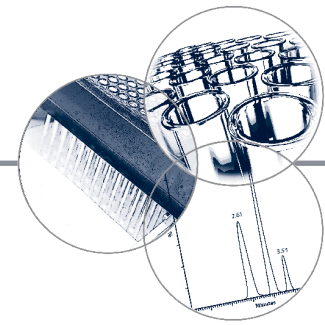
White Paper
Mini Focus Issue: Dried blood spots



Update of the EBF recommendation for the use of DBS in regulated bioanalysis integrating the conclusions from the EBF DBS-microsampling consortium

The European Bioanalysis Forum dried blood spots/microsampling consortium is reporting back from the experiments they performed on further documenting the potential hurdles of the DBS technology. Their experiments focused on the impact of hematocrit changes, IS addition, spot homogeneity, aging of spots and stability of fresh blood and cards. Results from these experiments demonstrate that the issues of DBS in regulated bioanalysis are real and that the technology will need additional improvements to be ready for use as a general tool for regulated bioanalysis. In addition, results on fresh blood and card stability were shared at international meetings and will be reported at a later date.

Keywords: dried blood spots ■ European Bioanalysis Forum ■ hematocrit ■ recommendation ■ spot homogeneity

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The technology of dried blood spots (DBS) does not need further introduction. Ever since the technology was reintroduced in pharmaceutical R&D in the early 2000s [1–3], numerous groups contributed to the broader understanding of DBS in different stages of drug development. Advantages, related to the 3R principles (replacement, reduction, refinement of animals in drug development), cost savings for bioanalysis – especially around sample shipment and storage – and patient comfort are acknowledged. Challenges, mostly related to the bioanalytical hurdles of the technique, have been and continue to be extensively described in peer reviewed literature or discussed at international conferences and workshops as part of the increased visibility of DBS in the regulated bioanalytical arena.

In recent years, the industry may have inadvertently created confusion or semantic contamination by using microsampling and DBS interchangeably, to the extent that even scientists now use both terms as synonyms. As part of this paper, the European Bioanalysis Forum (EBF) wants to re-emphasize that DBS is one of the many tools on the road to successful application of microsampling strategies (Figure 1), and that appreciation of DBS in this or previous papers from the European Bioanalysis Forum (EBF) should not be generalized for all microsampling technologies.

The EBF DBS consortium

Following up on the technology developments in regulated bioanalysis, the EBF has invested significant time and resources in trying to provide the industry with a broader perspective on the use of the technology. Taking advantage of the growing experience among its member companies, we connected experts and compared experimental data from many different compounds with application of DBS. In addition, the EBF organized its 1st Focus Meeting on the technology in 2010 [4], where we invited all stakeholders in the technology to connect around the most important scientific and regulatory questions for DBS. More importantly, in 2011, we reported back from our 2-year-long internal discussions on DBS by publishing an extensive science-based recommendation on the use of DBS in regulated bioanalysis [5]. In addition to the impact of the conclusions from this recommendation paper in industry, these conclusions gave rise to the formation of a dedicated group of companies to further work on DBS: the EBF DBS microsampling consortium. This new group, consisting of over 20 companies, carefully dissected the EBF recommendation to serve as a vehicle for continued sharing of data related to DBS. In addition, the recommendation also fuelled the consortium's proposal to industry

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to adapt a two-track approach to the DBS discussions:

- Stimulate a discussion on how to reposition the technology as a tool for microsampling in those areas of drug development where the technology is an immediate valid substitute or enhancement of liquid samples;
- Provide evidence-based and systematic documentation of the extent and scientific relevance of the (real or perceived) hurdles related to the technology in order to potentially reposition DBS – that is, stimulate the technology in those areas where it can deliver on promise and, in line with the first point, consider stopping or pausing the use of DBS in those areas where it is insufficiently established to add value.

The consortium officially started at an EBF organized Workshop in Brussels on 23 June 2011. At this workshop, scientists having experience with and vested interest in DBS or microsampling, discussed a way forward to unveil the relevance and impact of a number of pre-defined hurdles hampering the broader application and scientific acceptance of DBS.

