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Authors: Verstraete, J., Boffel, L., & Stove, C.

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Dried blood microsample-assisted determination of vitamins: recent developments and challenges

Jana Verstraete, Laura Boffel, Christophe Stove*

Laboratory of Toxicology, Department of Bioanalysis, Ghent University, Ottergemsesteenweg 460, B-9000 Ghent, Belgium

Corresponding Author

* Email: christophe.stove@ugent.be. Tel.: +32 9 264 81 35.

Abstract:

Although vitamin deficiencies are a well-known issue, monitoring of the vitamin status and diagnosis of vitamin deficiencies in remote regions is a complicated task. Dried blood microsampling, as an alternative sampling strategy, could offer a solution to the several drawbacks related to conventional venipuncture. Although highly relevant, the number of microsampling procedures that has been developed for assessing vitamins in dried blood samples is rather limited, indicating the challenging nature of the subject. This review discusses several challenges inherent to vitamin analysis in microsamples, thereby covering topics including sampling strategy, hematocrit (Hct) effect, sample preparation, calibration, assay sensitivity, stability, and clinical interpretation of the results.

Key words:

Vitamins; dried blood microsampling; dried blood spots; volumetric absorptive microsampling; quantitative analysis

1. INTRODUCTION

Vitamins are essential micronutrients for humans, as they play a crucial role in the production of hormones, enzymes and substances required for the development and growth of the human body (1). Vitamins are classified into two major groups: water soluble vitamins (the eight B vitamins and vitamin C) and fat soluble vitamins (vitamins A, D, E and K). As the endogenous synthesis of vitamins is absent and/or limited in the body, the body requires daily supplementation of these micronutrients through intake of vitamin-rich foods, except for vitamin D, which is synthesized from its precursor when the skin is exposed to sunlight (1, 2). Although only 'micro' amounts of vitamins are required for optimal growth and development, inadequate intake and, regarding vitamin D, insufficient exposure to sunlight, may lead to vitamin deficiencies.

Although vitamin deficiencies are a major health problem, affecting more than 25% of the global population, monitoring of the vitamin status in remote regions is a complicated task. Since the early symptoms of vitamin deficiencies are often nonspecific, early diagnosis relies on the determination of the vitamin(s) or other markers in blood, serum or plasma (3). Blood is commonly collected using conventional sampling procedures, which is time-consuming and invasive and therefore less suited in remote regions due to the absence of medically trained personnel and hygienic measures. Moreover, transport typically requires cooling and dedicated logistics. Most of these issues can be overcome by the analysis of dried blood microsamples (4-6).

Dried blood microsampling is a minimally invasive sampling procedure, where small volumes of blood (< 50 µL) are collected by a simple finger or heel prick and collected on a dedicated absorbent material – often filter paper, with the formation of dried blood spots (DBS). Due to the simplified collection technique, microsampling does not require dedicated clinical staff and represents limited biohazard, which is relevant in remote regions where HIV and malaria may be highly prevalent. In addition, in dried samples the stability of compounds is often improved (7). Consequently, microsamples can generally be transported and stored under ambient conditions. DBS are also amenable to fully automated analysis, allowing an increased sample throughput (8-10). Therefore, DBS sampling may facilitate large-scale epidemiological studies in remote areas (11, 12).

2. DETERMINATION OF VITAMINS IN DRIED BLOOD MICROSAMPLES

Despite the many advantages associated with the analysis of vitamins in dried blood microsamples, the number of microsample-based methods for the determination of vitamins is rather limited (Table 1), indicating the challenging nature of the subject. Therefore, based on literature and the authors' own experience, this review discusses an overview of the challenges that may be encountered during the set-up of methods for the determination of vitamins in microsamples, as well as (potential) strategies to cope with these challenges. This review solely focuses on the direct determination of the vitamins in dried blood microsamples. While the analysis of other biomarkers (which may e.g. indicate a vitamin deficiency) is beyond the scope of this review, several points of attention will also be applicable to those.

2.1. INFLUENCE OF SAMPLING STRATEGY

Parameters related to the initial sampling should be evaluated in an early stage of method development to avoid potential pitfalls in a later stage. For a detailed and thorough overview of the recommended aspects that should be evaluated during the set-up and validation of a DBS (or other dried blood microsample)-based method, we refer to the official international guideline by Capiau *et al.* (13).

Punch size and punch site

DBS analysis can either be done using whole blood spots or partial DBS punches. For whole blood spot analysis, a fixed volume of blood is volumetrically applied onto filter paper, whereafter the whole spot is punched out for analysis. Consequently, the hematocrit-(Hct) area-based bias, which is a heavily debated issue in quantitative DBS analysis and will be discussed in the next section '2.2 Hct-effect', is avoided. However, the deposition of a fixed sample volume onto DBS cards requires the accurate use of microcapillaries and pipets, which complicates the sampling procedure and limits the possibility for home-sampling (14, 15). Several solutions have become available or are still in development, such as HemaPen®, Capitainer® and dried plasma spot (DPS) systems (15-24). Currently, the most used approach involves the generation of subpunches from non-volumetrically applied DBS, as this allows a practical sampling procedure. However, the analysis of subpunches implies that parameters such as DBS volume and (in)homogeneity, in addition to the hematocrit (Hct), need to be taken into account. Recently, Majda *et al.* developed a strategy to calculate the blood volume in a subpunch based on the surfaces of the whole blood spot and subpunches (25). However, as the surface depends on the spotted amount of blood, such approach is only feasible when using volumetric applications, which is again less ideal in a

home-sampling setting. Finally, the punch size (e.g. 3 mm) may be determined by the analytical sensitivity that can or needs to be achieved.

