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Effect of Dried Blood Spot Quality on Newborn Screening Analyte Concentrations and Recommendations for Minimum Acceptance Criteria for Sample Analysis

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BACKGROUND: The analysis of dried blood spots has been used routinely for newborn screening since the early 1970s, and the number of disorders screened has expanded substantially in recent years. However, there is a lack of evidence regarding minimum blood spot quality acceptance criteria for sample analysis.

METHODS: Blood pools were spiked with phenylalanine, tyrosine, leucine, methionine, octanoylcarnitine, decanoylcarnitine, isovalerylcarnitine, glutarylcarnitine, thyroid-stimulating hormone, and immunoreactive trypsinogen to concentrations at the analytical cutoffs used in UK screening protocols. We evaluated the effect of sample volume applied to the card (10, 20, 50, 75, and 100 μL), punch location (central vs peripheral), and sample quality (double layering, applying blood to both sides of the filter paper, multispotting, applying insufficient sample, and compressing the sample after application).

RESULTS: Compression of blood spots produced significantly lower results (14%–44%) for all analytes measured ($P < 0.001$). Smaller blood spots produced significantly lower results (15%–24% for 10-μL vs 50-μL sample size) for all analytes at all concentrations measured ($P < 0.001$). Results obtained from peripheral punches were higher than those from a central punch, although this did not reach statistical significance for all analytes. Insufficient and multispotted samples demonstrated heterogeneous results.

CONCLUSIONS: All blood spots containing ≤20 μL (blood spot diameter <8 mm), those in which blood has not fully penetrated the filter paper, and all samples with evidence of compression should be rejected, since there is a risk of producing false-negative results.

Newborn screening (NBS)[3] is performed in many countries to allow early detection of conditions that are either life-threatening or can cause a clinically significant adverse outcome if left untreated *(1–3)*. Dried blood spots (DBS) have been used for NBS since the early 1970s because the sample is easy to collect and transport, and the sample volume required is relatively small *(4–7)*.

In the UK, 9 disorders are currently recommended by the UK National Screening Committee for screening at day 5–8 of life: phenylketonuria (PKU), medium-chain acyl-CoA dehydrogenase deficiency (MCADD), congenital hypothyroidism (CHT), cystic fibrosis (CF), sickle cell disease, maple syrup urine disease (MSUD), isovaleric acidemia (IVA), glutaric aciduria type 1 (GA1), and homocystinuria (HCU) *(8)*. Defined screening protocols contain condition-specific analytical and screening cutoffs *(9)*.

During routine collection of DBS for screening, blood from a heel prick is applied to filter paper attached to a screening card *(2, 4, 5)*. In the UK, PerkinElmer 226 filter paper, which contains four 10-mm-diameter circles, is used *(10)*. DBS quality is assessed subjectively by visual inspection in the screening laboratory, and repeat samples are requested on those deemed unsuitable for analysis. These samples are termed avoidable repeats, and UK standards recommend that the avoidable repeat rate be ≤2% *(1, 11)*. However, the rejection of samples is not standardized, since no specific guidance exists to define the minimum DBS quality acceptance criteria. The lack of consensus results in wide variation in practice, with different laboratories accept-

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[3] Nonstandard abbreviations: NBS, newborn screening; DBS, dried blood spots; PKU, phenylketonuria; MCADD, medium chain acyl-CoA dehydrogenase deficiency; CHT, congenital hypothyroidism; CF, cystic fibrosis; MSUD, maple syrup urine disease; IVA, isovaleric acidemia; GA1, glutaric aciduria type 1; HCU, homocystinuria; TSH, thyroid-stimulating hormone; IRT, immunoreactive trypsinogen.

ing or rejecting samples of differing quality, leading to confusion among sample collectors regarding what constitutes an acceptable sample and causing difficulty in comparing avoidable repeat rates.

Although guidance on sample collection states that the circles marked on the filter paper should be filled and evenly saturated with a single drop of blood *(4, 12–14)*, samples that do not meet this standard are regularly received by NBS laboratories. Poor-quality blood spots include multispotted samples (multiple small blood spots used to make 1 larger blood spot), blood applied to both sides of the card, layered blood spots, compressed blood spots, and insufficient blood on the card *(12)*.

Previous studies have assessed other factors that may affect results for some analytes, including punch location *(10, 15)*, hematocrit *(10, 15)*, sample volume *(6, 10, 15, 16)*, and filter paper type *(17)*.

To date, no study has investigated the effect of poor-quality samples on analytical results. Furthermore, previous studies assessing punch location and blood spot volume have not included all analytes measured in the current UK NBS program. Therefore, the aim of this study was to assess the effect of sample quality, sample volume, and punch location on analyte concentration in DBS to determine minimum acceptance criteria for analysis.

Materials and Methods

SAMPLE PREPARATION

We collected blood from 1 healthy adult male volunteer into lithium heparin tubes (BD Vacutainer®). The blood was pooled and baseline analyte concentrations were measured before spiking with the relevant analytes near to the analytical cutoff concentrations used in the UK screening protocols: 200 μmol/L phenylalanine and tyrosine (for PKU), 500 μmol/L leucine (for MSUD), 45 μmol/L methionine (for HCU), 0.4 μmol/L octanoylcarnitine (C8) and decanoylcarnitine (C10) (for MCADD), 0.56 μmol/L glutarylcarnitine (C5DC) (for GA1), 1.6 μmol/L isovalerylcarnitine (C5) (for IVA), 8 mU/L thyroid-stimulating hormone (TSH) (for CHT), and 60 ng/mL immunoreactive trypsinogen (IRT) (for CF) *(9)*.

We obtained amino acids from Sigma-Aldrich [phenylalanine (P-2126), methionine (M-2768), leucine (L8000-25G), tyrosine (T-3754)] and acylcarnitines from Herman ten Brink. IRT pools were created with anonymized, pooled serum from patients with high amylase. TSH (T9265-10UG) was purchased from Sigma-Aldrich. We repeated experiments with blood collected from a healthy adult female volunteer.