The consortium at work

From a longer list of potential hurdles or challenges related to the use of DBS in regulated bioanalysis, and using the aforementioned EBF Recommendation as a starting point, the consortium identified five major areas of focus to further document the validity of DBS for regulated bioanalysis:

- Effect of the hematocrit;
- Stability (of the blood prior to spiking and stability of the cards);
- Application of the IS to the samples;
- Sample dilution procedures;
- Spot homogeneity.

For the first four areas, a team consisting of experts from six to eight companies, each set out to design experiments to further document the scientific relevance of the (real or perceived) issue with respect to the acceptability of DBS in a regulated bioanalytical environment. Since spot homogeneity would be investigated as part of at least two of the other four identified areas (effect of the hematocrit and stability), the group agreed not to form a separate team for spot homogeneity.

In addition, all participating companies also committed to share blinded or nonproprietary data from ongoing experiments in their companies on the areas of focus as needed. Considering that the experiments could be executed in up to six different laboratories, the teams recognized that in order to draw valid conclusions from any planned experiment, they would need clear agreements on the experimental design. To prevent the inherent risk that such a standardization of experimental design would bias the results in one or the other direction, the teams took considerable time to agree on assay formats and compound selection. The outcome of these discussions was a set of well-designed experiments on multiple proprietary and nonproprietary compounds from a variety of chemical classes. With this paper, we are

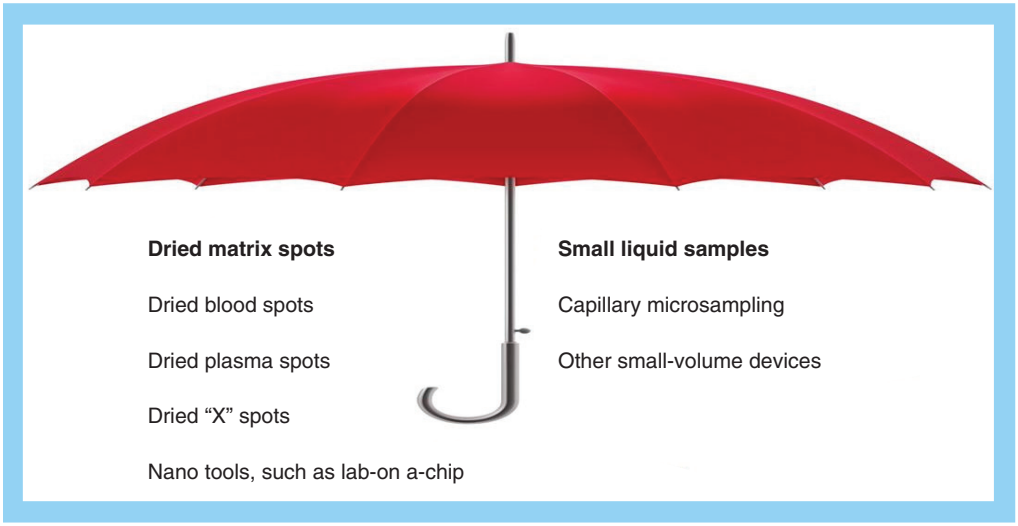

Figure 1. Dried blood spots versus microsampling.

providing a summary of the results from teams 1 and 3 and of the combined results that were gathered on spot homogeneity as an update of our previous recommendation from 2011. More detailed feedback of the results and individual conclusion from each of the teams' experiments is reported in separate papers [6,7,8].

Communication

To keep the scientific community abreast with progress of the consortium, a first status update was presented at the 4th EBF Open Symposium on 18 November 2011 in Barcelona (Spain) [9].

From fall 2011 onwards, all teams started with the execution of the agreed experiments related to their team goals. The team leads reconnected on a regular basis to ensure all teams remained on track, discuss emerging questions or uncertainties and share initial results. The data were discussed with all the team members at a second workshop held on 14 June 2012 in Brussels (Belgium) [10]. The review of the results triggered a few additional studies, especially related to the effect of aging of the spots and storage stability of the spotted cards. An executive summary of the results discussed in Brussels was presented at the 5th EBF Open Symposium on 15 November 2012 in Barcelona [101].