O'Broin *et al.* compared folate (vitamin B_9) levels in entire DBS samples and 6.35 mm DBS subpunches, punched both centrally and peripherally (26). As no significant differences in folate levels were observed, the authors concluded that folates were homogeneously distributed in the samples. Nevertheless, it is important to fix the position of the punch during the entire method validation and application to ensure reproducibility. Preferably, sampling of central punches is recommended as they can be sampled more reproducibly (13), which was also observed by Kvaskoff *et al.* when measuring vitamin D in subpunches (27). A few years later, O'Broin *et al.* investigated the distribution of serum folate (SF) in dried serum spots (DSS) (28). In contrast to their DBS method, they observed significantly higher SF concentrations in DSS punches taken at the edge compared to central DSS punches. Therefore, analysis of complete, volumetrically applied DSS, was preferred, to avoid variability due to the punch site.

For both vitamin A and D, a heterogeneous distribution in DBS samples was demonstrated by Erhardt *et al.* and Kvaskoff *et al.,* respectively (27, 29). Higher concentrations were measured at the periphery compared to the center. Similarly, a higher density of sodium, which is highly present in plasma/serum, was observed by Erhardt *et al.* at the periphery of a DBS sample, while the concentration of erythrocytes was higher at the center of the spot (29). This latter phenomenon (chromatographic effect) is the result of the easier migration of serum/plasma through the filter paper compared to the erythrocytes. Therefore, the inhomogeneity of both vitamin A and D in a DBS sample could be explained by the high presence of the vitamins in plasma/serum. A few years later, Kvaskoff *et al.* re-evaluated the distribution of vitamin D in DBS samples. Remarkably, this time, concentrations did not depend on the punch site (30). The authors hypothesized that differences in hydration or temperature may have influenced the analyte distribution. Therefore, the conditions applied at the time of drying must be similar during method development, validation and application.

Type of filter paper and spotting volume

Besides the punch site and punch size, the type of filter paper may influence the results obtained from DBS samples as well. Therefore, different types of filter paper should not be used interchangeably, unless equivalence has been demonstrated. The latter was nicely exemplified by Kvaskoff *et al*., who investigated two different types of filter paper (Whatman 903® and FTA®) and found that for depositing the same volume of blood, the spot area on FTA® paper was significantly smaller compared to Whatman

903® paper (27). In addition, peripheral punches from FTA® were also heavier. The authors concluded that on FTA® paper a larger volume of blood was absorbed per surface area. Consequently, 25-hydroxy vitamin D_3 ($25OHD_3$) levels on FTA® paper, quantified based on calibrators prepared on Whatman 903® paper, were overestimated, emphasizing the importance to use the same type of filter paper for the preparation of both calibrators and study samples. In addition, as spotting volumes below 50 µL led to significantly lower $25OHD_3$ levels (27), possibly owing to incomplete saturation of the filter paper, the authors recommended to spot blood volumes of minimally 50 µL. Typically, blood volumes, ranging from 20 µL to 70 µL, can be collected, therefore 50 µL, although already substantial for a drop of blood, can still be considered as a 'patient friendly' sample volume (13). Visual control over the collected blood volume can be achieved by using two concentric circles, with the requirement that the inner circle should be filled, while the outer circle should not be exceeded (31).

2.2. HCT EFFECT

The Hct (i.e. the volume proportion of red blood cells in a blood sample) determines the viscosity of blood, and, hence, the spreadability of the blood on filter paper. This results in varying spot sizes when a same volume of blood with different Hcts is applied onto filter paper. This Hct-based area bias needs to be taken into account when performing partial-punch DBS analysis (14, 15). As the volume of blood in a subpunch will vary depending on the Hct, analyte levels in subpunches from DBS samples with a higher or lower Hct (compared to the Hct of the blood to generate the calibration line) may be over- or underestimated, respectively. For analytes that are primarily (or exclusively) residing in serum, the inverse may be observed, as less serum will be contained in a high-Hct DBS subpunch, which may prevail the effect of decreased spreading. In line with this, Jensen *et al.* found that the concentrations of the highly plasma protein bound vitamin D were under- and overestimated in DBS subpunches from blood with a higher Hct (0.60) respectively lower Hct (0.30) (32). It should be noted, though, that it cannot be excluded that a Hct-dependent extractability bias may be responsible for the observed difference, in which an analyte is more easily extracted from a DBS prepared from blood with a low Hct (and *vice versa*). To correct for the variable plasma volume, depending on the Hct, Erhardt *et al.* determined the sodium content to calculate the plasma volume in a DBS sample, assuming a constant sodium concentration of 140 nmol/L in plasma (29). Another method using a blood electrolyte was developed by Capiau *et al.*, who demonstrated that the Hct of a dried blood sample can be predicted based on the K^+ content (31, 33, 34). However, as these strategies have to sacrifice part of the sample, Kadjo *et al.* and more recently, Dvorak *et al.* measured the electrical conductivity of a DBS extract as an equivalent

nondestructive method to determine the exact volume of a DBS sample (35, 36). Nevertheless, as mentioned by Velghe *et al.* (15), this technique is based on the strict regulation of electrolyte levels in the blood and can therefore not be used in patients with deviating electrolyte levels. Other nondestructive, and even noncontact Hct prediction methods have been recently developed, using diffuse reflectance or near infrared spectroscopy. These allow preservation of the entire DBS and do not require any sample preparation (37-39).

