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Is the hematocrit still an issue in quantitative dried blood spot analysis?

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1. Equally contributed

Abstract

Hematocrit-related issues remain a major barrier for (regulatory) acceptance of the classical dried blood spot (DBS) analysis in the bioanalytical and clinical field. Lately, many attempts to cope with these issues have been made. Throughout this review, an overview is provided on new strategies that try to cope with this hematocrit effect (going from avoiding to minimizing), on methods estimating a DBS volume, and on methods estimating or measuring the hematocrit of a DBS. Although many successful strategies have been put forward, a combination of different technologies still provides the most complete solution. Therefore, further efforts and the availability of a straightforward guideline for analytical and clinical method validation should help to overcome the hurdles still associated with DBS sampling.

Keywords

Hematocrit; Dried Blood Spot; Alternative sampling

1. Introduction

In the field of quantitative DBS analysis, many attempts to cope with the hematocrit (Hct) issue have been made over the past few years. The lack of a simple, universally applicable approach to overcome the Hct issue has been a main hurdle for the widespread implementation of this sampling technique in the clinical field. The potential applications are numerous and distributed over many different areas, amongst which newborn screening, therapeutic drug monitoring, toxicology, drug development [1-11]. As outlined below, the Hct issue is multifaceted, often also involving the need to convert(dried) blood-based results to reference values that are plasma-based[12, 13].

Tackling the Hct issue is quite challenging, since the impact of Hct is a compound-dependent matter which is affected by many factors. The latter was demonstrated by Abu-Rabie *et al.*, who concluded that the overall Hct-based bias can be subdivided into a Hct-based area bias, a Hct-based recovery bias and a Hct-based matrix effect bias [14]. The Hct-based area bias is the best documented bias and is the

physical consequence of a difference in spreading of whole blood with varying Hct over cellulose-based DBS cards. More specifically, blood with a high Hct (e.g. 50%) will spread less than blood with a lower Hct (e.g. 30%), this due to the differences in viscosity of the blood. This difference in spreading leads to a substantial difference in DBS area, which in turn leads to a difference in sample proportion when a fixed diameter sub-punch is taken from the DBS. The slower spreading of blood with high Hct was demonstrated by Chao *et al*., who investigated the effect of blood Hct on the kinetics of blood spreading on Whatman 903 filter paper, a commonly used filter paper for the generation of DBS [15].

As will be outlined further in this review, a whole punch analysis after volumetric application of a fixed volume of blood can nullify this Hct-based area bias. The recovery bias on the other hand, is a result of the fact that for conventional DBS extraction, the internal standard (IS) is typically only added during the extraction step. Therefore it is unable to correct for variations in recovery from the dried blood. Furthermore, a DBS sample with a different Hct can be considered as being a different matrix, which can give rise to a Hct-dependent matrix effect bias in procedures using liquid chromatography-(tandem) mass spectrometry (LC-(MS/)MS). Since this matrix effect bias can have an impact on the accuracy of an analytical result, it can lead to an under- or over-estimation, depending on the samples' Hct. In addition, both the Hct-based recovery bias and the Hct-based matrix effect bias can also affect the precision of an analytical result. Therefore, the inclusion of blood samples covering a broad Hct range is very valuable for the evaluation of recovery and matrix effects during method validation.

Many attempts have been made to develop new devices and strategies to collect samples for which the quantitative result is not (or less) influenced by the Hct (cfr. infra). However, the low cost and accessibility of a 'classical' DBS might cause some hesitation towards the use of these newer alternatives. Therefore, researchers have also explored ways to correct for the Hct-induced bias in quantitative DBS analysis. For this purpose, two strategies can be followed. The first one is to determine the volume of blood in a DBS (or sub-punch). Based on this volume, the result from a quantitative analysis can be adjusted. Hence, in this approach the Hct is not determined, but the Hct-based volume bias is dealt with. A second approach consists of determining or estimating the Hct of a DBS (or sub-punch). The determination of Hct can then be used to (1) apply a Hct dependent correction factor to correct the quantitative analysis; (2) determine if the Hct of the sample is within the validated Hct range of the method or (3) convert DBS concentrations into plasma concentrations.

An extensive description of the Hct, the Hct effect, and the possible impacts of deviating Hct values on quantitative DBS analysis can be found in a review by De Kesel *et al*. [13]. Therefore, these topics will not be extensively discussed throughout this review, which aims at providing an overview of the different strategies that have recently been suggested in order to cope with the Hct issue present in

quantitative DBS analysis. Here, a distinction will be made between attempts to avoid the Hct issue, strategies to minimize the Hct issue and approaches to measure or estimate the volume and/or Hct of a DBS.