We measured hematocrit on an ABX Pentra ML (Horiba Medical). Whole blood pools were adjusted to a hematocrit of 47% (1%) by plasma removal. A hematocrit of 47% was selected as the approximate value for neonates at day 5 of life *(18)*. Whole blood pools were mixed and spotted onto PerkinElmer 226 filter paper (lot 102274/313449) with a calibrated pipette. Samples were dried overnight at ambient temperature. We took 3.2-mm-diameter punches from central and peripheral locations of each blood spot for analysis (Fig. 1A).

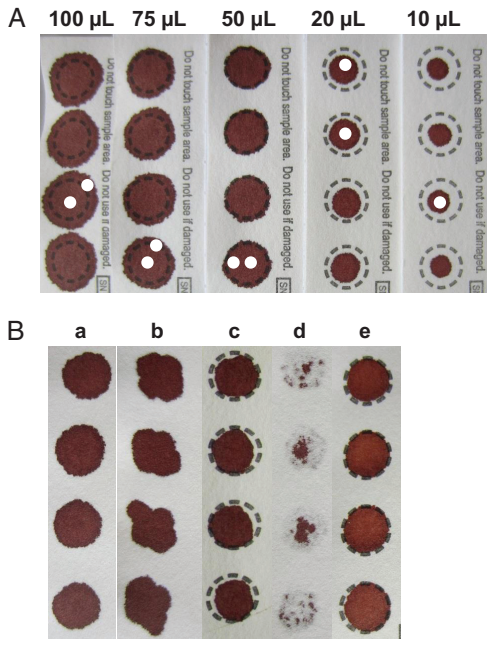

Fig. 1. Sample volume and quality factors.

(A), Effect of sample volume on blood spot diameter. The white circles represent central and peripheral punch locations taken from each blood spot during this study. (B), Examples of poor-quality blood spots: a, double layered/applied to both sides of card; b, multispotted samples; c, insufficient sample applied (view of front of card); d, insufficient sample applied (view of back of card); e, 20-μL spots compressed.

INVESTIGATION OF BLOOD SPOT VOLUME

To examine the effect of sample volume on the various analytes, we applied sample volumes of 10, 20, 50, 75, and 100 μL to filter paper. Only central punches were taken from 10-μL samples, since the area was too small for >1 punch (Fig. 1A). Blood spot diameter for each volume was measured to the nearest 0.5 mm with a magnifying ruler.

PREPARATION OF POOR-QUALITY BLOOD SPOTS

To assess the effect of poor-quality samples on analyte concentrations, we prepared a series of blood spots (Fig. 1B) as described below.

For samples that were double layered immediately and after 4 min (total sample volume applied 50 μL),

25 μL was applied to the front of the card, then a further 25 μL was reapplied 30 s (immediately) or 4 min after the initial application (Fig. 1B, a). We selected 4 min because this was the approximate time it took to apply blood to create the initial 30 replicates.

For samples spotted on the front and back of the card immediately and after 4 min (total sample volume applied 50 μL), blood was applied as above, but the second blood spot was applied to the back of the card. These samples were visually comparable to those in Fig. 1B, a.

For multispotted samples (total sample volume applied 50 μL), four 12.5-μL blood spots were added to the 4 corners of each circle (Fig. 1B, b).

For insufficient samples (total sample volume applied 20 μL), blood was applied while maintaining contact between the pipette tip and the filter paper, thereby preventing a hanging drop of blood from forming and the blood from fully penetrating the filter paper (Fig. 1B, c and d). Punches were taken from the best location (in the center where possible), with the second punch taken peripherally (where the least blood had soaked through to the back of the card). The first punch was not always central, since it was important to ensure that the best location was used to reflect current laboratory practice.

For compressed samples (total sample volume applied 20 μL), the effect of compression was assessed in 2 separate series of experiments. For both experiments, 20 μL blood was applied to the center of each of the 4 circles marked on the filter paper. In the first experiment, the filter paper was placed in a glassine envelope, and each blood spot was compressed by pressing the center of the blood spot with a thumb. In the second experiment, blood spots were compressed by 2 different individuals and by rolling a glass bottle over the blood spots to assess the effect of different forms of compression (Fig. 1B, e).

BLOOD SPOT ANALYSIS METHODS

We used the AutoDelfia® fluoroimmunoassay (Perkin-Elmer) for analysis of TSH (lot 632664) and IRT (lot 631698). Samples for TSH and IRT were prepared per the manufacturer's instructions. We analyzed amino acids and acylcarnitines as underivatized species with our in-house routine screening method. A working internal standard solution was prepared by reconstituting NSK-A, NSK-B, and NSK-B-G solutions (Cambridge Isotope Laboratories) in LC-MS grade methanol (Romil, H050). Final concentrations were 1.02 μmol/L for [$^{13}C_6$]phenylalanine, [$^{13}C_6$]tyrosine, [$^{2}H_3$]leucine, and [$^{2}H_3$-]methionine; 0.031 μmol/L for [$^{2}H_9$]C5 and [$^{2}H_3$]C8; and 0.062 μmol/L for [$^{2}H_3$]C5DC. C10 concentration was calculated with [$^{2}H_3$]C8. Samples were punched into 96-deep well microplates (VWR), and 100 μL of working internal standard solution was added to each well as the extraction solvent. Plates were covered with a plate-sealing cap and shaken for 20 min. All of the eluent was then transferred to 96-well Nunc microplates (Thermo 249946) and dried down under nitrogen. Samples were reconstituted with mobile phase [80% acetonitrile (Romil, H411), 20% deionized water, 0.1% formic acid (Greyhound, 069141A6)] before being analyzed by flow injection electrospray ionization tandem mass spectrometry in positive ion mode, with a Waters Xevo TQ. Ion transitions for the metabolites were as recommended in the UK NBS Protocols *(9)*. We used Neolynx software to calculate concentrations of samples and controls.