■ Integrating the conclusions on hematocrit, IS addition & spot homogeneity

Overall, the experimental conclusions from the different teams confirm that the issues that were the basis of our work are real. This should not be a surprise, since most experiments were designed to investigate and better document earlier reported and recognized potential issues with the technology.

In presenting our conclusions, we need to acknowledge the fact that we homed in on bioanalytical issues to expose and visualize the issues more clearly. This can lead to an increased or exaggerated feeling of discomfort with the technology. Nevertheless, the frequency and extent of results that do not meet the acceptance criteria we are used to in regulated bioanalysis for small molecule bioanalysis [11,102] requires the attention from the bioanalytical community to carefully reflect on the use of DBS in regulated, and in extension also nonregulated bioanalysis. In what follows, our recommendations are focused on the use of the technology for PK studies for small-molecule LC–MS/MS assays using currently routinely used treated and untreated cards.

■ Plasma versus blood

Prior to discussing the results of the different teams, it is appropriate to highlight one of the downstream consequence of DBS: a renewed interest in the discussion on the use of plasma or blood as the matrix to document the PK of compounds.

This discussion also reopened the discussion in industry stating that any changes of the hematocrit also impacts the plasma or serum volume in traditional assays and, thus, the plasma/serum concentrations prepared from these blood samples. This is true, but not the key area of the challenge of DBS with hematocrit changes. The impact of the hematocrit changes on the performance of a DBS assay is different compared with above volume changes. For DBS, the effect of hematocrit changes introduces an additional covariant. When spotting blood on a card, the hematocrit determines how the blood behaves on the card. Qualitatively, we see a wider and/or faster distribution over the surface with lower hematocrit ('thinner blood') resulting in a bigger spot size for blood with a low hematocrit. These different spot sizes for identical volumes of blood spiked with different hematocrits introduces bioanalytical effects, which cannot be simply explained by the spot size. These effects described and concluded on in this paper cause a number of challenges that are currently not yet fully understood or resolved by the bioanalytical community.

The bioanalytical experience with plasma assays is many orders of magnitude larger than that with whole-blood assays. A head-to-head comparison of whole-blood assays and DBS assays or of dried plasma spots versus DBSs is perhaps a better starting point of a back to back technology comparison. That kind of comparison will probably reveal the issues related to liquid versus dried matrix or blood versus plasma assays in a more comprehensive way.

Last but not least, the industry (and regulators) are also more experienced with plasma PK versus blood PK. The Emmons paper did certainly give some crucial insights into plasma and blood-level relations from a PK perspective [12]. No comparable consideration is yet available for DBS.

■ Individual conclusions & recommendations for hematocrit

As mentioned above, our experiments were designed to document the impact of hematocrit on the accuracy and precision of blood concentrations generated using

the DBS technique from multiple angles. These bioanalytical effects are the core of the discussions in the bioanalytical community, and should not be confused with the PK effect mentioned above for plasma. It is imperative for the development of the technology that the bioanalytical community fully investigates all aspects of the impact of hematocrit changes for DBS analysis.

Our experiments show that hematocrit changes remain the single most important parameter defining compound behavior and DBS assay performance. Referring to the focused experiments performed by the EBF teams as well as data from other groups, the overall impact of hematocrit changes on spot formation, spot homogeneity, accuracy and precision and recovery in both fresh and aged spots is significant. In addition, these effects are compound dependent, which makes documenting and managing them an integral part of the method establishment of a compound and not of the use of a card type or the technique in general. We tested the contribution of clinically relevant hematocrit changes [13] on the above parameters for a broad range of compounds. Concluding from the supporting paper [5], hematocrit changes have a significant impact on the accuracy and precision of the back-calculated concentration of analyte. As a reference point, all concentrations at different hematocrits were normalized against the analyte response at 45% hematocrit. The impact of hematocrit values ranging from 20 to 70% on the response compared with 45% hematocrit, which is a measure for the inaccuracy of the assay, was beyond acceptable values for regulated bioanalysis for many compounds and for hematocrit changes, which are realistic in a clinical setting. Similarly, when comparing the relative response and different clinically relevant hematocrit values for aged spots to a normalized value of freshly extracted spots for the same hematocrit value, again these changes in response impact the accuracy of the assay beyond acceptable values for regulated bioanalysis, albeit to a lesser extent. A potential explanation is that recovery can change upon aging of the spots, depending on the analyte, thus impacting the absolute value measured when a method is not optimized towards extraction recovery of the aged spots.