2. New strategies to cope with the Hct issue

2.1. Avoiding the Hct issue

Recently, different alternative sampling strategies have been designed that allow to maintain the benefits of DBS but eliminate the Hct-based area bias coupled to DBS. A common theme here is that the dried blood sample is generated volumetrically, followed by analysis of the entire dried blood sample. Besides, Abu-Rabie *et al*. have demonstrated that the overall Hct-based bias can be avoided when this whole spot analysis is combined with a strategy that nullifies the Hct-based recovery bias [14]. As mentioned above, the recovery bias is inherent to the addition of the IS in the extraction solvent. Therefore, different IS application techniques were investigated for their ability to allow quantitative coextraction of analyte and IS, to eliminate the recovery bias [14]. Three different IS addition strategies were actually able to eliminate the recovery bias: (1) spiking of the IS into blood before spotting of the blood, (2) spray addition of the IS onto DBS samples before sample extraction, using the IS spray module integrated within a commercially available DBS direct elution instrument (CAMAG DBS-MS500), and (3) spray addition of the IS onto the DBS card prior to blood application [14]. However, only the second nullifying-option is likely to be practical for most applications. Furthermore, Hempen *et al*. showed that the recovery can also be improved by using heated flow-through desorption [16]. Combining the latter with whole spot analysis also enables a Hct-independent automation (by using the commercially available flow-through desorption-solid phase extraction system provided by Spark Holland) of the entire DBS workflow [16].

As already mentioned by De Kesel *et al*., volumetric application by using anticoagulant-coated microcapillaries or an accurate pipetting technique will in practice only be possible in situations where dedicated staff (e.g. an experienced nurse or trained laboratory personnel) is available (e.g. patients in a hospital setting, postmortem sampling or preclinical studies) [13]. A recent, more user friendly application of precision capillaries, is the HemaPEN® technology (Trajan Scientific and Medical, Australia). This is a device that consists of 4 end-to-end capillaries, dispensing a fixed volume of 2.74 µL of capillary blood onto 4 pre-punched DBS paper spots (Figure 1). Since patients only need to make contact between a blood drop (obtained by a fingerprick) and the tip of the device, the problem of accurate deposition on filter paper is overcome. Hence, this collection seems far better suited for patient self-sampling compared to conventional precision capillary-assisted preparation of DBS. However, still, as also noted by Neto *et al*., attention should be paid to a difference in collection and

transfer velocity of the glass capillaries, depending on the Hct of the collected blood (collection took 1.2 s, 3 s or 6-8 s, for 25, 43 and 61% Hct samples, respectively). While a promising proof-of-concept has been published, making use of an experimental setup comparable to the working of the HemaPEN® [17], further research, including real life applications, are necessary to demonstrate the (Hct-independent) applicability and robustness of the proposed technology.

In 2014, Neoteryx introduced the Mitra® microsampling device based on volumetric absorptive microsampling (VAMS™) technology. These devices consist of an absorbent polymeric tip connected to a plastic handler (Figure *2*), which allows a straightforward collection of an accurate and precise amount (10, 20 or 30 µL) of a liquid sample across a broad Hct range [18, 19]. Careful handling of these devices is necessary in order to exclude a variation in the amount of blood retained on the absorbent tips. E.g., Denniff *et al*. demonstrated that misuse of the tips can lead to a higher or lower amount of blood retained, by touching the tips before sampling with greasy fingers or with poorly dried hands, respectively. Furthermore, incorrect handling of the tips *after* sampling resulted in a sample loss of up to 19%, depending upon the absorptivity of the materials to which the wet tips were touched [20]. In the context of the Hct effect, Spooner *et al*. demonstrated that the VAMS technique is able to minimize or eliminate the volumetric Hct effect coupled to DBS sampling. They did so by investigating the volume of blood with varying Hct values (20, 45 and 65%) and from multiple species (rat and human) that was absorbed by the tips. This Hct-independent uptake of blood was later confirmed using authentic patient samples [21]. However, even though the blood uptake is Hct-independent, a divergent Hct value can still have an impact on other parameters, e.g. analyte recovery or matrix effect. We -as well as others- found that VAMS samples, as compared to DBS, may even be somewhat more susceptible to an impact of the Hct on the analyte recovery [21-23]. In this context, Mano *et al*. found that the inclusion of a sonication step within the sample extraction procedure can help to (partially) overcome the Hct-based recovery bias [24]. In a procedure for quantitative analysis of conventional anti-epileptics, extraction of the tips at elevated temperature (60 °C) provided the best results in terms of robust absolute recovery [22]. Others showed that combining a sonication step with an elevated extraction temperature and extended vortex mixing, may also be a good option to obtain a Hct-independent recovery [25]. Furthermore, various extraction solvents should be comprehensively evaluated in order to achieve a good and consistent recovery across a broad Hct range [26]. Importantly, as shown by Xie *et al*., improving the recovery (>80%) is not only necessary to avoid a significant Hct effect, but also to minimize apparent stability issues that are actually related to an unoptimized extractability, having a negative impact on aged VAMS samples [27]. Furthermore, we demonstrated that when using the same procedure for extracting paracetamol from blood-filled VAMS, no recovery issues were encountered, whereas this was the case when using water-filled VAMS,

concluding that an extractability-mediated recovery bias can also be matrix-dependent [Delahaye et al., submitted for publication].

In conclusion, VAMS helps to overcome the volumetric Hct effect coupled to DBS sampling whilst maintaining all the benefits coupled to regular DBS analysis. However, Hct-dependent recovery can be an issue. To (partially) resolve this issue, thorough optimization of the extraction procedure is necessary. This is particularly the case for compounds with a limited absolute recovery, since the Hct-based recovery bias has been demonstrated to be more prominent in such cases [14]. Furthermore, as the complete tip is used during sample extraction, analysis is simplified when compared to partial-spot DBS analysis. Moreover, as recently demonstrated by Verougstraete *et al*. analysis of 'wet' VAMS samples is a possibility as well, offering opportunities in a hospital as well as a home-based setting [28, 29].