Interassay CVs were <9% for all analytes. CVs were calculated with the internal QC data obtained for all newborn screening runs undertaken during the period of sample analysis.

STATISTICAL ANALYSIS

Analysis was carried out with 1-way ANOVA with post hoc Tukey test, calculated with SPSS (version 16). We used the results from a central punch from a 50-μL blood spot as the standard control value for comparison. This volume was selected because it filled the 10-mm printed circle on the NBS card. We used a central punch from a 20-μL blood spot as the control for insufficient and compressed samples since, in practice, such samples would be smaller in volume. Results are presented as percentage bias compared with control values. Thirty replicates were analyzed for each variable; exceptions were made for TSH and IRT because of restrictions on plate capacity on the AutoDelfia or insufficient volume of the initial whole blood pools to spot all cards (Figs. 2–4). Twenty replicates were analyzed for volunteer 2. We carried out all statistical analyses on the raw data and not on percentage differences.

For the second compression study, analysis was carried out with Mann–Whitney test, since 10 replicates were analyzed for each type of compression. A 20-μL central punch was used as the control.

Results

EFFECT OF BLOOD SPOT VOLUME APPLIED TO FILTER PAPER

Smaller blood spot volumes (10 and 20 μL) produced significantly lower results for all analytes at all concentrations compared with blood spots of volumes ≥50 μL ($P < 0.05$) (Fig. 2). The mean (SD) diameters of the 10-, 20-, 50-, 75-, and 100-μL volume blood spots were 6 (0.5), 7.5 (0.5), 11.5 (0.5), 14 (0.5), and 15.5 (0.5) mm, respectively.

EFFECT OF PUNCH LOCATION

Analyte concentrations obtained with a peripheral punch were higher than those obtained from a central punch. Although this trend was observed for the majority of analytes, these differences rarely reached statistical significance (Table 1).

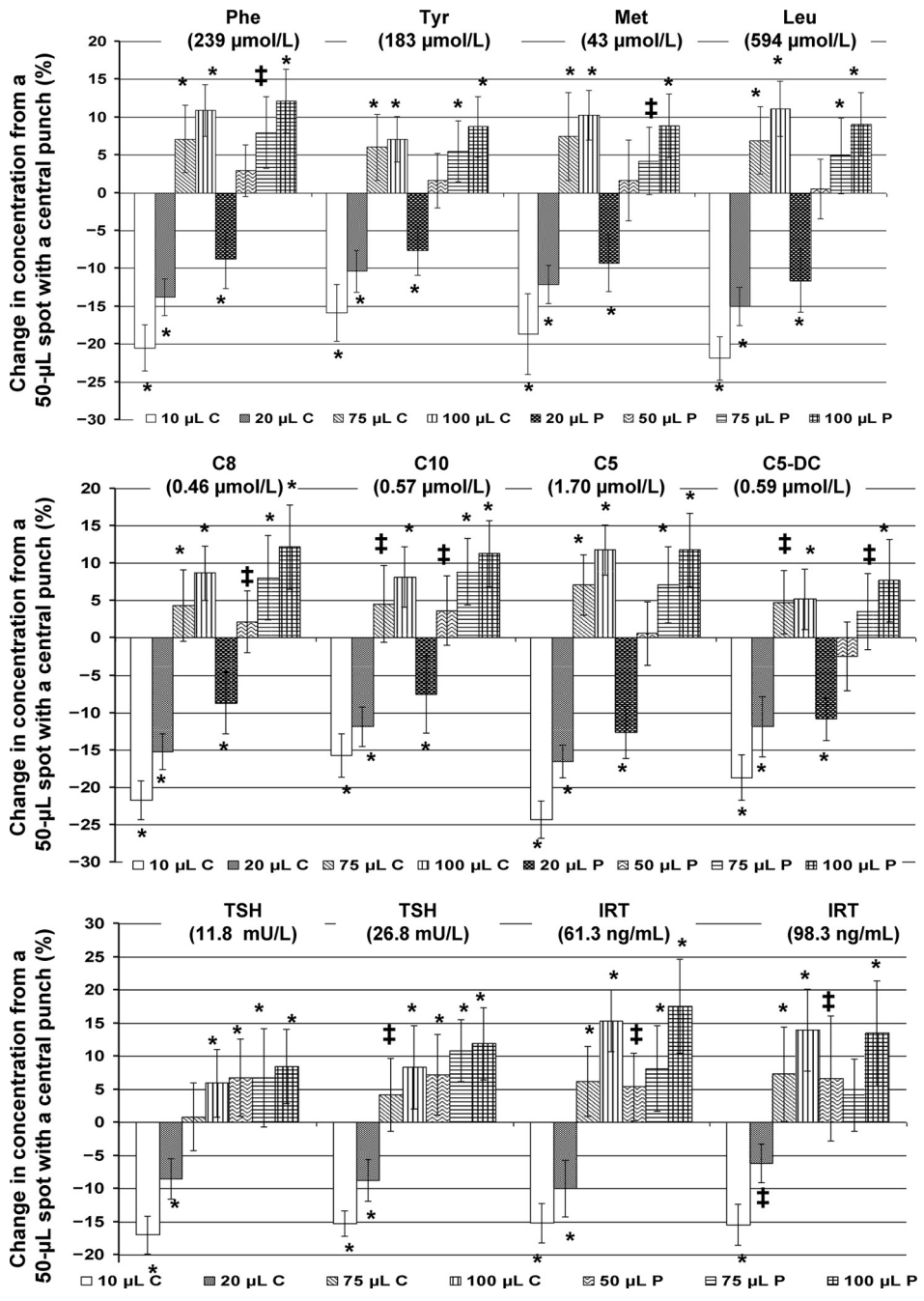

Fig. 2. Effect of blood volume and punch location on analyte concentrations.