Alternatively, entire blood spot analysis may be used to eliminate the Hct-based area bias. In addition, alternative microsampling techniques have been developed (15), such as volumetric absorptive microsampling (VAMS). VAMS devices contain a polymeric tip that absorbs a fixed volume of whole blood, independent of the Hct (40, 41). In 2017, Kopp and Rychlik described the determination of 5-methyltetrahydrofolic acid (5-MTHF) in VAMS samples (42). Even though analysis of whole DBS and VAMS samples eliminates the Hct-based area bias, the extraction efficiency of analytes from dried samples may still be impacted by the Hct. As mentioned before, analytes may be less efficiently extracted from blood with a higher Hct, due to the higher relative amount of erythrocytes, which may form an extraction barrier in dried microsamples (14, 15, 41). Since the internal standard (IS) is typically only added during the extraction step, it is unable to correct for variations in recovery. Remarkably, from the methods listed in Table 1, only two research groups described the evaluation of the influence of Hct on the recovery. Both Jensen *et al.* and Mathew *et al.* found Hct-independent recoveries for 25-hydroxy vitamin D (25OHD) and thiamine diphosphate (TDP), respectively (32, 43). If a Hct-dependent recovery would occur, several approaches to avoid the Hct-related recovery bias have been described, such as the inclusion of a sonication step or the co-extraction of the IS from dried microsamples (44, 45).

Furthermore, blood with different Hcts can be considered as a different matrix, as the relative amount of plasma to erythrocytes differs. Therefore, varying Hcts may potentially affect the matrix effects (ME) in methods using LC-(MS/)MS, and, consequently, may influence the accuracy of the analytical results if no suitable stably labelled IS is included to compensate for possible matrix effects (14). For vitamin D determination in DBS, all published LC-MS/MS procedures made use of a deuterated analogue (ranging from d_3-d_6) of vitamin D as IS (Table 1).

In addition, there is also a physiological aspect to the Hct which relates to the blood/plasma (B/P) ratio of an analyte (14). This is of high importance since microsample-based data are usually converted to equivalent plasma levels, as reference intervals are commonly established in plasma or serum. The degree to which the Hct influences the B/P ratio depends on whether an analyte is predominantly

residing in the plasma, potentially bound to plasma proteins, or is mainly present in the erythrocytes. Since vitamin A and D are completely excluded from red blood cells and are mainly bound to plasma proteins, these vitamins have a low B/P ratio and, for a given plasma concentration, this ratio will decrease with increasing Hct. Contrarily, for vitamins mainly present in the erythrocytes, such as polyglutamylated folates and the active metabolite of thiamine, thiamine diphosphate (TDP), the B/P ratio will increase with increasing Hct. Consequently, the Hct may influence the correlation between DBS and plasma concentrations. This latter topic is more elaborately discussed in section 2.7. 'Clinical interpretation'.

For a more detailed in-depth overview of the Hct issue in dried blood microsamples, and how to cope with it, we refer to De Kesel *et al.* and Velghe *et al.* (14, 15).

2.3. SAMPLE PREPARATION

In addition to the sampling aspect, the use of dried microsamples can also be seen as a strategy to facilitate sample preparation, as analytes can be selectively extracted from the card or device, consuming lower amounts of reagents (14). Sample cleanup can be established by selective extraction, or simple protein precipitation (PP), possibly combined with more elaborate procedures such as liquid-liquid extraction (LLE) or solid phase extraction (SPE).

Most methods presented in Table 1 applied a simple PP, using organic solvents such as methanol (MeOH) or acetonitrile (ACN), followed by LLE with hexane, to extract the lipophilic vitamin D from a DBS sample. However, PP and LLE have been reported to be insufficient to remove phospholipids (PLs), which are typically co-extracted from blood when using nonpolar extraction solvents (30). Kvaskoff *et al.* performed, in addition to PP with ACN, a solid phase ion-exchange sample cleanup using ZrO_2/TiO_2 medium (30). This medium selectively binds the phosphate groups of phosphatidylcholines, which are the major compounds of PLs. This additional purification step led to an overall reduced PL content and enhanced vitamin D signal. Although this involved an additional step during sample preparation, multiple samples could be processed in parallel using a 96-well format. Zakaria *et al.* described the use of supported liquid extraction (SLE) to reduce PL interference (46). Although the authors observed an increased sensitivity, it was not formally demonstrated that this was really caused by the removal of PLs, as hexane, in which PLs are co-extracted, was still used as the elution solvent. It might be that the increased sensitivity was caused by the increased extraction of vitamin D using SLE, rather than by the removal of PLs.

In contrast to vitamin D, none of the methods measuring vitamin A described interferences by PLs, even though vitamin A is also a lipophilic vitamin. However, retinol, bound to its binding protein (RBP), was initially eluted from the paper using polar solvents, followed by denaturation of the proteins and liberation of retinol by an organic solvent. Therefore, the PLs, due to their lipophilic character, were likely not co-extracted with the RBP complex and hence, did not interfere with the analysis of retinol. As retinol is sensitive to oxidation, extraction solvents contained antioxidants such as ascorbic acid (AA), diethylenetriamine pentaacetic acid (DTPA), butylated hydroxytoluene (BHT) or hydroquinone (29, 47-50).