Recently, Nakahara *et al*. reported on a volumetric absorptive paper disc (VAPD) and mini-disc (VAPDmini), which combine the principles of classical DBS and VAMS. These devices consist of a filter paper disc, which can hold an accurate volume of blood, fixed with adhesive tape within a filter paper sheet containing slightly larger holes than the disc [30]. It was demonstrated that, by using clozapine and its metabolites as model compounds, analyte recovery was Hct independent, since the percentage difference between a 30%, 40% and 60% Hct sample, compared to a 50% Hct sample, was within 15% [30]. Therefore, it was concluded that these devices could serve as an alternative for the VAMS tips or as a Hct independent alternative for classical DBS analysis. However, these findings should be confirmed using other compounds, since it is known that the recovery of compounds from VAMS sampler tips may be Hct-dependent and this could also be the case for these devices.

Another device, recently designed by KTH Royal Institute of Technology (KTH, Stockholm, Sweden) to overcome the volumetric Hct effect coupled to regular DBS analysis, is the Capitainer-B device (Figure *3*). This device consists of an inlet port to which a drop of blood (produced by a fingerprick) is added, which fills a capillary microchannel, with a defined volume of 13.5 µL. When the capillary channel is filled, a thin film at the inlet dissolves, ensuring that the excessive amount of blood is absorbed by a paper matrix, resulting in a separation of the excess blood and the filled channel. Finally, by complete dissolution of a thin film at the outlet, the capillary channel is emptied through capillary forces, leading to the absorption of 13.5 µL of blood by a pre-perforated paper disc [31]. Once dried, the pre-perforated paper discs can easily be removed using tweezers, for further sample processing. A proof-of-concept was provided by Spooner *et al*., who demonstrated that these devices are able to precisely dispense an average blood volume of 13.5 µL across a broad Hct range (25 to 65%) [32]. Furthermore, by evaluating the recovery of radiolabeled material they could also conclude that recovery from the

derived DBS was Hct-independent. In addition, we also demonstrated the Hct-independence of the devices, when measuring caffeine and its metabolite paraxanthine, using authentic patient samples with a broad Hct range (18-55%) [33]. Also for the direct alcohol marker phosphatidylethanol 16:0/18:1 a good agreement was found when comparing the results of the Capitainer-B devices with those of liquid whole blood [34]. Our data also suggest that there is no impact of the amount of blood, pipetted to the inlet of the device on the accuracy and precision of the dispensed amount of blood at the device outlet [33]. Further research is required to demonstrate the user-friendliness and robustness of the device in real-life applications, including the direct application of unknown amounts of blood, obtained via fingerprick.

The HemaXis DB device (DBS System SA, Gland, Switzerland) is another example of a device for generating volumetric DBS (Figure *4*). This is a plastic foldable microfluidic-based device, comprising of a standard DBS paper card and a microfluidic chip containing 4 capillaries. The sampling procedure is straightforward and should easily lend itself to patient self-sampling [35]. Following contact between the capillary inlet and a drop of blood, the capillary channel fills itself with a fixed amount of blood (5.5 or 10 µL) [35, 36]. After repeating this step 4 times, the book-like plastic cover can be closed and needs to be pressed for 10 seconds to enable quantitative transfer of the fixed amount of blood from the outlet of the capillary channels to the opposing DBS card [35]. When followed by whole spot analysis, these devices are also able to nullify the volumetric Hct effect coupled to conventional DBS analysis. In contrast to the above-mentioned HemaPEN®, Capitainer-B and Mitra® devices, this sampling technique still maintains the format of a classic DBS card, which allows compatibility with the automated systems available for DBS analysis, as demonstrated by Verplaetse *et al*. [35]. Furthermore, DBS samples prepared by a volumetric pipet and samples prepared by using the HemaXis DB devices were shown to be comparable in providing acceptable data, independent of the Hct of a sample [35, 36].

Besides the volumetric application of DBS, the Hct bias can also be eliminated by using plasma instead of whole blood for the generation of dried matrix spots, resulting in dried plasma spots (DPS). The use of DPS requires a longer sample preparation time, since -normally- a centrifugation step is necessary. Hence, this also implies the impossibility of patient self-sampling. On the other hand, certainly for therapeutic drug monitoring, using plasma instead of blood definitely has benefits (e.g. reference ranges of several drugs are set in plasma) and a good correlation has been described between DPS results and results obtained from liquid plasma analysis [37-43]. In 2012, Astra Zeneca introduced capillary plasma microsampling (CMS), in which 8 µL of plasma was obtained by centrifugation of a Hct tube, containing 32 µL of whole blood, followed by a manual cutting step in which the plasma