C, central punch; P, peripheral punch. n = 30 replicates in each volume experiment (exceptions: n = 24 for TSH for 10-μL C and n = 18 for IRT concentration 1 for 100-μL C and P). Results are mean (SD). *$P < 0.001$, ‡$P < 0.05$.

EFFECT OF OVERSPOTTED SAMPLES

A positive bias was observed for the majority of analytes when samples were layered (whether the blood was applied to the front or back of the card) (Fig. 3). This was not statistically significant when the second blood spot was applied immediately, but did reach significance for the majority of analytes when the second layer was spotted after 4 min (Fig. 3). Central

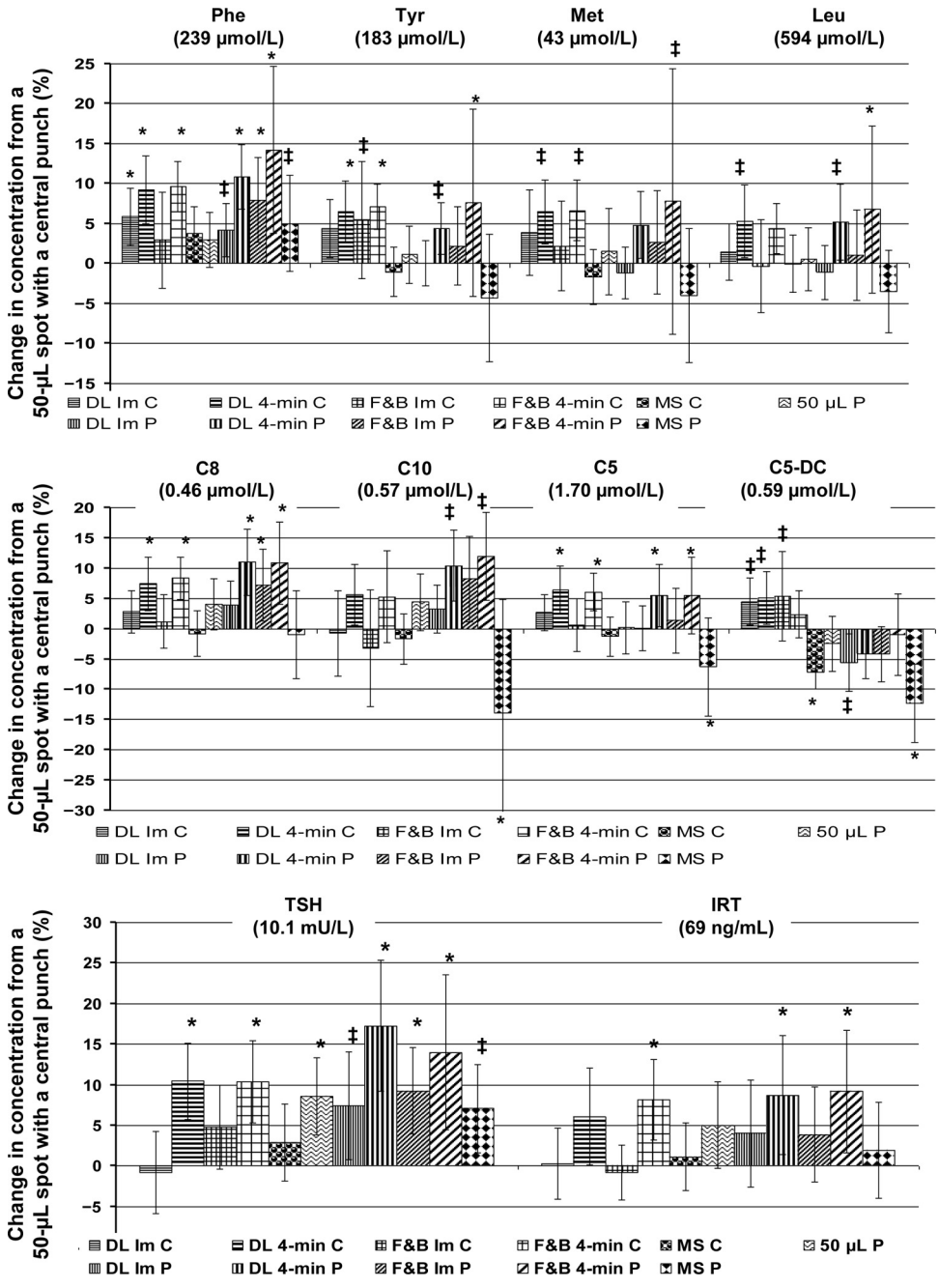

Fig. 3. Effect of overspotting and punch location on analyte concentrations.

DL, double layered; F&B, front and back; MS, multispotted; Im, immediately; Min, minutes; C, central punch; P, peripheral punch. n = 30 replicates in each experiment (exceptions: n = 26 for TSH and IRT for multispotted C). Results are mean (SD). *$P < 0.001$, ‡$P < 0.05$.

punches from multispotted samples were not significantly different from controls, except for C5DC. However, for peripheral punches, a significant negative bias was observed for C10, C5, and C5DC.

