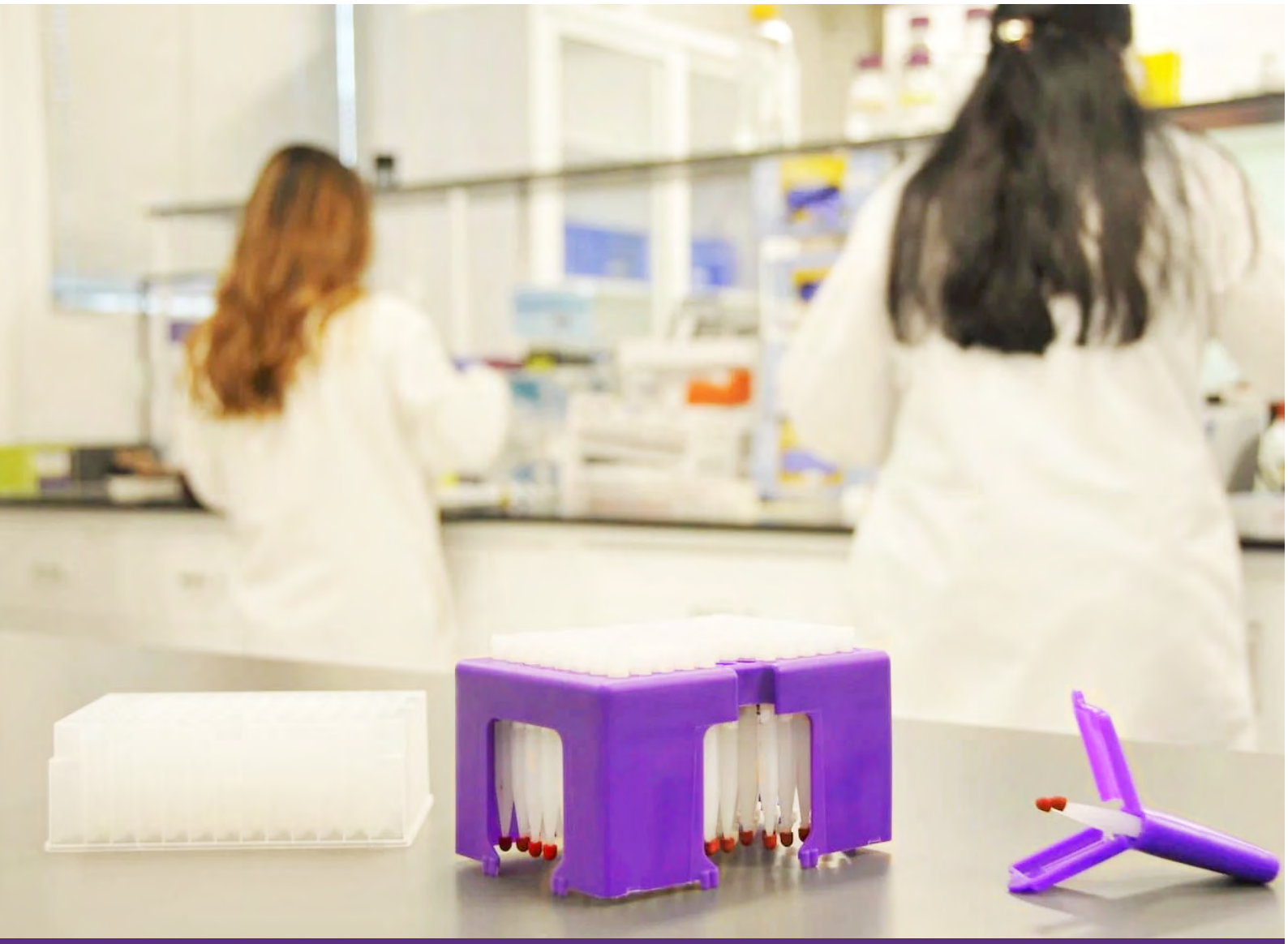
neoteryx®

The promise of microsampling, delivered



The Mitra Microsampling User Guide

How to Extract and Process Biological Microsamples in a Research Laboratory

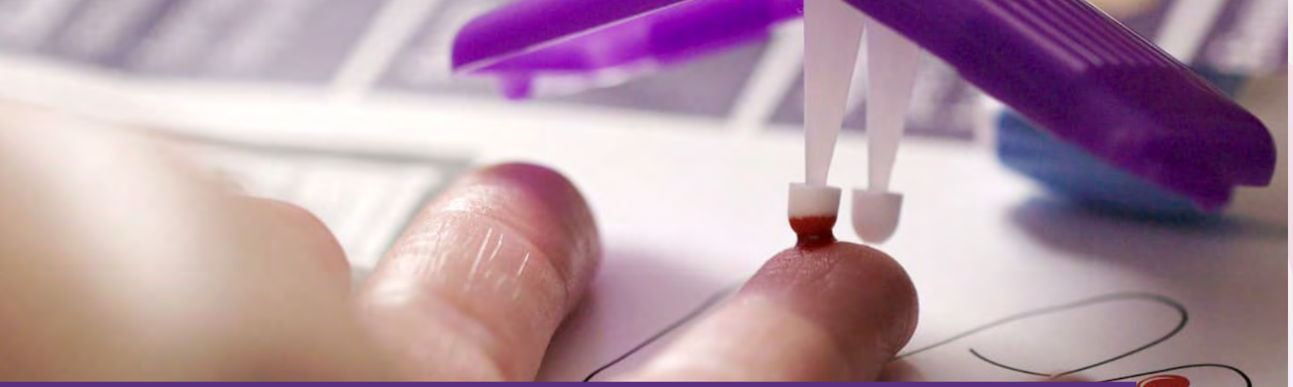
CONTENTS

Introduction

Thank you for choosing Mitra® devices based on VAMS® microsampling technology for your research! We hope this user guide will help get you going in the right direction with volumetric absorptive microsampling.

This user guide doesn't focus on any one type of analyte, matrix or analytical equipment. Instead, it guides you in making initial decisions that smooth the way towards solid analytical validations for your research projects. Drawing from nearly 200 peer reviewed publications on Mitra with VAMS, we have distilled best practices from a wide range of disciplines within the field of bioanalytical research. These publications can be accessed via our comprehensive online library of manuscripts. To get started with microsampling, we invite you to read our user guide!

Mitra devices are intended as a specimen collector and for the storage and transport of biological fluids. They are CE-IVD self-certified in the UK and EU, a Class 1 IVD in Australia, Brazil and China, Class B in South Africa, and registered with health agencies in Canada, Thailand, and Ukraine. In the United States, Mitra devices are for Research Use Only (RUO). In some countries, Mitra devices may be used in clinical diagnostic laboratory systems after the laboratory has validated their complete system in compliance with relevant rules and regulations.



Considerations before embarking on Mitra® microsample extractions for research studies

What is volumetric absorptive microsampling and how does it relate to dried matrix sampling?

Microsampling is defined as collecting very small amounts of a biological fluid (<50 µL) with the intention of analyzing components from these in a bioanalytical laboratory. Volumetric absorptive microsampling further refines this concept, allowing for the collection of a fixed volume of a bio-fluid, such as blood, without the need for cumbersome glass capillaries and filter paper. Volumetric sampling is a critical factor to enable quantitative measurements that are both accurate and precise. Mitra® devices based on VAMS® technology simplify volumetric sampling by leveraging capillary forces to absorb biofluids into the VAMS tip. When the VAMS tip of the Mitra device comes into contact with a bio-fluid, it rapidly absorbs it. Once each VAMS tip is visibly filled, the microsamples are ready to send to a lab for analysis.