compartment within the tube was separated from the red blood cell (RBC) compartment [44]. Later, GlaxoSmithKline commercialized, in cooperation with Drummond Scientific, an application on the CMS introduced by Astra Zeneca. Here, a 75 µL glass capillary tube was developed containing a thixotropic gel, which is able to create a physical barrier between the plasma and RBC fraction after centrifugation, excluding the need for cutting the capillary [45]. However, since the workflows of both CMS methods include many manual steps and require an extensive training of lab technicians, the techniques were not widely accepted outside the preclinical area. Therefore, the interest arose in the development of a centrifugation-independent device for the collection of DPS. In 2015, Sturm *et al*. reported the autoDPS Card, which was designed as an improved version of the Yorktest plasma separator, used by Li *et al*. [46, 47]. The prototype autoDPS Card consists of a RBC filtration membrane connected to a plasma collection material, consisting of four 2 or 4 mm diameter collection wells, separated from one another via hydrophobic wax barriers. Over 50 materials were tested during card development and asymmetric polymer membranes turned out to be the best option for RBC filtration, whilst cellulose-based materials were selected for plasma collection [46]. Evaluation of the analytical performance revealed that the card is able to produce accurate and precise results at a Hct level of 45% [47]. However, to date, the autoDPS Card is not able to produce acceptable bioanalytical data independently from a patients' Hct. A positive bias was observed when comparing the extraction of a whole plasma spot originating from 30% Hct blood with a spot from 45% Hct blood, whilst a negative bias was observed when using 60% Hct blood. The latter can be caused by a varying volume of plasma generated by blood with a different Hct [47]. Therefore Sturm *et al*. concluded that a redesign of the card is necessary in order to deliver a Hct independent device [46]. A Hct compatible alternative is the NoviPlex plasma extraction card (Novilytic LLC, IN, USA) [48]. In contrast to the above-mentioned autoDPS card, DPS are not collected on a card but on pre-punched discs, which can be considered a disadvantage when aiming at integration with systems for automated DBS analysis. To date, two different formats of the card exist: the original NoviPlex cards containing 1 plasma spot and the NoviPlex Duo cards which generate 2 plasma spots from a single blood deposition. The NoviPlex cards are able to generate a fixed amount of plasma (i.e. 2.5 or 3.8 µL, for the original and Duo card, respectively), starting from a variable amount of (capillary) blood [49]. The top layer of the NoviPlex card contains a 'test area' to which an unmeasured amount of (capillary) blood needs to be applied. When the volume of this drop is sufficient, a control spot should appear. In a next step, the blood rapidly dissipates across a spreading layer, before 2.5 or 3.8 µL of plasma is generated by a separation membrane. The generated plasma is collected on a reservoir, which can be removed after a drying time of 15 minutes. Kim *et al*. gravimetrically tested the impact of Hct (20, 41 and 71%) on the sample volume. The 20 and 41% Hct samples had a within card volume variation of less than 1%, whilst the 71% sample had a variation of 3.4%. Furthermore, a %RSD of less than 1% was reported for the 3

different Hct levels, suggesting the Hct compatibility of the NoviPlex card [48]. However, Sturm *et al*. detected an unacceptable Hct bias when applying 20 µL of whole blood onto the NoviPlex cards when using 30 or 60% Hct blood, compared to samples with a 45% Hct. On the other hand, application of 50 µL of whole blood resulted in an acceptable bias for 30, 45 and 60% Hct samples [46]. In conclusion, the NoviPlex card may serve as a user-friendly plasma collection technology, with as limitation that an application of 50 µL of whole blood is a necessity. Another device is the HemaXis DX (DBS system SA, Gland, Switzerland), which should also be able to generate pure plasma or serum, starting from whole blood, without a centrifuge [50]. To date, the device is not yet available for research evaluation and therefore no data is available about the analytical performance of the device. Finally, there is the HemaSpot™-SE device (Spot on Sciences, Austin Texas), which consists of a spiral-like filter paper within a plastic holder [51]. The design should allow the separation of whole blood in serum and blood cells, resulting in a high concentration of red blood cells, platelets and leucocytes in the center of the filter paper, whilst serum and serum components are found at the end of the spiral [51]. However, to date, no data have been published concerning this device. It will be important e.g. to determine whether chromatography effects take place in the paper. A common benefit of the plasma generating systems is that they eliminate the need for a centrifugation step, making the technique accessible for anyone, anywhere, without the need of a phlebotomist. It needs to be demonstrated on a case-by-case basis that the DPS concentrations are effectively equivalent to those in plasma – also when starting from blood with atypical Hct values.

2.2. Minimizing the Hct issue

Besides different strategies to avoid the Hct issue, different approaches that try to minimize the Hct issue have been put forward by various stakeholders. One of the proposed approaches is the development of special filter material or special filter paper formats. In this context, HemaSpot™-HF (Spot on Sciences, Austin, Texas) was developed and claims to have a reduced Hct effect. This collection device consists of a cartridge, containing an 8-spoked filter paper disk and desiccant covered by an application surface with a small hole allowing blood to enter [52]. However, to date, there are only few reports available and no data concerning a possible reduction in Hct effect is available [53, 54].

Furthermore, Mengerink *et al*. presented the use of a hydrophilically coated woven polyester fiber as a Hct-independent substrate for DBS analysis, named Qyntest cards (Qynion, Geelen, The Netherlands). It was demonstrated that the cards generate spots with a Hct-independent size within a Hct range of 20 to 70%, allowing to use partial spot analysis without the Hct volume bias [55]. A clear drawback here is that this is a completely distinct material, which also may be more expensive to

produce, as compared to conventional cellulose-based cards. No follow-up reports have been published during the last few years.

Chitosan and alginate foams, two non-paper substrates that can be dissolved, may also offer the potential to overcome Hct-related recovery issues due to generally higher recovery values compared to classical commercial DBS cards [56].