Unlike vitamin A and D, hydrophilic vitamins cannot be extracted using pure organic solvents. Therefore, trichloroacetic acid or a mixture of MeOH/H_2O were described to precipitate the proteins within the sample, while still ensuring extraction of the hydrophilic vitamins (B_1, B_2, B_6) (43, 51, 52). Folates (B_9) were extracted using an aqueous solution containing AA as antioxidant, as folates are sensitive to oxidation. Consequently, the advantage of DBS or VAMS as a tool to facilitate sample preparation was lost and additional sample purification was needed, such as SPE or filtration. Moreover, by using aqueous extraction solvents, samples cannot be concentrated via evaporation and reconstitution, which further challenges assay sensitivity.

In contrast to manual sample preparation, automated extraction, which can be both off- and on-line, allows high sample throughput and reduces the risk of human errors (8-11, 53). Recently, Lin *et al*. developed an automated on-line extraction method for the determination of riboflavin (vitamin B_2) in DBS (52). Riboflavin was extracted from DBS by pumping the extraction solvent (MeOH/H_2O (7/3)) through the filter paper, whereafter the eluent was directly injected into the LC-MS/MS system. The extraction platform was cleaned, followed by extraction of the next sample, which overlapped with the LC-MS/MS analysis of the preceding sample. Therefore, sample processing and analysis could be reduced to the time required for LC-MS/MS analysis, enhancing throughput.

2.4. ENDOGENOUS ANALYTES

The endogenous presence of vitamins in whole blood poses a major challenge during the entire method set-up. Important validation parameters, such as selectivity, determination of the Lower Limit of Quantitation (LLOQ), recovery, ME and the set-up of a calibration curve, typically use a blank matrix (54, 55), which is hard to achieve with native samples.

Calibrators are ideally prepared in the same matrix as the study samples to avoid differences in extraction efficiency and ME. However, it is very difficult, if not impossible, to obtain vitamin-free blood. Different approaches to overcome the issue of endogenous analytes have been described, e.g. standard addition and the use of an adapted (e.g. washed or charcoal-treated) or surrogate analyte/matrix (56-58). Both standard addition and the use of a surrogate matrix have been widely used to quantify vitamins in microsamples. Alternatively, Fallah *et al.* constructed a calibration curve based on the endogenous retinol plasma concentration: DBS calibrators were prepared by replacing plasma with artificial plasma to obtain lower retinol concentrations (48). However, consequently, the highest calibrator is limited to the endogenous analyte level.

Standard addition is one of the most commonly used approaches to quantify vitamins in microsamples. Briefly, standard addition is performed by spiking increasing concentrations of the vitamin of interest to aliquots of the biological matrix, from which DBS or VAMS samples are prepared and a standard curve can be constructed. This approach is not compatible with the *direct* application of a drop of blood from the fingertip onto filter paper. As an alternative, Huang *et al.* spiked increasing concentrations of retinol standard solution to already prepared and dried DBSs, after which the spiked spots were dried (49). However, this approach requires caution as there should be no difference in extraction efficiency between calibrators and native samples, which is of relevance for the highly protein-bound retinol. A major benefit of standard addition is that a calibration curve is set up in the same matrix as the study samples, avoiding differences in ME. However, as the endogenous vitamin content is incorporated into the calibrators and may vary between different matrix sources, it may be less feasible to fix an assay range (59). Moreover, the analyte's endogenous presence complicates the evaluation of the LLOQ and hence the determination of low analyte levels (60).

In addition to standard addition, a surrogate matrix-approach, in which the authentic matrix is substituted, has been applied to circumvent the endogenous presence of vitamins. Leaney *et al.* evaluated different surrogate matrices (phosphate buffered saline (PBS), PBS with 2% bovine serum albumin (BSA) or 5% BSA and washed erythrocytes with PBS and 2% or 5% BSA) for the quantification of $25OHD_3$ in VAMS samples (61). Remarkably, when pure PBS was used, $25OHD_3$ was retained on the device, as a poor recovery of $25OHD_3$ was observed. Interestingly, Delahaye *et al.* also observed a reduced recovery of paracetamol from VAMS, when applied in pure water (62). This suggests that analyte-polymer interactions, compromising extractability, may take place in the absence of matrix. In the case of $25OHD_3$, the recovery from VAMS samples was shown to be highly dependent on the amount

of proteins present in the surrogate matrix, increasing with increasing protein content. As vitamin D is highly plasma protein bound, the analyte is probably extracted from the VAMS sample attached to plasma proteins (when using an aqueous extraction solvent), indicating the importance of having a sufficient amount of protein in the surrogate matrix, to ensure reliable quantification. Therefore, as vitamin A and D are mainly present in plasma/serum, the surrogate matrix to quantify vitamin A or D commonly consists of washed erythrocytes, mixed with PBS containing BSA or human serum albumin (HSA) (50, 60, 63). Importantly, since vitamin A and D are endogenously bound to albumin, BSA and HSA batches may contain traces of those vitamins. Hence, Leaney *et al.* stressed the importance to screen BSA batches for their low presence of vitamin D prior to use (61). Finally, to demonstrate the suitability of a surrogate matrix, Matuszewski *et al.* showed that the relative ME between different matrices can be assessed by comparison of the slopes of the calibration lines established in the different matrices (64). Zhang *et al.* applied this approach to demonstrate the suitability of a surrogate matrix (washed erythrocytes mixed with PBS containing 2% BSA) to quantify retinol in DBS samples (50).

