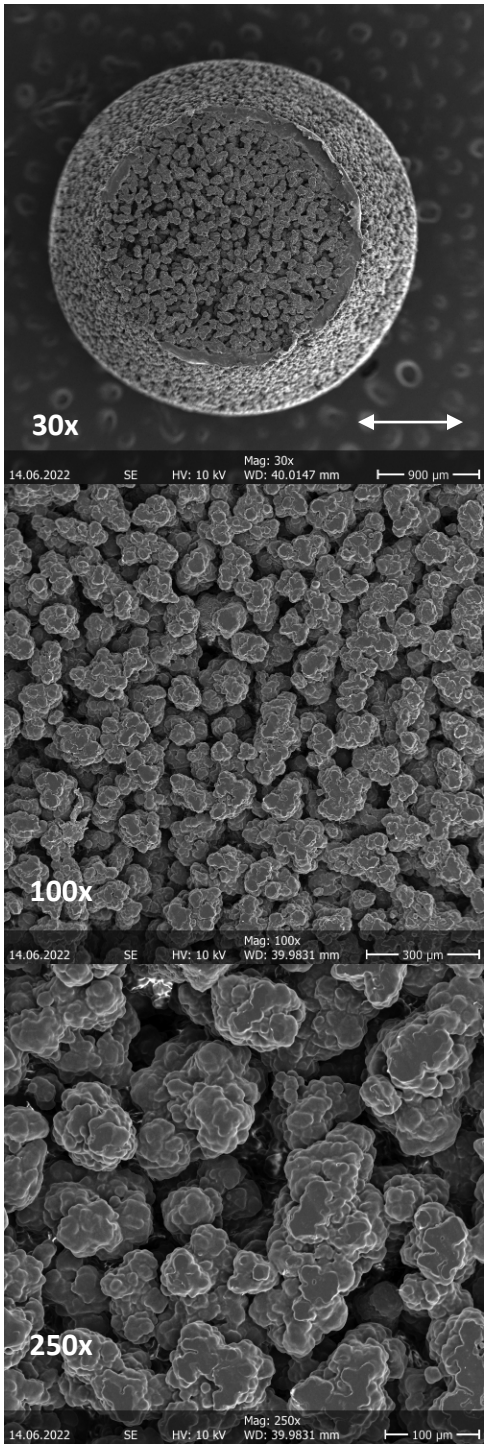
30x
Mag: 30x
14.06.2022 SE HV: 10 kV WD: 40.0147 mm 900 µm
100x
Mag: 100x
14.06.2022 SE HV: 10 kV WD: 39.9831 mm 300 µm
250x
Mag: 250x
14.06.2022 SE HV: 10 kV WD: 39.9831 mm 100 µm

**Table 1**.

| Average curve characteristics | 10 µL CRM | 10 µL Aqueous | 10 µL DBS on 222 | 10 µL DBS on 226 |
|---|---|---|---|---|
| **Slope** | 0.00373 | 0.00358 | 0.00354 | 0.00331 |
| **Slope 95% CI** | 0.00368 to 0.00378 | 0.00353 to 0.00363 | 0.00350 to 0.00359 | 0.00323 to 0.00338 |
| **Y-intercept** | 0.0207 | 0.0490 | -0.0089 | -0.0243 |
| **Y-intercept 95% CI** | -0.0255 to 0.0669 | 0.0007 to 0.0973 | -0.0498 to 0.0319 | -0.0949 to 0.0463 |
| **$R^2$** | 0.999 | 0.998 | 0.998 | 0.995 |
| **% difference from CRM** | - | **-4.0%** | **-5.1%** | **-11.3%** |
| **% difference from Aqueous** | - | **-** | **-1.1%** | **-7.3%** |

**Table 2a.**

| | 222 | | | 226 | | |
|---|---|---|---|---|---|---|
| | Mean (µmol/L) | SD (µmol/L) | CV (%) | Mean (µmol/L) | SD (µmol/L) | CV (%) |
| **Low** | 104 | 3 | 2.9 | 92 | 4 | 4.1 |
| **Medium** | 337 | 7 | 2.2 | 303 | 13 | 4.4 |
| **High** | 744 | 13 | 1.8 | 673 | 28 | 4.2 |

**Table 2b.**

| | 222 | | | 226 | | |
|---|---|---|---|---|---|---|
| | Mean (µmol/L) | SD (µmol/L) | CV (%) | Mean (µmol/L) | SD (µmol/L) | CV (%) |
| **Low** | 102 | 3 | 3.3 | 94 | 4 | 4.6 |
| **Medium** | 332 | 11 | 3.3 | 304 | 11 | 3.7 |
| **High** | 735 | 19 | 2.6 | 679 | 28 | 4.1 |