As readily discussed by De Kesel *et al*., one of the easiest strategies may be to use calibrators prepared from blood with a Hct close to the expected median value of the target population [13]. Although one can never exclude that there may still be Hct extremes, this approach will make sure that the Hct impact will be minimized for the majority of the investigated population.

2.3. Measuring or estimating the volume of a DBS

As the Hct-based area bias (or volume bias) can be nullified by simply determining the volume in a DBS or DBS punch, strategies will be discussed below, where the volume of a DBS is determined or estimated, without determination of the Hct as such. The final aim of these strategies is to correct the analytical result based on the volume of the DBS (punch). The reasoning behind is that it shouldn't matter whether an analyte is measured in e.g. 10 µl dried or liquid blood – the result should be the same. A general remark concerning methods that aim at allowing a correction for the Hct-based area (or volume) bias, is that they do not take into account the other possible effects of Hct on a quantitative measurement (i.e. the Hct-based recovery bias and matrix effect bias). Therefore, during method development, special attention should be paid to the effect of the Hct on these parameters.

A first approach to estimate the volume of blood in a DBS was developed by Liao *et al*. Here, the extent of suppression of ionization in LC-MS/MS, caused by early eluting non-volatile salts in blood, is used to estimate the blood volume on a DBS card. The degree of suppression is measured by means of a post-column infused internal standard (PCI-IS). The reciprocal of the minimum response of the PCI-IS is calculated and correlated to the blood volume in a DBS via a calibration curve. To demonstrate the applicability of this method, the researchers compared the quantitative determination of voriconazole from DBS and plasma samples from 26 patients. A good correlation was found between the concentrations in DBS and plasma samples, and furthermore, Hct variation did not have a significant effect on the estimation of the DBS volume. One of the main advantages, according to the authors, is the additional function of the PCI-IS for the correction of matrix effect-caused quantification errors [57]. However, other variations that may occur during sample analysis are not accounted for by this approach, and therefore an additional IS, which is included early in the sample preparation procedure, remains necessary. Moreover, as this approach measures the extent of ion suppression, it is only

applicable when the analyte of interest is quantified via liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS(/MS)), as the phenomenon of ion suppression is specific for this technique. In addition, a more elaborate LC development is necessary and the selection of the IS and optimization of the PCI parameters are key for the success of this method. While in this report only whole spot analysis was performed it remains to be determined whether this approach can also be used with DBS sub-punches.

Kadjo *et al*. introduced a different technique, based on the electrical conductivity of a DBS extract, measured by a ring disk electrode (RDE). This technique to determine the blood volume of a DBS, is based on the strict regulation of electrolyte concentrations in human blood. As electrolyte concentrations are more or less constant between and within individuals, the electrical conductivity of a DBS extract could indicate the blood volume present in the DBS punch. The conductivity of the DBS extract is dependent on the composition of the extract, but also on the solution depth and the extraction solvent. Therefore, the authors also investigated the minimum required liquid depth necessary to obtain reproducible results with a RDE type electrode [58]. A custom made RDE was fabricated, since commercially available electrodes were too large for measurements in a typical 1.5 mL microcentrifuge tube. The extraction solvents tested in the experiments were 100 µL water, 100 µL 50:50 methanol-water, and 100 µL methanol. All extraction solvents performed well, provided that calibration of the electrode is done with standard solutions generated in the same solvent as the extraction solvent. The authors concluded that 100 µL, when put in a 1.5 mL microcentrifuge tube, provides sufficient immersion depth for reproducible measurements with this specific type of RDE. The developed method was also applied on a limited set of patient samples, to demonstrate the repeatability and reproducibility. However, no real comparison was made with a reference method for the estimation of blood volume in a DBS sub-punch from the patient samples. The benefits of this method are its non-destructiveness (the extraction solvent can be used further for sample preparation) and the automatability of the conductivity measurement. On the contrary, this method cannot be used for patients with deviating electrolyte levels, which can occur in certain diseases.

2.4. Measuring or estimating the Hct of a DBS

A first spectrophotometric technique used to tackle the Hct issue was described by Miller IV *et al*. This technique uses the UV-VIS reflectance of a DBS to estimate the Hct of the blood. First, the researchers investigated if there was a correlation between the Hct of the DBS and the reflectance at 540 and/or 570 nm, two hemoglobin(Hb)-specific wavelengths. A correlation at these wavelengths was not found, but a relationship was seen between the background scattering at 980 nm and the Hct of a DBS. The researchers determined the correlation between the reflectance and the Hct, and between the Hct

and the sample volume. Thereby, they were able to estimate the sample volume in a 3 mm DBS punch based on the reflectance at 980 nm. The authors pointed out that this technique should only be used for DBS of similar size, since spotted volume can also have an influence on the reflectance measured from spots with the same Hct. Therefore, they recommend it to be used for spots generated on pre-printed filter paper, so that an evaluation of spot size is possible [59]. Importantly, an evaluation of the effect of the age of a DBS on the measured reflectance was not included in the validation. Since the color of a DBS changes with age, this can have a major influence on the reflectance [13].