2.5. ASSAY SENSITIVITY

A major challenge associated with the use of dried blood microsamples to determine vitamin levels is the assay's sensitivity. Due to the low sample volume and low analyte abundance, highly sensitive analytical methods are required. Moreover, when the analyte of interest is mainly present in serum/plasma, as is the case for vitamin A and D, sample volumes are even lower (32). Consequently, current methods are often only able to determine the major vitamin forms such as $25OHD_3$, 5-MTHF and TDP. However, in some cases, such as metabolism studies which aim at obtaining more insight into genetic variants, it is also relevant to look at other (minor) vitamin forms such as 24,25-dihydroxyvitamin D (24,25-diOHD), 1,25-dihydroxyvitamin D (1,25-diOHD), tetrahydrofolate (THF), 5,10-methylenetetrahydrofolate (5,10-CH_2THF), 5-formyltetrahydrofolate (5FoTHF), nonphosphorylated thiamine, thiamine monophosphate (TMP), etc., which are now rarely determined in dried microsamples.

The lipophilic nature and the absence of functional groups that facilitate ionization complicate the MS-based analysis of vitamin A and D (65). Only Zhang *et al.* described an LC-MS/MS method for the determination of retinol in DBS, while others typically used LC-UV detection (29, 47-50). In contrast, vitamin D has commonly been measured via LC-MS/MS (Table 1), with chemical derivatization as the most common solution to improve sensitivity, via increasing its ionization capacity. However, this implies an additional time-consuming step and also complicates chromatography as two stereoisomers are formed. Therefore, several groups circumvented this derivatization step by analyzing whole DBS or

multiple subpunches, thereby increasing the sample size, to reach the required sensitivity (Table 1). This strategy may not be feasible in all instances. Kvaskoff *et al.* increased assay sensitivity by selectively removing PLs via a new sample cleanup procedure (30), while Jensen *et al*. performed 2D-chromatography, allowing to inject a higher sample volume while minimizing ME via online sample cleanup (32).

From the authors' own experience, TDP determination via LC-MS/MS has proven to be challenging due to the analyte's high polarity and ionogenic characteristics. Typically, TDP is determined via HPLC-fluorescence using phosphate buffer as mobile phase, as recently done by Mathew *et al.* (43). However, due to its nonvolatility, this phosphate buffer is incompatible with LC-MS/MS. While 80% of thiamine in blood is present as TDP, nonphosphorylated thiamine and TMP are only present at very low concentrations. Moreover, the latter two thiamine vitamers are mainly present in plasma, which results in an even smaller sample volume available for analysis. Zhang *et al.* obtained sufficient sensitivity for the determination of nonphosphorylated thiamine from DBS by analyzing complete 40 μL DBS and injecting a high sample volume (40 μL) (51).

Kopp and Rychlik described an LC-MS/MS method for the determination of 5-MTHF in DBS and VAMS samples (42, 66). Despite the smaller sample volume of VAMS (10.8 μL) compared to DBS (50 μL) samples, similar LLOQs were attained. This increase in assay sensitivity was mainly attributed to omitting the SPE step during sample preparation of VAMS samples. This SPE step was responsible for 43% loss of analyte level in DBS analysis. Furthermore, the use of a longer column resulted in better separation of the matrix compounds, reducing possible ME, which may decrease the analyte signal.

2.6. STABILITY

The assessment of vitamins in dried blood microsamples greatly depends on their stability during the entire analytical procedure, i.e. drying, transport and storage. Although dried blood microsampling has been described to increase stability, the latter depends on the analyte characteristics and should be evaluated on a case-by-case basis at the different conditions (temperature, relative humidity, time, etc.) representative for the eventual study conditions (13).

For vitamin A and D, acceptable stability was observed at different storage conditions by several research groups. This may be related to their highly protein-bound nature, which may protect them from degradation. Zhang *et al.* evaluated the effect of a protectant, added to freshly prepared DBS, on retinol stability during drying (50). While retinol losses were observed in freshly prepared DBS (> 20%), they

found that treatment with AA or BHT could protect retinol from oxidation and therefore significantly improved stability during drying. This kind of approach, where antioxidants are deposited onto freshly prepared DBS samples, although feasible in centralized sampling settings, are not workable in the context of home-sampling. Alternatively, pre-impregnation of filter paper with the antioxidants may be evaluated. However, such approach may raise additional issues as the impregnated cards come in close contact with the pricked fingertip and may affect the spreading of the blood.

By contrast, thiamine and its phosphate derivatives are amongst the most unstable water-soluble vitamins. Mathew *et al.* evaluated the stability of TDP in DBS and found the analyte to be stable during 8h storage at room temperature (RT) (43). It should be noted that this latter study was performed in India, with ambient conditions of 34°C and 75% relative humidity. Within this time frame, samples should be transferred to -80°C to ensure long-term stability (6 months). Zhang *et al.* evaluated the stability of nonphosphorylated thiamine, riboflavin and pyridoxal-phosphate (PLP) in vacuum treated DBS (51). While thiamine was stable for 15 days of storage at RT, riboflavin and PLP were stable up till 1 year. Furthermore, all analytes were stable during 7-day storage at 40°C and 50% humidity, mimicking the conditions in remote areas. Remarkably, while riboflavin levels decreased > 20% during 2h drying at RT, thiamine and PLP remained stable. Since riboflavin levels remained stable after drying during storage under vacuum, the authors applied an adjustment factor to account for this initial loss in riboflavin content.