The above results are not new to bioanalytical scientists. But when discussed in the peer community, these effects are often minimized as long as they do not impact the result beyond ±15%, as this is considered to still fall within the normal acceptance criteria of a bioanalytical assay. However, we should emphasize that the bias introduced by hematocrit differing from the hematocrit of the blood used for validation in most cases results in an incorrect concentration on which to apply the inaccuracy and imprecision of the bioanalytical method and not interpreted as being part of this. This error may be small/irrelevant and not affecting the outcome of the study for some compounds or small hematocrit changes, but they can exceed 20% if the compound is susceptible to the impact of hematocrit changes and/or the hematocrit value of the incurred samples differs significantly from the values used during the validation. Considering that the potential magnitude of the error is both compound and hematocrit dependent, this magnitude will require upfront documentation during method establishment. In addition, it may result in documenting the hematocrit values of incurred samples.

How do our findings on the impact of hematocrit changes influence the method establishment of DBS assays? Since we have an assignable cause of the bias introduced (i.e., hematocrit changes and aging of samples compared with hematocrit value/age of validation samples and calibration samples), it will be possible to accurately document these effects on assay performance as part of the assay validation. However, the resulting consequences on requirements for method validation, and in continuation on applying the assay for study samples, may imply that the resources needed to document the impact of hematocrit changes makes method establishment tedious and costly in comparison with the liquid assay method validation requirements. For example, in order compensate for the effects of hematocrit changes and aging on the accuracy of the assay, it may be necessary to include validation experiments at discrete hematocrit values reflecting the boundaries of hematocrits anticipated in the study and ensure the assay gives the same result, the same extraction recovery in fresh and aged spots at different hematocrit values.

Patient/animal data on the extent and variability of hematocrit changes will be needed to guide the required validation experiments. As a consequence, bioanalysts should have access to the anticipated hematocrit values and later on, at the time of analysis also the actual hematocrit values per sample or hematocrit boundaries of the study samples. This in turn raises the questions: do we need to have a validated assay

for hematocrit determinations available as well? Will (clinical) sites be equipped to measure this for regulated bioanalysis purposes and what is the impact on the resource needed during sampling?

In addition to, and as a consequence of, the above observations, bioanalytical laboratories may need to have the availability of pre-spotted (aged) CAL and QC samples mimicking the age and/or hematocrit value (or boundaries) of the incurred samples.

All of the above may not be needed for some applications or compounds, but in general, the impact of hematocrit changes on the performance of the DBS assay can create an undesirable and resource demanding validation exercise compared to an equivalent liquid assay. It requires a careful balance of the potential advantages such as patient comfort, sample handling or 3R to compensate for these analytical requirements