What are the benefits of volumetric absorptive microsampling?

Within this guide we will be referring to the term ‘dried matrix’. The ‘matrix’ is the material (normally a biofluid) that contains your analyte of interest. Essentially any biofluid or matrix can be collected and dried on Mitra to create the ‘dried matrix’. There are times when there are specific biofluids discussed, where specific strategies are required to address certain challenges when working with that matrix.

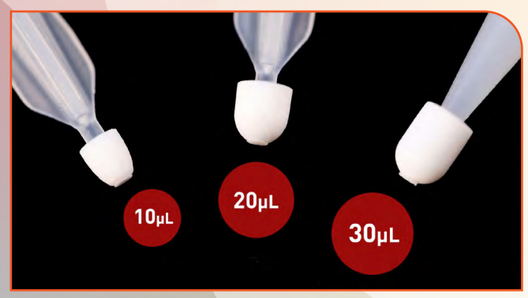
Dried microsamples don’t require costly cold-chain shipping or cold storage. This is beneficial to budgets and simplifies logistics for research studies conducted in low-resource areas, as well as those in which study subjects can participate remotely by self-sampling at home. Microsamples collected with Mitra devices don’t need to be left out to air dry before shipping, as is the case with other dried matrix sampling tools. Mitra devices are housed in a protective cartridge that can be closed immediately after the samples have been collected. The closed cartridges are sealed inside a specimen pouch that includes drying desiccant, which allows the microsamples to dry in transit to the lab.

For more information on how to successfully collect a Mitra sample, including video demonstrations, please visit: https://www.neoteryx.com/how-to-properly-take-a-blood-sample-using-the-mitra-microsampler-vams.

What microsampler volume do I need for my assay?

Currently there are 10, 20 and 30 µL VAMS microsampling tips available on Mitra devices (Figure 1) and your choice of tip volume depends primarily on how sensitive the assay needs to be. As a good rule of thumb, 10 µL tips are sufficient for use on LC-MS instruments, as mass analyzers are both highly sensitive and specific. When considering ligand binding assays, 20 µL tips tend to be more popular as they provide more dried matrix for desorption. Finally, 30 µL tips are recommended when high input volumes are needed. For example, next-generation DNA sequencing benefits from DNA input masses around 200 ng (dependent on length, white cell count and instrumentation), and 30 µL tips have been proven to yield more than 200 ng.