O'Broin *et al.* evaluated the stability of vitamin B_{12} in DSS stored for 7 days at 4°C, 20°C and 37°C and found that vitamin B_{12} was stable at all conditions (67). The same research group investigated the stability of total folate in both DBS and DSS (28, 68). While AA negatively affected folate stability in DBS, it improved the stability in DSS. The authors explained this phenomenon by AA's ability to rapidly lyse erythrocytes, with as a consequence less protection against degradation then when folates remain incorporated in the erythrocytes. Folates in untreated DBS were only stable for a few days of storage at ambient temperature, while levels remained stable in DBS stored up till 1 year at -20°C. Folates in DSS were stable for 1 week at 20°C and 2 weeks at 4°C, from which the authors derived its potential for shipment by regular mail. However, actual temperatures may be a lot higher during summer. Zimmerman *et al.* evaluated long-term total folate stability during 9 months at different conditions (69). Folate concentrations substantially decreased when samples were stored at higher temperature (24-27°C) and greater humidity (53-68%). Kopp and Rychlik evaluated the short-term stability of 5-MTHF in both DBS and VAMS samples (42, 66). 5-MTHF in VAMS samples was stable during 2.5h drying at RT.

Moreover, upon storage at -20°C, 5-MTHF was stable for two, respectively three weeks in DBS and VAMS samples.

2.7. CLINICAL INTERPRETATION

Vitamin levels are typically measured in plasma/serum (vitamin A, D, B_2, B_6, B_9 (short-term status) and B_{12}), erythrocytes (vitamin B_9, long-term status) or whole blood (TDP).

To allow the comparison of microsample-based results with existing results from previous studies and cut-off values in plasma/serum or erythrocytes, a conversion of microsample-based results is often required. Alternatively, new microsample-based cut-off values could be established. However, this will be very expensive and time consuming and furthermore, inconvenient for physicians as they are familiar with existing cut-off levels (70). The B/P ratio is an important factor concerning the correlation between dried blood microsample and plasma or erythrocyte concentrations. This ratio is to an important extent determined by a sample's Hct (14). Ideally, the individual Hct of each sample is applied to convert the results from DBS/VAMS samples to equivalent plasma/serum or erythrocyte levels. However, often an average Hct value of the study population is used, e.g. women, men, neonates. Yet, this latter approach is only acceptable when the Hct range of that specific population is quite narrow. Karvaly *et al.* reported wide Hct ranges within a subpopulation; 0.42 (0.25-0.51) for males and 0.4 (0.26-0.44) for females, indicating the inappropriateness of using a single correction factor (71). Furthermore, the utilized average Hct value often differs between methods, even though similar target population may be considered, which further complicates method comparability. For example, within the discussed vitamin D publications, the average Hct applied for women ranged from 0.4-0.43, for men from 0.45-0.47 and for neonates from 0.5-0.6. Makwoski *et al.* and Muller *et al.* even used a single Hct value, independent of the gender (60, 72). Zhang *et al.* and Heath *et al.* set up a model to convert DBS-based concentrations for retinol and 25OHD, respectively (50, 73). The latter two research groups used two sets of DBS samples with their matching plasma/serum samples, a 'test' set and a 'training' set. The test set was used to establish a model describing the correlation between DBS and plasma/serum results, with evaluation of key predictors of vitamin D status (e.g. age, sex, month of sample collection, etc.) for their individual contribution to the model fit. The training set was used to test the model's ability to predict plasma levels from DBS results. Heath *et al.* observed that the use of a sex-specific model did not fully account for differences between DBS and plasma results (73). Even though the Hct is strongly influenced by the sex, there are also physiological variations in Hct. Fallah *et al.* determined the individual Hct levels from a few additional drops of blood using a point-of-care test, the Hemocue B Hemoglobin Photometer (48).

However, this strategy is hard to conceive in a home-sampling context, as every patient would need such a device (14). Alternative methods to determine the Hct of a dried blood microsample can be used, as mentioned in section 2.2. and recently reviewed by Velghe *et al.* (15).

Red blood cell folate (RCF) is recommended to assess the long-term folate status (74). Shreckengost *et al.* determined the hemoglobin (Hb) content of a DBS using the sodium laurylsulfate (SLS) microplate method (75, 76). Consequently, folate concentrations may be expressed as Hb folate (HF) (nmol/g) by dividing the DBS whole blood folate concentration by the measured Hb concentration. Subsequently, the corresponding RCF can be deduced from the HF using either a specific or population-based mean corpuscular hemoglobin concentration (MCHC) value.

Once microsample-derived results are converted to their equivalent plasma/serum or erythrocyte results, the correlation between both methods (alternative *vs.* conventional) should be evaluated to demonstrate the potential of the alternative sampling strategy and the validity of the obtained results (13). To compare two methods, regression analysis (Deming, Passing-Bablok) should be performed, followed by an agreement and bias estimation test (Bland-Altman) (77-79). Although a Bland-Altman plot is shown in most of the discussed publications, this should be accompanied by a correct interpretation of the results, which is often lacking (80). The mean bias should be accompanied by its 95% confidence interval, ideally containing zero. If this is not the case, it should be discussed whether the observed bias is still acceptable, which highly depends on the purpose of the method. Furthermore, the limits of agreement (LoAs), which will statistically cover 95% of the results, should be calculated. The span covered by these LoAs is an indication of the agreement between two methods, and pre-set (clinical) criteria should be applied to discern whether the observed differences are acceptable. For example, for vitamin D, most of the published methods present wide limits of agreement, illustrating high variation of the results. However, Jensen *et al.* showed that all of their sample comparisons fell within the total allowable error (TAE) (32). Factors contributing to the high variation observed in many papers may possibly be the use of a single correction factor or mean Hct value to convert DBS results to equivalent plasma/serum results.