Individual conclusions & recommendations

■ IS application

Our evaluation of the application of the IS to the DBS samples shows that prudence is required here as well. It was already known that the current 'best practice' of applying the IS in solution together with the extraction solvent to the punched sample spots was more in line with external standardization. Indeed, this process of adding an IS in solution may not cover for all the desired features of an IS, such as mimicking the analyte recovery from the punched filter paper. Other techniques have been or are being investigated to overcome this problem, with none of them yielding solid results to date. As described in the paper from van Baar *et al.*, we compared the above 'best practice' with several other techniques of applying the IS [7]. All proposed procedures appeared to have their analytical and procedural advantages or disadvantages. Referring to the results and discussion from this paper and the proceeding conclusions, with the exception of the impractical method of adding the IS to the (incurred) blood sample prior to spiking, no other tested method of applying the IS, including the current 'gold standard', gave satisfactory results to comply with current expectations as required in regulated bioanalysis for all the compounds tested. In line with the observations for hematocrit, we see a compound-dependent impact on the accuracy and precision. The observations from the IS team should boost the awareness in the bioanalytical community of the impact of process and compound dependency of applying the IS for DBS. As for the findings of the hematocrit, our experiments indicate that focussed experiments should be executed during method development and subsequently in method validation to document the IS behavior for any particular compound and any particular mode of application. We acknowledge that extraction recovery was not fully optimized for our experiments and we suggest as part of our recommendation to develop an assay where extraction recovery is constant under all conditions (e.g., different hematocrit and aged vs fresh).

In line with and in addition to our conclusions on hematorit in the above paragraph, most of the observations for the IS application for any chosen process will probably be resolvable, but that will lead to increased time/cost/resources needed for full validation of the DBS assay compared with a liquid assay.

■ Spot homogeneity

Spot homogeneity was difficult to separate out as a distinct parameter because it is influenced by many factors. As for hematocrit, and mostly driven by it, spot homogeneity will impact the bioanalytical strategy for DBS altogether: spotting, selection of cards-type, size and/or coordinates of where to punch. The conclusion from the stability team and hematocrit team on spot homogeneity, either originating from focused experiments to visualize spot homogeneity using radioactivity or from experiments where spot homogeneity was gathered using LC–MS/MS of multiple spots regionally punched across one sample, provide a clear view of potential issues related to spot nonhomogeneity.

In conclusion, spot homogeneity, either visualized using radioactive spotting or as a relative measurement expressed as center/perimeter ratio is influenced by both the card type and hematocrit to a level it may impacts the accuracy and precision to fall outside the current acceptance criteria for regulated bioanalysis. In contrast with the recommendation for hematocrit or IS addition, it may be more difficult to include experiments on spot homogeneity in validation. At first sight, there may be a simple way out of the spot nonhomogeneity issue, by taking the complete spot instead of small punches from the spot. We should face the downstream

bioanalytical consequences of such a strategy: it will be difficult to perform any reanalysis unless we have a second spot. Do we currently have enough data to conclude that any second spot on the same card is identical to the first spot? How to proceed with ISR?

Also, additional bioanalytical challenges are introduced: it will require the clinical laboratory to sample and spot an accurate blood volume at the bedside. This is certainly not impossible, but will add a next level of complexity to any (pre)clinical study that should be covered during validation.

A separate conclusion on spot homogeneity may be difficult to make, since it builds on many factors. As such, the overall effect leading to spot inhomogeneity may be the parameter, which is less easy to control or document.

Effects of aging

We need to acknowledge that the current experience in industry is still relatively limited with respect to storing DBS cards for a significant time after sampling (6 months or more). Taking into account that we already see the impact of stored/aged spots under controlled circumstances as part of our experiments, we need to be careful and need more data on the impact of prolonged storage, including the potential effect temperature or relative humidity may have. This may especially be the case if the technology is applied in longer-duration Phase III clinical trials where storage conditions may surpass 6 months, and where there is still limited experience with storage variability and compliance. In addition, and not investigated by the EBF, we need to acknowledge that aging may introduce superimposable effects, which may be difficult to separate. Failed long-term stability experiments in DBS may be caused by compound degradation, changes in extraction recovery upon storage or a combination.

Overall conclusion

In conclusion, and supported by the additional data that were generated by the EBF DBS/microsampling consortium, we have updated our 2011 recommendation on the use of DBS in regulated bioanalytical environment as follows: parameters impacting the performance of a DBS assay investigated with respect to spot homogeneity, hematocrit changes and IS addition indicate that significant efforts will be required to validate a DBS assay towards currently agreed acceptance criteria for small-molecule-regulated bioanalysis. Most, if not all, of the challenges we observed have an assignable cause and will be scientifically manageable. The number and extent of additional experiments required may either be impractical or not affordable compared with validation of liquid assays. As a consequence, DBS will probably not be a competitive tool to be used as a generally applicable technology in regulated bioanalysis in the short term. Nevertheless, if documented appropriately, the observed issues should not preclude the use of DBS in those cases where there is no viable or preferable alternative to DBS (e.g., impossibility to store or ship liquid samples).