EFFECT OF POOR-QUALITY SAMPLES

The results obtained for central punches from insufficient blood spots were not significantly different from controls, with the exception of methionine, C10, TSH,

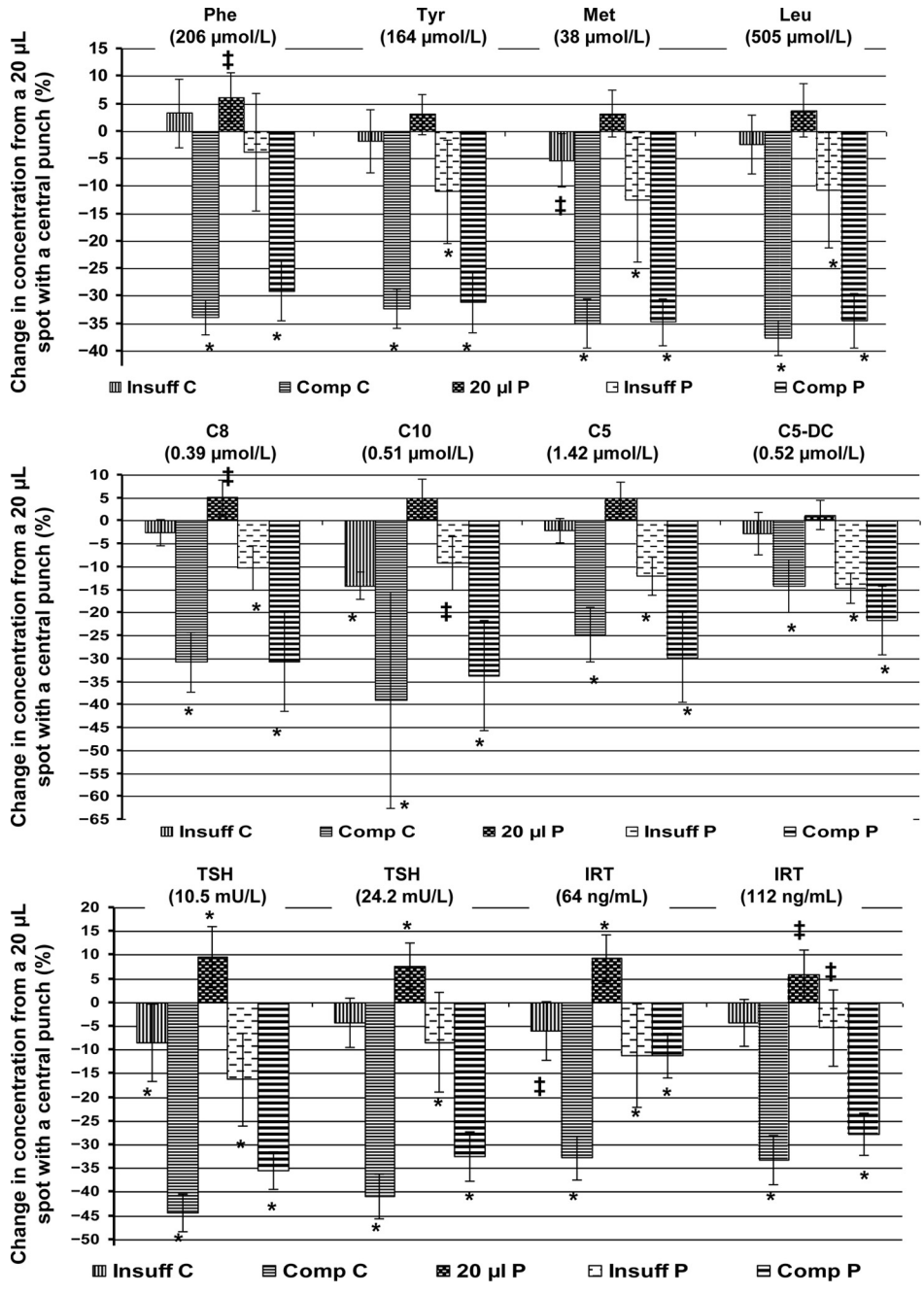

Fig. 4. Effect of poor-quality blood spots and punch location on analyte concentrations.

Insuff, insufficient; Comp, compressed; C, central punch; P, peripheral punch. n = 30 replicates in each experiment (exceptions: n = 28 for TSH at both concentrations and IRT concentration 2 for 20-μL peripheral multispotted C and n = 26 for insufficient C for IRT concentration 1). Results are mean (SD). *$P < 0.001$, ‡$P < 0.05$.

and IRT (Fig. 4). However, there was a significant difference for all analytes when samples were analyzed from volunteer 2 (see Supplemental Fig. 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue3). There was a significant negative bias for all analytes (with the exception of phenylalanine for volunteer 1) when peripheral punches were taken (Fig. 1B, c and d, and online Supplemental

Table 1. Effect of punch location on measured analyte concentration for different sample volumes.[a]

Analyte	20 µL			50 µL			75 µL			100 µL		
	Central	Peripheral	*P*	Central	Peripheral	*P*	Central	Peripheral	*P*	Central	Peripheral	*P*
Phenylalanine, µmol/L	206 (5.9)	219 (9.2)	<0.001	239 (8.5)	247 (8.2)	<0.05	256 (10.6)	258 (11.3)	NS	265 (8.2)	268 (10.2)	NS
Tyrosine, µmol/L	165 (5.1)	169 (5.9)	NS	183 (6.5)	187 (6.6)	NS	194 (7.9)	193 (7.4)	NS	196 (5.5)	199 (7.3)	NS
Leucine, µmol/L	505 (15.0)	525 (24.7)	<0.05	594 (22.5)	597 (23.3)	NS	635 (26.4)	624 (29.8)	NS	660 (21.7)	648 (25.0)	NS
Methionine, µmol/L	38 (1.1)	39 (1.6)	NS	43 (1.6)	44 (2.3)	NS	46 (2.5)	45 (1.9)	NS	47 (1.4)	47 (1.8)	NS
C8, µmol/L	0.39 (0.01)	0.42 (0.02)	<0.001	0.46 (0.02)	0.47 (0.02)	<0.05	0.48 (0.02)	0.50 (0.03)	NS	0.50 (0.02)	0.52 (0.03)	NS
C10, µmol/L	0.51 (0.02)	0.54 (0.03)	<0.05	0.57 (0.03)	0.60 (0.03)	<0.05	0.61 (0.03)	0.63 (0.04)	<0.05	0.63 (0.02)	0.64 (0.05)	NS
C5DC, µmol/L	0.52 (0.02)	0.53 (0.02)	NS	0.59 (0.02)	0.58 (0.03)	NS	0.62 (0.03)	0.62 (0.03)	NS	0.63 (0.02)	0.64 (0.03)	NS
C5, µmol/L	1.42 (0.04)	1.48 (0.06)	<0.05	1.70 (0.06)	1.70 (0.07)	NS	1.81 (0.07)	1.81 (0.09)	NS	1.89 (0.06)	1.89 (0.08)	NS
TSH, mU/L	NA	NA	NA	11.8 (0.57)	12.6 (0.69)	<0.001	11.9 (0.60)	12.6 (0.87)	<0.001	12.5 (0.60)	12.8 (0.66)	NS
IRT, ng/mL	NA	NA	NA	61 (3.1)	65 (3.1)	<0.05	65 (3.2)	66.2 (3.9)	NS	71 (2.9)	72.1 (4.3)	NS