For TDP (vitamin B_1), which is already routinely measured in whole blood, the results obtained from dried blood microsamples should still be compared with those from conventional liquid blood. E.g., as capillary blood consists of a mixture of venous and arterial blood and interstitial fluid, there might be a difference between venous and capillary results. However, such differences were mostly reported for exogenous substances as these require a distribution phase (13).

Ideally, the validity of a particular method can be demonstrated using commercially available secondary (matrix-based) reference materials (SRM) with a known vitamin content. Different SRMs, containing specific vitamins, are commercially available through different institutes. NIST provides reference serum material for vitamin A (SRM 968f, SRM 1950), vitamin D (SRM 972a, SRM 1949, SRM 1950, SRM 2973), vitamin B_6 (SRM 1950, SRM 3950) and folates (SRM 1950, SRM 3949). The National Institute for Biological Standards and Control (NIBSC) offers reference material for vitamin B_{12} (03/178, serum) and folates (03/178, serum; 95/528, whole blood). Ideally, these SRMs resemble the native samples as close as possible (i.e. unadulterated, non-spiked human material) and are compatible with dried microsample analysis. As vitamins B_1, B_6 and B_9 are mainly present in the erythrocytes, reference whole liquid blood material is preferred to evaluate dried blood microsampling methods. However, this material is only available for vitamin B_9 (NIBSC 95/528). Yet, none of the microsample-based folate methods evaluated the application of this blood reference material onto DBS cards or VAMS. For vitamin B_{12}, O'Broin *et al.* spotted the compatible liquid serum reference material IRR 81/563 onto filter paper to evaluate their DSS method (67). Although no compatible reference materials (i.e. because of a different matrix) were available for the determination of vitamin A and D in whole blood microsamples, serum reference material was mixed with washed erythrocytes, after which the prepared whole blood was spiked onto blank DBS cards (32, 50). Also for other analytes, serum reference material has successfully been used to allow the generation of dried blood microsample quality controls to demonstrate a method's validity (81).

It is important to note that blood SRMs are not necessarily compatible with dried microsampling methods, as commutability issues may arise (13, 82). These issues may be caused by (i) effects resulting from lyophilizing and reconstituting blood, (ii) differences in viscosity compared to fresh blood, which may result in incorrect conclusions when a DBS sub-punch is analyzed, and (iii) differences in extraction efficiency compared to native samples. A good way to assess the trueness of calibration and the compatibility of a SRM for a specific dried microsampling method is the parallel analysis of native materials and SRMs, both in their dried and liquid form. If the results for the liquid SRM samples are acceptable, but those for their dried equivalents are not, the SRM may be unsuited for the specific microsample application. The same holds true when the results of liquid and dried SRM match, whilst those of native samples don't (pointing at a commutability issue).

3. CONCLUSION

While dried blood microsampling offers some important advantages to diagnose vitamin deficiencies in e.g. remote regions, the number of published methods in this field is relatively limited, likely owing to the (many) associated challenges. Therefore, we discussed some important issues that may be encountered during the set-up of a method for the determination of vitamins in microsamples.

LC-MS/MS is the method of choice to analyze microsamples. Yet, achieving sufficient sensitivity to determine low vitamin levels in micro-volumes of blood remains challenging. Additional measures such as extensive sample cleanup, derivatization and increase of sample size can be evaluated to attain the required sensitivity. Moreover, the endogenous presence of vitamins complicates their quantification. Although standard addition has been described as a strategy to cope with this latter issue, this approach is not compatible with the direct application of a drop of blood from the fingertip onto filter paper. Furthermore, standard addition challenges the determination of the method's sensitivity or LLOQ. Therefore, alternative procedures, such as the use of a surrogate matrix, should be considered.

Microsample-specific parameters, such as punch site, spotting volume, etc. should be evaluated in an early stage of method development, and should be fixed during the entire analytical process, as they may influence the final analytical results. In general, the use of dried microsamples can be seen as a strategy to facilitate sample preparation, as analytes can be selectively extracted from the card or device, consuming lower amounts of reagents The specific extraction procedure depends on the analyte's characteristics and the aimed sensitivity. More extensive procedures such as solid phase extraction may be recommended when sensitivity issues occur due to matrix effects. Dried microsampling definitely showed a positive impact on the stability of all vitamins described. However, it remains essential to further evaluate stability on a case-by-case basis at the different conditions (temperature, relative humidity, time, etc.) representative for the eventual study conditions.

Not only may the Hct influence important analytical parameters such as accuracy, recovery and ME, the Hct may also affect the B/P distribution of an analyte and, consequently, the clinical interpretation of the results. As most clinical reference ranges are set in plasma/serum or whole liquid blood, the results obtained by the alternative method should be critically compared with those obtained by a conventional method to demonstrate the suitability of microsampling as a valid alternative.

