The overall goal of this doctoral thesis was to help bridge the gap between research and routine in quantitative dried blood spot (DBS) analysis by tackling some of the hurdles that hinder(ed) more widespread implementation of this technique. These hurdles included the lack of a guideline for DBS method validation and the lack of adequate ways to cope with the hematocrit (HT) effect.

A general introduction to DBS sampling and analysis is provided in **General background, structure and objectives.** DBS are prepared by collecting a (typically non-volumetric) drop of blood onto a filter paper. This drop of blood can be obtained *via* a finger or heel stick, depending on the patient’s age. DBS sampling provides several advantages over traditional venipuncture such as minimal invasiveness, the very small sample volume that is required, increased analyte stability as well as the ease of sample collection, transport and storage. DBS analysis has been used in many fields for the analysis of a wide range of compounds. However, this sampling technique also faces several challenges and issues, of which the HT effect is considered to be an essential one. The HT effect can be subdivided in analytical and physiological aspects. The analytical aspects refers to the fact that the analytical result may depend on a patient’s HT, whereas the physiological aspect refers to the potential impact of the HT on the interpretation of the DBS-based result. In this section volumetric absorptive microsampling (VAMS) is also discussed. Using this sampling technique, a fixed volume of blood is wicked up from a non-volumetric drop of blood using an absorbent tip connected to a plastic handle. Therefore, VAMS-based results should be unaffected by a sample’s HT.

A more in-depth overview of dried blood sample techniques, as well as other alternative sampling strategies is provided in **Chapter 1.** These alternative sampling strategies refer to the patient-friendly sampling of non-conventional matrices as well as to the unconventional sampling of traditional matrices such as whole blood, serum and plasma. Matrices that are discussed include DBS, VAMS samples, dried plasma spots, dried matrix spots, oral fluid, interstitial fluid, hair, tears, exhaled breath, sweat and nasal mucus. For each matrix specific advantages, challenges and limitations are discussed as well as some relevant applications. These applications encompass the fields of therapeutic drug monitoring (TDM), newborn screening, endocrinology, toxicology, phenotyping, proteomics and metabolomics. Special attention is given to dried blood sampling strategies, as this is the main topic of this work. Blood remains the matrix of choice, since there is typically a correlation between an analyte’s
blood level and its (therapeutic) effect. TDM-related applications are discussed more elaborately, as TDM is the domain in which quantitative DBS-based methods are closest to routine implementation.

To help ensure the quality of DBS-based assays, a DBS method validation guideline was set up in collaboration with the Alternative Sampling Strategies Committee of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT). This guideline is the subject of Chapter 2. To successfully incorporate DBS-based methods in routine practice, good quality methods are a prerequisite. Since the quality of a method starts with its design, a sound method set-up not only ensures the method is suitable for a given application, it also increases the chances of a successful method validation. The quality of a method needs to be assessed both during analytical and clinical validation and should be compared with pre-set acceptance criteria. Most importantly, each aspect of a method should be evaluated in a way that reflects the situation in which it will eventually be applied. Throughout this guideline special attention is given to matrix-specific issues such as the HT, volume and chromatographic effect, and suggestions are provided on how to deal with these. Although this guideline was specifically constructed for the validation of quantitative DBS-based methods that use LC-MS/MS in the context of TDM, many of its aspects are also valid for dried blood microsample analysis in general.

In the following chapters the HT effect was tackled. This was done on the one hand by developing several strategies to determine (predict) the HT of dried blood microsamples. On the other hand, a ‘HT-independent’ alternative sampling strategy, i.e. VAMS, was evaluated. The first method we developed to derive the HT of a DBS, which is described in Chapter 3, estimated the HT based on a DBS’s K⁺ content. This electrolyte was chosen as a HT marker as its intracellular concentration is about 35 times higher than its extracellular concentration and its levels are under tight physiological control. As red blood cells are the predominant cells in blood, these are the major contributors to the total blood K⁺ concentration. The method requires a two-step extraction of a 3-mm DBS punch with MilliQ water containing 2.5 mM KCl and the K⁺ levels were measured via indirect potentiometry, using a routine chemistry analyzer. After successful analytical validation the method was applied to 111 venous patient DBS with a wide HT range (0.19 – 0.63), yielding excellent results. The developed methodology can be easily introduced into any automated clinical laboratory, due to its simplicity, speed
and the fact a routine analyzer can be employed. The predicted HT can be used to assess whether the HT of the sample is within the validated HT range or to compensate for the anticipated HT effect by introducing a HT-dependent correction factor. Furthermore, it may allow conversion of DBS-based results to the corresponding plasma or serum values.