[a] Data are mean (SD). NA, not analyzed; NS, not significant.

Fig. 1). All other experiments showed comparable results between volunteers 1 and 2 for the majority of analytes.

Compression of blood spots caused a significant negative bias (14%–44%) for all analytes (Fig. 4). This effect was replicated when samples were compressed by different methods, causing results to fall below the analytical cutoffs (Fig. 5). Central and peripheral punches taken from the compressed samples were not found to be significantly different.

Discussion

The aim of NBS is to allow early identification of specific disorders to enable presymptomatic treatment, thereby improving clinical outcome *(1–3)*. It is important to evaluate all aspects of blood spot sample quality to determine minimum acceptance criteria for DBS analysis. In this study, we established experimental procedures to simulate the varying quality of samples received routinely in the NBS laboratory to assess the effect on analytical results.

Our results show that the quality of the DBS has a significant impact on the reliability of the screening result produced, to the extent that there is a risk of false-negative and false-positive results for some of the conditions screened. The most significant findings were the large negative biases observed when blood spots were compressed. Compression resulted in the greatest risk of a disorder being missed if concentrations in affected individuals were near to the cutoffs (Fig. 5). The application of pressure caused the 20-μL blood spots to fill the circle (Fig. 1B, e), mimicking the size of a 50-μL blood spot (Fig. 1A). Visually, compressed blood spots contain a pale center with a darker ring around the periphery, which can be difficult to detect in practice. However, if there is evidence of possible compression, such as the presence of blood on the glassine envelope used to protect the card or the compressed appearance of the blood spot, the sample should be rejected.

We confirmed previous findings that a smaller-volume DBS will produce lower analyte concentrations *(4, 10, 15, 16)*. The smaller the volume applied to the card, the further the blood spreads relative to a sample of higher volume. Therefore, the higher the volume of blood applied, the more concentrated the blood will be within the punch. We recommend that samples of $\leq$20 μL ($<$8 mm diameter) be rejected, secondary to the observed negative bias.

We also confirmed that results obtained with peripheral punch locations are often higher than those from a central punch location *(10, 15)*. However, in contrast to previous studies, the differences were not significantly different for all analytes. Despite the results not being statistically significant, we recommend that the initial punches taken for each assay should be from a peripheral