Capiau *et al*. also developed a spectroscopic technique, based on diffuse UV-VIS reflectance, for the prediction of Hct from a DBS [60]. In the approach that was developed, broadband light from a halogen source is guided to the surface (5.9 mm-diameter spot) of the DBS and a reflectance spectrum is recorded. Three Hb derivatives, oxyhemoglobin, methemoglobin, and hemichrome, are taken into account to determine the total Hb content. This is important, since over time -as the DBS dries and ages- the relative amounts of these derivatives change. Comparison of different anticoagulants during method development showed that the choice of the anticoagulant for the set-up of a calibration curve is important, since not all yielded the same reflectance spectrum as non-anticoagulated blood. The influence of spotted volume and DBS age was investigated, and no significant effect on the predicted Hct value was found. Application on patient samples showed very good agreement (95% of the predicted Hct values were within ±20% of the true Hct) between the actual Hct (measured via routine whole blood analysis) and the predicted Hct via this method [60]. The downside of this method is that a specialized software program and complicated algorithm are necessary to estimate the amount of each of these three Hb derivatives. Therefore, Capiau and colleagues continued to improve this method and developed a method that can determine the Hb content via a single-wavelength (589 nm) reflectance measurement. The reflectance at this wavelength (1) remained constant for DBS with equal Hct but different age (2 h up to 5 months) and (2) could be used to accurately predict the Hct. Application on patient samples yielded even slightly better results than the more complicated full spectrum-based method in terms of Hct prediction (more than 98% of the predicted Hct values were within ±20% of the true Hct). The predicted Hct was used to correct caffeine concentrations from DBS analysis with a Hct-dependent correction factor, resulting in a drastic improvement of the accuracy of the caffeine determinations (i.e. 54.5% of the samples had a %difference within 20% before correction, whilst 95.5% after correction) in DBS samples, which had a broad Hct range (20 to 50%) [61]. The main benefits of this technique are that it is nondestructive, does not require any sample preparation and in principle should be easily automatable. Incorporation in a stand-alone instrument or even in an automated DBS analyzer may be possible. However, as for now, such dedicated equipment is not yet commercially available.

Not only UV-VIS spectroscopy can be used for the estimation of Hct, but also near infrared (NIR) spectroscopy can be employed for this purpose. Oostendorp *et al*. developed an NIR-spectrum based method for the determination of Hct from DBS samples [62]. The Hct values obtained with this method were compared with Hct determinations via routine hemocytometry and both methods showed good correlation. The authors investigated the influence of drying time, albumin concentration, age and sex of patients, but none of these parameters were identified as significant covariates. For the development of this method actual patient samples were used to set up calibration curves. As application on only a limited set of DBS samples was reported, statements based on these results should be interpreted with caution. Also here, the nondestructive nature and absence of sample preparation are the major advantages of this method.

A different approach to estimate the Hct of a DBS, also based on Hb quantification, was developed by Richardson *et al.* [63]. These authors used a method based on the formation of a sodium lauryl sulphate-Hb complex (SLS-Hb), which has an optimal absorbance range between 500 nm and 560 nm, and is therefore very well suited to be measured with simple UV-VIS spectrophotometry at 550 nm. For the formation of this complex, a commercially available SLS containing reagent (Sulfolyser) is added to 10 µL of an aqueous DBS extract. A 6 mm DBS-punch, extracted with a total volume of 100 µL water, was used to reach the optimal sensitivity. The influence of spotted volume, punch location, storage time, and storage condition was evaluated. None of these parameters had a significant influence on the predicted Hct. The good long term stability (up to 6 months when stored at 4 °C) is due to the ability of SLS to bind with all forms of Hb. Application of this method on a set of 59 patient samples, with Hct varying from 27 to 51%, demonstrated the ability of the method to predict the Hct of a DBS compared to a routine analysis in a clinical lab [63]. The basic instrumentation and chemicals that are required for this method allows it to be implemented in every laboratory. Although it is a destructive method, the major part of the extract remains available for further analysis. However, the extraction can only be done with water, or combinations of water-organic solvent via which proteins are extracted from the DBS. This can be a disadvantage, since a protein precipitation step can (often) be avoided by working with extraction solvents with high percentages of organic solvents. Furthermore, the effect of using organic solvents in the extraction solvent on the absorbance measurements and Hct prediction was not evaluated. In addition, a 30 min extraction was necessary to ensure complete desorption of the blood from the DBS. Furthermore, it might be challenging to implement this procedure in an automated DBS analyzer, as part of the extraction solvent needs to be accurately separated. On the other hand, this method is easily automatable with a liquid handler system, which is present in many clinical labs.

In addition, also LC-MS/MS analysis –the gold standard technique for the quantification of small molecules- has been used for quantification of Hb in DBS [64]. Although the method was not developed with the aim to cope with the Hct issue, the determination of Hb is a good surrogate for Hct, as demonstrated by the methods described above, and this method could in theory also serve that purpose. However, as Hb is a protein, the sample preparation is very labor intensive, requiring protein digestion with trypsin. Therefore, its practical use will probably be rather limited, as the determination of the Hct will take much more time and effort, compared to the determination of the small molecule analyte. Furthermore, this technique is destructive, and part of the DBS has to be sacrificed for the Hct determination. Since more and more proteins are being quantified from DBS, this approach is not unlikely to be incorporated in such analysis, as simultaneous determination of Hb and the protein of interest might be possible. However, it remains to be demonstrated that determination of Hb with this method effectively allows to correct for the Hct-induced bias.