We do not make final conclusions on the impact of spot aging of DBS assay performance, but data from our experiments suggest that more investigation is required to better understand the effects of prolonged storage DBS samples.

Other parameters may have an important impact on the quality of the data in DBS. In our recommendation we focused on what we considered important hurdles of the use of DBS in regulated bioanalysis, as highlighted in our recommendation of 2011 and identified by the DBS teams. We did not touch on aspects such as blood/plasma ratios or poor spot quality as part of unacceptable spotting processes in a (non) clinical laboratory.

As an industry, we should look carefully at how we assign acceptance criteria for new technologies. Using the example of DBS, where we rapidly adopted acceptance criteria from chromatographic (small-molecule) assays, it may be beneficial to widen the acceptance criteria from 15 to, for example, 20%, provided this does not affect patient safety. Having less stringent criteria will not necessarily remove the need of additional validation experiments as mentioned in this paper, but could alleviate or remove some downstream consequences of a method allowing slightly wider accuracy or precision (e.g., no need to measure hematocrit values in study samples, if for instance, the impact of hematocrit changes does not exceed a certain value such as 10% across a range of clinical relevant hematocrits).

Although this is not a bioanalytical decision, we would like to re-emphasize that a pivotal downstream consequence of the choice for DBS is that PK will have to be evaluated in blood. This needs a well-documented upfront evaluation (at the start of a project or during protocol writing) on the choice of blood. In

case either plasma or blood concentrations are generated throughout a project, a decision is required on how to bridge of plasma and blood PK as part of the life cycle of a project.

We would continue to consider DBS as a developing tool and look forward to innovations that can bring a better balance in the advantages of the technique versus the its current limitations.

We should be careful not to copy any conclusion on DBS to other microsampling techniques yielding a liquid plasma, serum or blood sample,

Finally, the learnings from DBS should stimulate continued critical scientific thinking on other techniques for the benefit of the patient.

Future perspective

EBF considers DBS as a developing technology and awaits further innovations and improvements to better balance the advantages of the technique versus its current limitations. As a consequence, we do not see an immediate use of the technology as a general alternative for current liquid (plasma, serum or blood) assays. However, if documented appropriately, the current hurdles should not preclude the use of DBS in those cases where no viable alternative to DBS is available (e.g., impossibility to store or ship liquid samples). Moving forward, the scientific community should be careful not to copy any conclusion on DBS to other microsampling techniques yielding a liquid plasma, serum or blood samples.

Disclaimer

The views expressed in this article are those of the European Bioanalysis Forum and do not necessarily represent the views of its individual member companies.

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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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Executive summary

- The European Bioanalysis Forum concludes that we are not yet ready for the use of dried blood spots (DBS) as a general tool for regulated bioanalysis in the short term.
- Most, if not all, of the challenges we observed on spot homogeneity, hematocrit changes and IS addition have an assignable cause and are scientifically manageable.
- To overcome the challenges, significant efforts/experiments are required to validate a DBS assay towards current acceptance criteria for regulated bioanalysis. These additional experiments may be impractical or not affordable compared with validation of liquid assays.
- Sparse data demonstrate an impact of spot aging suggest that more investigation is required to better understand the effects of prolonged storage.
- A pivotal downstream consequence of DBS is that PK/TK/PD will be evaluated in blood. In case both plasma/blood data are generated in a project, a strategy on how to bridge PK data as part of the life cycle of a project is needed.
- Adopting acceptance criteria from regulated bioanalysis chromatographic assays for DBS may have been premature. Wider acceptance criteria, not affecting patient safety, should be considered. This would not remove the need of additional validation experiments, but could simplify or remove some experiments or consequences.

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