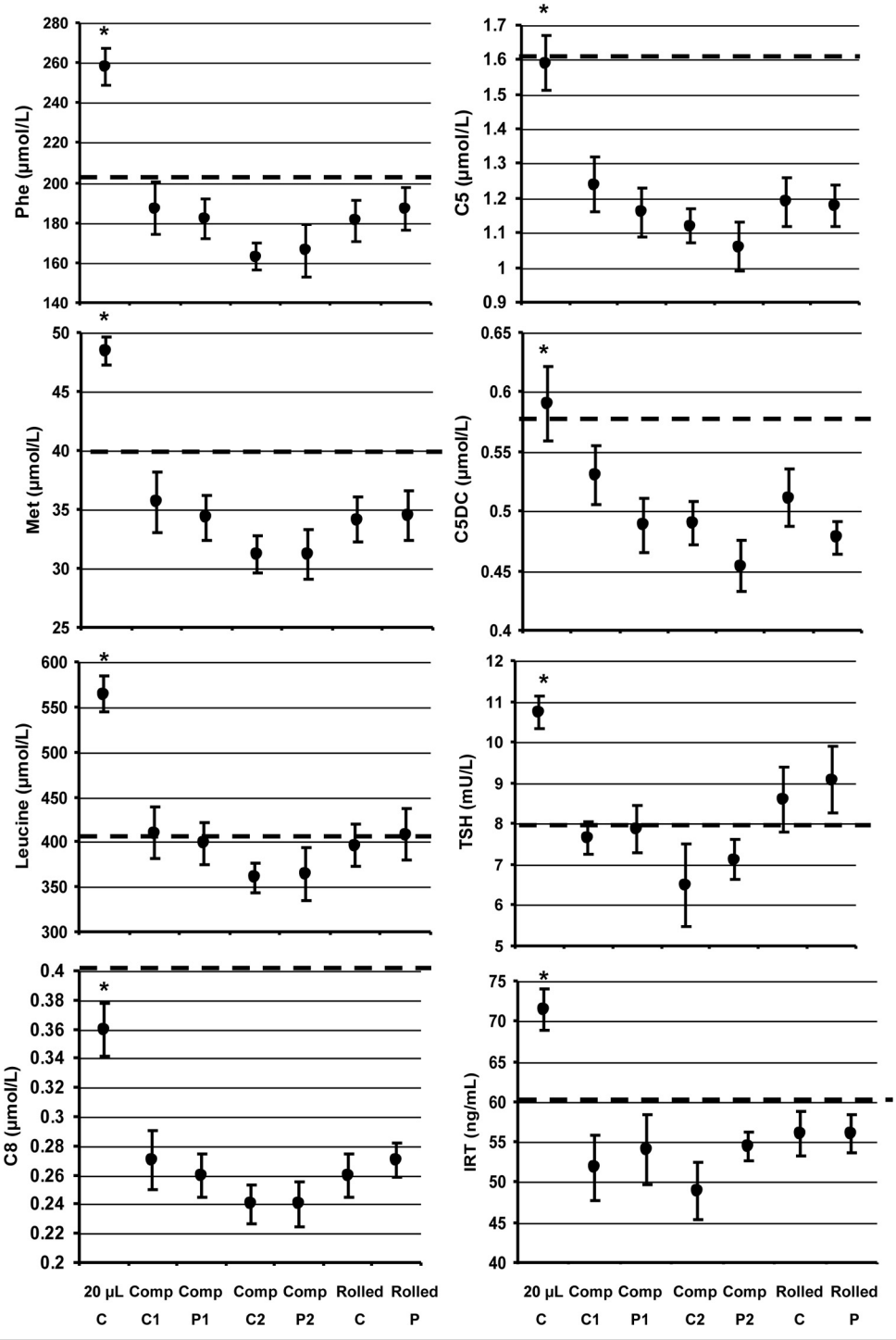

Fig. 5. Effect of compression and punch location on analyte concentrations.

Comp, compressed; C, central punch; P, peripheral punch; C1, central punch from volunteer 1; C2, central punch from volunteer 2; P1, peripheral punch from volunteer 1; P2, peripheral punch from volunteer 2. n = 10 replicates in each experiment. Results are mean (SD). *$P <$ 0.001. The dashed line represents the analytical cutoffs used in the UK NBS program. The initial concentrations for some of the whole blood pools (e.g., C8) were not above the cutoff before compression.

location. If results are above the defined analytical cutoffs, the repeat punches should then be taken from a central location from 2 different blood spots (provided the blood spots are of acceptable quality).

Although statistically significant, the negative biases observed for small-volume or compressed blood spots are not clinically significant for all analytes. The phenylalanine concentration in an infant with classic PKU will be significantly higher than the screening cutoff, and therefore a case is unlikely to be missed. However, this becomes more clinically relevant for disorders such as MCADD and MSUD, for which the results in affected infants may be near the screening cutoffs. A recent case of MCADD (compound heterozygote for 2 disease-causing mutations, c.985A>G and c.250C>T) had a C8 concentration of 0.55 μmol/L. The card contained 4 filled and evenly saturated circles of blood. If the negative biases observed in this study were applied, the results would have been below the screening cutoff if the sample had been ≤20 μL, and below both the analytical and screening cutoffs if the sample had been compressed, resulting in a false-negative case. Furthermore, in the CF screening program, babies with CF often have results close to the screening cutoff. We reviewed cards from false-negative cases over a 7-year period (n = 6), and in 4 cases the cause could potentially be attributed to insufficient or poor-quality samples.

Overspotted samples generally showed a positive bias in results, which was greater when the initial blood applied started to dry before the second application. This is secondary to the differing diffusion pattern observed with immediate double layering, where the blood from the second application becomes homogeneous with the first. Therefore, the amount of blood in a 3.2-mm punch will be variable when samples have started to dry. In practice, these samples may be difficult to detect, but can appear with a darker ring within the spot. The prominence of these rings will increase as the interval between applications increases. A positive bias may result in a false-positive case, which should be avoided to prevent psychosocial harm *(1, 19)*. However, because a case will not be missed, we recommend that double-layered samples be accepted because repeat sample collection can also cause stress and anxiety to parents *(2)*. However, this should not be the case if blood spot samples are to be used for diagnostic or monitoring purposes or for screening tests where a screen-positive result is defined by a result being below, rather than above, a designated cutoff, e.g., when screening for biotinidase deficiency, glucose-6-phosphate dehydrogenase deficiency, and severe combined immunodeficiency.

Results obtained from insufficient and multispotted samples showed greater heterogeneity. This finding is secondary to the nonuniformity of blood distribution, which causes variability in results when punches are taken from different locations within or between blood spots. This heterogeneity for insufficient samples was demonstrated by the greater negative bias observed for samples taken from volunteer 2. Because there is a risk of a negative bias and substantial heterogeneity in results for these samples, we recommend that these blood spots be rejected.

The UK NBS program has expanded, with the addition of MCADD, MSUD, IVA, GA1, and HCU since 2009. This expansion has resulted from the use of tandem mass spectrometry, which allows simultaneous detection of multiple analytes from a single punch *(3, 14, 20)*. The use of this technology provides scope for additional disorders to be added in the future *(3, 20)*, and as such may include other disorders that have the potential for false-negative results if samples are not of the recommended quality *(21)*. The recent program expansion has resulted in the inclusion of more clinically severe conditions, which increases the likelihood that further second-tier testing will be required on the DBS. For MCADD, IVA, and GA1, this includes diagnostic testing (derivatized acylcarnitine profile), and for HCU, homocysteine, both of which can be analyzed on the initial NBS sample. Availability of these results allows greater certainty of the screening result and therefore can prevent unnecessary parental anxiety *(3, 14)*. However, these analyses may not be possible if sample volume or quality is poor.

Poor-quality samples can pose additional issues during the screening process. This includes delayed reporting of results while a repeat sample is obtained, delayed referral of a screen-positive case, and unnecessary rebleeding of babies. Increased laboratory and midwifery workload and costs are associated with repeat sampling *(2)*, as well as concern where parents decline repeat sampling or where the repeat sample is not taken *(1)*.