Table 1. Overview of published methods for the determination of vitamins in dried microsamples. 5-MTHF, 5-methyltetrahydrofolate; $25(OH)D_2$, 25-hydroxy vitamin D_2; $25(OH)D_3$, 25-hydroxy vitamin D_3; $24,25(OH)_2D_3$, 24,25-dihydroxy vitamin D_3; DBS, dried blood spot; DBSP, plasma concentration measured in dried blood spots; DPS, dried plasma spot; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLOQ, lower limit of quantification; NS, not specified; TDP, thiamine diphosphate; TMP, thiamine monophosphate; UV, ultraviolet; VAMS, volumetric absorptive microsampling.

Analyte	Reference	Analytical technique	LLOQ	Microsample type	C = Complete analysis P = Punch
Vitamin A					
Retinol	(47)	HPLC-UV	NS	DBS	P: 6.35 mm Ø punch from 50 µL spot
Retinol	(29)	HPLC-UV	0.5 µmol/L	DBS	P: 6.35 mm Ø punch from 30 µL spot
Retinol	(48)	HPLC-UV	NS	DBS	C: 60 µL spot
Retinol	(49)	HPLC-UV	0.05 µg/mL	DBS and DPS	DBS: P: 8.5 mm Ø punch from 40 µL spot DPS: C: 40 µL spot
Retinol	(50)	LC-MS/MS	0.04 µg/mL	DBS	C: 40 µL spot
Vitamin B_1					
Thiamine	(51)	LC-MS/MS	0.5 ng/mL	DBS	C: 40 µL spot
TDP	(43)	HPLC-fluorimetry	10 ng/mL	DBS	C: 40 µL spot
Thiamine, TMP, TDP	(83)	HPLC-fluorimetry	1.5-3 nmol/L	DBS	P: 6.35 mm Ø punch from 40 µL spot
Vitamin B_2					
Riboflavin	(51)	LC-MS/MS	0.2 ng/mL	DBS	C: 40 µL spot
Riboflavin	(52)	LC-MS/MS	2 ng/mL	DBS	C: 20 µL spot
Vitamin B_6					
Pyridoxal 5'-phosphate	(51)	LC-MS/MS	0.5 ng/mL	DBS	C: 40 µL spot
Vitamin B_9					
Total folate	(68)	Microbiological assay	NS	DBS	C: 50 µL spot

Analyte	Reference	Analytical technique	LLOQ	Microsample type	C = Complete analysis P = Punch
5-MTHF	(66)	LC-MS/MS	25 nmol/L (DBS)	DBS and DPS	C: 50 µL spot for DBS and DBSP
Total folate	(69)	Microbiological assay	NS ~~6.3 nmol/L (DBSP)~~ 4.4 nmol/L (DPS)	DBS	P: 6.35 mm Ø punch C: 30 µL spot for DPS
5-MTHF	(42)	LC-MS/MS	26 nmol/L	VAMS	C: complete sample tip
Vitamin B_{12}					
Vitamin B_{12}	(67)	Microbiological assay	NS	DSS	C: 50 µL spot
Vitamin D					
25(OH)D_2, 25(OH)D_3	(84)	LC-MS/MS	NS	DBS	P: 4 x 6 mm Ø punch
25(OH)D_2, 25(OH)D_3	(85)	LC-MS/MS	7.7-10.7 nmol/L	DBS	P: 3.2 mm Ø punch
25(OH)D_2, 25(OH)D_3	(86)	LC-MS/MS	4 ng/mL (LOD)	DBS	P: 6 mm Ø punch
25(OH)D_3	(63)	LC-MS/MS	3.0 ng/mL	DBS	P: 2x 3 mm Ø punch
25(OH)D_3	(27)	LC-MS/MS	NS	DBS	P: 3.2 mm Ø punch
25(OH)D_3	(87)	LC-MS/MS	1.5 ng/mL	DBS	P: 3.2 mm Ø punch
25(OH)D_2, 25(OH)D_3	(88)	LC-MS/MS	32.5 nmol/L	DBS	P: 2 x 3.2 mm Ø punch
25(OH)D_2, 25(OH)D_3	(30)	LC-MS/MS	5 nmol/L	DBS	P: 3.2 mm Ø punch from 50 µl spot
25(OH)D_2, 25(OH)D_3, 3-epi-25(OH)D_3	(72)	LC-MS/MS	1.5 ng/mL	DBS	P: 2x 6.0 mm Ø punch
3α-25(OH)D_3, 3β-25(OH)D_3	(60)	LC-MS/MS	0.1 – 1 ng/mL	DBS	P: 5 mm Ø punch from 50 µL spot
25(OH)D_2, 25(OH)D_3	(71)	LC-MS/MS	3.85 – 4.62 ng/mL (DBS) 1 ng/mL (DSS)	DBS and DSS	P: 3 x 5.5 mm Ø punch from 80 µL spot
25(OH)D_2, 25(OH)D_3	(32)	LC-MS/MS	10.9 - 12.8 nmol/L	DBS	P: 3.2 mm Ø punch
25(OH)D_2, 25(OH)D_3, 3-epi-25(OH)D_3 and 24,25(OH)$_2D_3$	(65)	LC-MS/MS	0.04 - 0.48 ng/mL	DBS	P: 2 x 3 mm Ø punch
25(OH)D_3	(46)	LC-MS/MS	0.5 nmol/L	DBS	P: 3.2 mm Ø punch from 50 µL spot
25(OH)D_3	(61)	LC-MS/MS	1 ng/mL	VAMS	C: complete sample tip

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