As discussed by De Kesel *et al*., potassium (K^+) is also a suitable candidate as a marker of Hct [13]. The first method using K^+ for the prediction of Hct from non-volumetrically applied DBS was developed by Capiau *et al*. [13]. In short, the K^+ content of a 3 mm DBS-punch extract, measured with a routine clinical chemistry analyzer, was shown to correlate with the Hct. A successful prediction of the Hct was demonstrated by application on patient samples. The influence of punch location and spotted volume was investigated, but neither one of these parameters had a relevant effect on the K^+ measurement. The Hct range covered by this method (19 to 63%) represents the majority of the patient population [65]. In a follow up study, De Kesel and colleagues evaluated the use of this method for a Hct-correction in the analysis of caffeine and its major metabolite paraxanthine from DBS [66]. These two analytes were chosen as model compounds since they are susceptible to a Hct-dependent bias [67]. First, a simple K^+-based algorithm was derived by plotting the whole blood/DBS caffeine concentration ratio against the K^+ concentration (as a surrogate for Hct) for a sub-set of samples. These samples were obtained from healthy volunteers, as well as from hospitalized patients. Application of this algorithm on DBS samples from an independent test set (n=50), for caffeine as well as paraxanthine, largely alleviated the clear Hct bias observed in [68]. The major benefit of this method is the use of standard instrumentation, available in any clinical laboratory and not requiring high-end instrumentation such as LC-MS/MS, which may not be present in resource limited settings. While a downside is the need for an additional 3-mm punch for the K^+ measurements, in most cases it is possible to obtain (at least) two 3-mm punches from 1 capillary DBS. However, some analytes require a larger punch or multiple 3-mm punches, which implies that an additional DBS is necessary for the K^+ measurement. This K^+-based strategy has been applied by several groups [69, 70].

Capiau *et al*. also investigated the use of this K^+-based Hct prediction starting from dried blood samples obtained via VAMS devices [Capiau *et al*., unpublished data]. Both an aqueous and organic extraction method were evaluated, and both were able to estimate the Hct within pre-defined acceptance criteria based on international guidelines. However, the organic extraction procedure was only performed with a limited set of patient samples and should still be further evaluated, Moreover, for the organic extraction procedure, evaporation of the organic extract is necessary, as the routine clinical analyzer is not compatible with organic solvents. On the other hand, for the analysis of most compounds, the organic extraction procedure decreases the workload. Hence, taking both analyses into account, the organic extraction procedure might be preferred. Again here, this process should be easily automatable. In addition, Bloem *et al*. also demonstrated the applicability of K^+ as a good marker for the estimation of the Hct when making use of VAMS devices [71].

Den Burger *et al*. used a modified method for the correction of creatinine measurements from DBS [72]. The adaptations of this method compared to that of Capiau *et al*. encompassed, amongst others, the measurement of the analyte and K^+ from one 8-mm DBS punch. A part of the DBS extract was used for the quantification of creatinine via LC-MS/MS and a part was used for the K^+ measurement. Also here, the influence of spotted volume and punch location were investigated, but the effects were not noteworthy. Also in this report, an improvement was seen in the correspondence between DBS and whole blood analysis when DBS concentrations were corrected for the Hct [72], although the Hct range was more limited (18 to 44%) in comparison to that of Capiau *et al*. On the other hand, both measurements could be performed starting from one 8-mm DBS punch, which requires less total sample preparation.

In addition, Rufail *et al*. investigated whether a perimeter ring sample from a DBS can serve for the measurement of K^+ and subsequent Hct prediction, as an alternative for the regularly used center sub-punch. To this end, a fixed volume (40 µL) DBS spot was prepared, the area of the center punch and perimeter ring were determined via image analysis, and K^+ was measured from both samples. It is important to note that the very outer edge of the DBS was also included in the perimeter ring sample. Next, a value of K^+ per area (α) was calculated. Both the circular center punches and the perimeter ring samples showed a good correlation between α and Hct, but values for α were higher for perimeter ring samples compared to central sub-punches. This resulted in a higher resolution for α vs Hct, and hence a smaller standard deviation when α was used for Hct prediction [73]. This result is not surprising, as it is known that some accumulation of RBCs occurs at the very outer edge of a DBS (known as the "volcano" effect). As these perimeter ring samples are typically not used for analyte determinations, their use for Hct prediction could eliminate the necessity of an additional DBS. A hurdle here is the requirement for volumetric DBS application and the requirement to measure the DBS area. This

measurement is easily automatable, but adds a supplementary step, and complicates the implementation of DBS in clinical practice.

Finally, Liao *et al*. used a lipidomics profiling strategy to identify markers for Hct estimation [74]. Via LC-MS the most abundant RBC membrane lipids, including phosphatidylcholines and sphingomyelins, were investigated as potential Hct estimation markers. Three sphingomyelins were identified as potential Hct estimation markers. As a next step, the measurement of these markers was applied to estimate the Hct values of two sets of DBS, DBS generated from blood with artificially prepared Hct on the one hand, and DBS collected from patients on the other hand. All three sphingomyelin compounds were able to estimate the Hct from these patient samples, within ±20% limits. However, the Hct range of the patient samples was rather limited (20 to 40%). Furthermore, the performance of the method with DBS punches from non-volumetrically applied DBS was not demonstrated, which is essential for its implementation in clinical practice. As this method uses LC-MS, it may be possible to determine analyte concentration and the suggested Hct markers simultaneously. To achieve this, more elaborate optimization of the extraction conditions and chromatographic separation may be necessary, in particular for more polar compounds, which are very different from the lipophilic sphingomyelin markers.