This K⁺-based HT prediction method was adapted to be applicable to VAMS samples as well. Although a VAMS-based result should be HT-independent, this is not always the case, as this matrix is susceptible to HT-dependent extraction efficiency issues. Therefore, it is valuable to check whether the HT of a VAMS sample is within the validated HT range. Alternatively, the HT may be required to convert VAMS-based results to the corresponding plasma or serum concentrations. In Chapter 4, two straightforward methods are described that allow to derive the HT from a VAMS sample based on its K⁺ content. One of these methods uses an aqueous extraction procedure, whereas the other one requires an organic extraction. Both methods have the potential to be seamlessly integrated with most existing VAMS analyses, allowing both target analyte quantitation and K⁺ analysis on a single VAMS extract. These methods were successfully validated and applied to 95 venous patient VAMS samples with a broad HT range (0.21 – 0.45).

Although the K⁺-based HT prediction method is straightforward, reliable, cheap and requires instrumentation that is available in every clinical laboratory, it does necessitate the destruction of (part of) a dried blood sample. As the sample volume that is available for analysis is already very limited with DBS analysis, a non-destructive alternative was developed. This method, which is described in Chapter 5, predicts the HT of a DBS based on its total Hb content and uses non-contact diffuse reflectance spectroscopy. The DBS are illuminated with halogen light, which is guided to the DBS surface via a fiber probe, and the light which is reflected by the DBS is transported via the same probe to a spectrometer. The reflectance spectra are fitted to a light transport model which takes into account the presence of oxyhemoglobin (OxyHb), methemoglobin (MetHb) and hemichrome (HC). This is essential, since Hb is originally present as OxyHb, which is then oxidized to MetHb and further denatured to HC upon storage (cfr. the change in color of a DBS upon aging). The fitting algorithm assigns a value with arbitrary units to each of the above-mentioned Hb derivatives and the sum of those values is used as a surrogate measure of total Hb and HT. After an elaborate analytical validation, the method was successfully applied to 233 venous patient DBS. This non-contact
diffuse reflectance spectroscopy-based method overcomes the need for sample preparation, which in turn reduces the analysis time, minimizes the possibility of errors and, importantly, eliminates the need for sample destruction. Basically, mere scanning of a DBS suffices to derive the HT of a DBS.

In Chapter 6 a simplification of this non-destructive method is described. In this simplified method the HT is calculated using the reflectance at a single wavelength, located at the quasi-isosbestic point of 589 nm. At this wavelength the reflectance is insensitive to the Hb degradation and only scales with the total amount of Hb and, therefore, the HT. This simplification eliminates the need for a complicated algorithm to derive the total Hb content from the DBS's reflectance spectrum. Interestingly, this simplified method even slightly outperforms the original, spectrum-based one. Furthermore, it is demonstrated, using caffeine as a model compound, that this HT prediction method can be effectively used to implement a HT-dependent correction factor to DBS-based results to alleviate the HT bias.

As mentioned above, apart from developing strategies to compensate for the HT effect, an alternative sample collection technique was evaluated which tries to avoid the HT effect altogether. More particularly, in Chapter 7, a quantitative VAMS-based method is described to determine Co using inductively coupled plasma coupled to mass spectrometry (ICP-MS) for the follow-up of metal-on-metal (MoM) prosthesis patients. Co is monitored in this patient population as a marker of the degree of implant wear. The development of this method revealed that VAMS-based results may still be subjected to a HT effect, more particularly to HT-dependent extraction efficiency issues. This observation stresses the importance of evaluating the HT effect in VAMS methods as well, using both fresh and aged samples. After adequate optimization of the sample preparation procedure, a robust, HT- and VAMS age-independent method was obtained. Furthermore, application to patient samples (n = 78), revealed a good agreement between the venous VAMS results and those obtained on the corresponding liquid whole blood samples. Yet again, special attention was paid to develop a method which is simple, applicable to realistic sample volumes and compatible with the capabilities of most clinical laboratories equipped with ICP-MS analysis.

In the ‘Broader international context, relevance and future perspectives’ section an overview is given of other strategies that have been developed during recent years to deal with the HT
effect in DBS analysis. In general there are three main trends: i) the development of several devices that allow to generate fixed volume DBS (or other dried blood samples) starting from a non-volumetric drop of blood; ii) the development of devices that allow the *in situ* generation of DPS; iii) the development of other methods to estimate and/or correct for the HT of dried blood samples. Importantly, there does not seem to be a ‘best’ way to deal with HT effect. Multiple strategies have proven to be valuable, and a combination of different approaches still provides the most complete solution. Which (combination of) approach(es) is best suited, depends on the context in which the dried blood microsample analysis will be employed. Aside from the recent developments with regard to dealing with the HT effect, also other advances that may facilitate routine implementation of dried blood sample-based methods are briefly discussed. Furthermore, a non-exhaustive overview is provided of real life DBS applications in various settings. In addition, to demonstrate the current general interest in home-based sampling, several other sampling formats that may facilitate this, are reviewed.