In this study, factors that could cause variability in the results were minimized where possible. The erythrocytes were not washed to maintain sample integrity. All samples were spotted, punched, and analyzed by the same scientist, and all samples being compared were analyzed in a single batch to exclude interassay variability. The effects of filter paper type and humidity *(22)* were eliminated by using the same lot of filter paper and by preparing and drying samples in the same laboratory simultaneously. Plate effects were assessed by carrying out some analytical runs in reverse order. Two volunteers were used to eliminate intraindividual variation, and the hematocrits of all pools were adjusted to 47% (1%). The effect of hematocrit was not assessed in this study, since routine measurement is currently impractical in DBS. Therefore criteria cannot be set for accepting or rejecting samples on the basis of this parameter. A variable that could not be accounted for was the use of lithium heparin samples, which would not be identical to taking a heel

prick. This may cause differences in results; however, it would not be possible to replicate routine sample-taking due to the large volume of blood required to complete all tests.

On the basis of the results from our study, our recommendation for screening laboratories is that the following samples should be rejected:

- compressed samples;
- samples ≤20 μL (<8-mm diameter);
- insufficient samples; and
- multispotted samples.

However, these recommendations may not apply to screening tests in which a screen-positive case is identified as a result being below, rather than above, a designated cutoff.

In addition, our study has extended previous findings on the effect of punch location and sample volume to include all analytes measured in the current UK program. Sample quality is essential to obtain valid results to ensure that disorders are not missed, and to prevent parental anxiety caused by false-positive results. Our findings may have wider implications, since DBS are being increasingly used for diagnostic (e.g., inborn errors of metabolism) and monitoring (e.g., therapeutic drug monitoring) purposes *(4, 7, 14)*. Therefore, sample collectors should ensure that all marked circles on the filter paper are filled and evenly saturated by 1 drop of blood *(4, 12–14)*. The introduction of blood spot quality guidelines, on the basis of our evidence and recommendations, should improve the quality of the service and allow standardization of sample rejection.

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References

1. Bello S, Ferguson C, Wallis R. Improvements to the newborn bloodspot screening service are required to meet national standards. J Med Screen 2010;17:114–20.
2. Shepherd AJ, Glenesk A, Niven CA, Mackenzie J. A Scottish study of heel-prick blood sampling in newborn babies. Midwifery 2006;22:158–68.
3. Lehotay DC, Hall P, Lepage J, Eichorst JC, Etter, ML, Greenberg CR. LC-MS/MS progress in newborn screening. Clin Biochem 2011;44:21–31.
4. Li W, Tse FLS. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. Biomed Chromatogr 2010;24:49–65.
5. Miller JH, Poston PA, Rutan SC, Karnes T. An on-card approach for assessment of haematocrit on dried blood spots which allows for correction of sample volume. J Anal Bioanal Tech 2013;4:162.
6. Mei JV, Alexander JR, Adam BW, Hannon WH. Use of filter paper for the collection and analysis of human whole blood specimens. J Nutr 2001;131:1631S–6S.
7. Sharma A, Jaiswal S, Shukla M, Lal J. Dried blood spots: concepts, present status and future perspectives in bioanalysis. Drug Test Anal 2014;6:399–414.
8. Public Health England. 2013 Jan 1 (first published). Newborn blood spot screening: programme overview. https://www.gov.uk/newborn-blood-spot-screening-programme-overview (Accessed February 2016).
9. Public Health England. A laboratory guide to newborn blood spot screening for inherited metabolic disorders. https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/444978/IMD_Laboratory_Handbook_May_2015_v1.0.pdf (Accessed February 2016).
10. Lawson AJ, Bernstone L, Hall SK. Newborn screening blood spot analysis in the UK: influence of spot size, punch location and haematocrit. J Med Screen 2016;23:7–16.
11. Public Health England. 2014 Jan 1 (first published). Standards for NHS newborn blood spot screening. https://www.gov.uk/government/publications/standards-for-nhs-newborn-blood-spot-screening (Accessed February 2016).
12. UK Newborn Screening Programme Centre. 2012. Guidelines for newborn blood spot sampling. https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/390963/A4_Guidelines_2012_for_web.pdf (Accessed February 2016).
13. CLSI. Blood collection on filter paper for newborn screening programmes; approved standard–sixth edition. Wayne (PA): CLSI; 2013. Document No. NBS01-A6.
14. Keevil BG. The analysis of dried blood spot samples using liquid chromatography tandem mass spectrometry. Clin Biochem 2011;44:110–8.
15. Holub M, Tuschl K, Ratschmann R, Strnadová KA, Mühl A, Heinze G, et al. Influence of haematocrit and localisation of punch in dried blood spots on levels of amino acids and acylcarnitines measured by tandem mass spectrometry. Clin Chim Acta 2006;373:27–31.
16. Adam BW, Alexander JR, Smith SJ, Chace DH, Loeber G, Elevers LH, Hannon WH. Recoveries of phenylalanine from two sets of dried-blood-spot reference materials: prediction from hematocrit, spot volume and paper matrix. Clin Chem 2000;46:126–8.
17. Slazyk WE, Phillips DL, Therrell BL, Hannon WH. Effect of lot-to-lot variability in filter paper on the quantification of thyroxin, thyrotrophin and phenylalanine in dried-blood specimens. Clin Chem 1988;34:53–8.
18. Jopling J, Henry E, Weidmeier SE, Christensen RD. Reference ranges for hematocrit and blood hemoglobin concentration during the neonatal period: data from a multihospital health care system. Pediatrics 2009;123:e333–7.
19. Moran J, Quirk K, Duff AJA, Brownlee KG. Newborn screening for CF in a regional paediatric centre: the psychosocial effects of false-positive IRT results on parents. J Cyst Fibros 2007;6:250–4.
20. Chace DH, Kalas TA, Naylor EW. Use of tandem mass spectometry for multianalyte screening of dried blood specimens from newborns. Clin Chem 2003;49:1797–817.
21. Peng M, Liu L, Peng L. Evaluation of factors influencing accuracy in the analysis of succinylacetone in dried blood spots. Clin Chim Acta 2012;413:1265–9.
22. Hill JB, Palmer P. Filter paper blood collection and punching as a means of quantification. Clin Chem 1969;5:381–9.