3. Conclusion

While the advantages of DBS sampling over classical venous sampling have been generally recognized, the Hct-dependent assay bias (Hct-based area bias, Hct-based recovery bias, and Hct-based matrix effect bias) has been an important barrier for (regulatory) acceptance of DBS.

In summary, the Hct-based area bias can be overcome in two ways: either by whole sample analysis preceded by volumetric application of a fixed volume of whole blood or by correction for the bias introduced by varying Hct levels.

Many suggestions to overcome the Hct-based recovery bias have been proposed. As demonstrated by several researchers, this recovery bias can have an important impact on the total assay bias. In most cases, this issue can be tackled via thorough optimization of the sample extraction method, resulting in a maximum absolute recovery of the analyte(s) from the dried sample.

Compared to the area and recovery bias, the Hct-based matrix effect bias has generally no significant contribution to the total assay bias. However, its evaluation during method validation remains required.

Besides the attempts to overcome the total Hct bias coupled to the use of whole blood, efforts have been made to develop devices enabling the generation of DPS starting from capillary blood. In theory the use of DPS instead of DBS inherently offers the advantage of avoiding all Hct-related issues – at least, if DPS concentrations are equivalent to those in plasma.

Moreover, the Hct-based area bias can be coped with by determining the volume of blood present in a DBS via several analytical techniques, followed by full-spot analysis. Still, attention should be paid here to a potential Hct-based recovery bias.

Rather than trying to minimize or avoid the Hct bias, the Hct of the blood contained within a DBS can also be measured or estimated. Different strategies have been proposed, based on the measurement of endogenous compounds. As some of these techniques can potentially be incorporated within stand-alone instruments or even in commercially available automated DBS analyzers, we believe that these incorporations may become standard in the future, also given the non-destructive nature of some of the proposed technologies. Importantly, incorporation of some technologies might even be used to serve as a “quality control”, either by controlling whether the Hct of a given DBS lies within the validated range, or by providing an assessment of DBS quality as a whole, which is relevant in all fields of DBS analysis, irrespective of whether it’s e.g. newborn screening or therapeutic drug monitoring.

Although many successful efforts have led to the introduction of several new alternative sampling devices and/or methods to correct for the Hct, the implementation of these methods within clinical practice remains limited. A more widespread implementation of the presented alternatives, resulting in a cost reduction, will definitely help extensive spreading of the DBS technique across laboratories. Furthermore, novel capillary blood collection devices (e.g. from seventh sense biosystems, fluisense®, and BloodTrackR device) in combination with volumetric absorptive microsampling strategies can serve as a patient-friendly alternative in the future. The availability of a straightforward guideline for analytical and clinical method validation [Capiau and Veenhof *et al*., to be submitted] should also help to streamline DBS analysis and help to overcome the hurdles associated with DBS sampling and analysis.

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Figure 1 HemaPEN device (with permission from Trajan Scientific and Medical, Australia.)

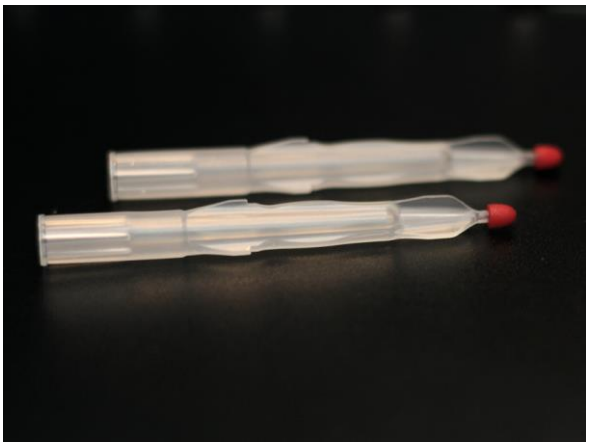

Figure 2 the Mitra® microsampling devices.

Figure 3 The Capitainer-B device.
Picture 1 (left) showing the inside of the device containing user instructions; Picture 2 (middle) demonstrating the application of a drop of blood at the inlet port; Picture 3 (right) displaying 4 filled pre-perforated paper discs.

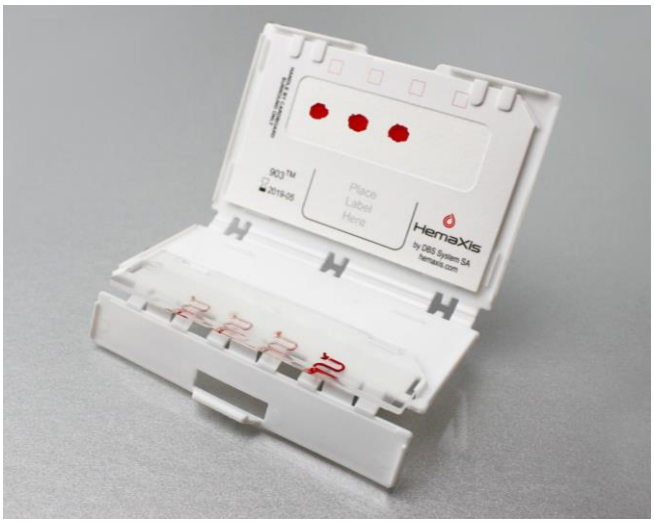

Figure 4. HemaXis DB device (with permission from DBS System SA.)