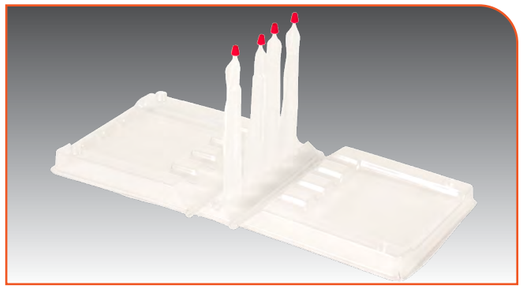
Figure 1 - Three sizes of VAMS tips on Mitra devices



How do I process microsamples in the laboratory?

Remotely collected Mitra microsamples arrive in the lab encased in protective housings. There are two types of housing, the clamshell which is aimed for researcher led collection and is available in 2- and 4-sampler versions. The cartridge houses 2 samplers and is designed for user-friendly collection by anyone for research studies, due to its ergonomic design. All three tip sizes are available in both the clamshell and cartridge.

Figure 2 - Mitra Cartridge (Left) and Clamshell (Right)



After sampling with Mitra devices, there are two primary methods of processing Mitra microsamples for extraction. The first and most straightforward is by using the 96-Autorack™ (Figure 3).

Figure 3 - 96-Autorack

The concept behind the 96-Autorack is that collected Mitra samples are transferred from their outer housings into the 96-Autorack in preparation for sample extraction. If the Mitra devices are barcoded (optional), then they are scanned into a LIMS before accessioning (Figure 4A). Once filled (Figure 4B), the 96-Autorack is then ready for extraction by placing it over a 96-well plate prefilled with extractant (Figure 4C). In totality, the process includes transferring individual Mitra samples to the 96-Autorack, preparing the extractant in a 96-well plate, placing the filled 96-Autorack over the 96-well collection plate, and extracting the anlayte of interest (typically vortexing / shaking and/or sonication; Figure 4D). Details of extraction methods are covered later in this guide.

Figure 4 - Processing Mitra samples from accessioning to extraction

The second way to extract Mitra samples is to remove the VAMS tip from the Mitra device body (which looks like a pipette tip) and place the tip into a tube. There are several ways to do this. One way is to trap the tip in between a tube and its lid, then pull and allow the tip to drop into the tube (Figure 5). Another way is to use pre-slitted covers, such as 96 well rubber silicone sealing mats. Detachment is achieved by poking the tip through the seal and pulling back so that the tip drops into the well of a plate.

Figure 5 - A VAMS tip being detached into a tube.

What analyte classes are compatible Mitra microsamping for research studies?

Most analyte classes are compatible for use with Mitra devices. These include small trace elements, small organic molecules like drugs, and large biological molecules like proteins and nucleic acids. Care must be taken in validations to ensure analytes are stable in the dried matrix of choice (e.g., blood, saliva, urine). Further, the journey of the sample also needs to be considered, including environmental temperatures and humidity during journey transport, for example. It must be noted that some molecules may be too volatile for dried matrices, with a heightened risk of losses during the drying process.

What instruments are compatible with Mitra extracts in research studies?

Most bioanalytical instruments are compatible with Mitra extracts to varying degrees. This depends on the following factors: the matrix, analyte and level of sample preparation needed for instrument compatibility, sensitivity and specificity. Below we have listed the most popular instruments / techniques for measuring Mitra extracts.

(U)HPLC/MS-MS

This is by far the most popular technique; (U)HPLC-MS/MS (LC-MS) is ideally suited to measuring analytes from complex matrices due to the excellent specificity and sensitivity it offers. Analytes that are popular for this technique are: small organic molecules such as drugs, environmental contaminants, and biomarkers. In addition to small molecules, LC-MS is also well suited to large molecules, such as proteins. However, this depends on an appropriate choice of MS analyzer. For example, the time of flight (TOF) analyzer can measure at high mass accuracy and at high mass ranges, so is more ideally suited to these macromolecules. The triple quadrupole, however, is still the most popular choice of mass analyzer due to is high dynamic range for quantitation. Yet, the mass range for this type of analyzer is limited to around m/z of 10-100 at the lower end to 1000-4000 at the upper end, dependent on the instrument. As a result, analytes larger than this need to be fragmented. This can be achieved in the MS source, but many people in the field of proteomics, for example, prefer to partially digest the protein with enzymes such as trypsin. Indeed, this can be achieved as part of an extraction from Mitra. Another benefit of LC-MS is that many analytes can be simultaneously detected, making it an ideal analyzer for limited sample volumes.

The main disadvantage with using LC-MS is that the instruments can be very expensive. Furthermore, LC-MS instruments require highly skilled operators to run and maintain them. Another consideration when using LC-MS instruments is that post-extraction sample preparation is often required. This prevents blockages of the fluidics, including the (U)HPLC column and / or contamination of non-volatile substances on the source. Sample preparation can be as simple as protein precipitation and then centrifugation or SPE.

ICP-MS

Similar to LC-MS, ICP-MS is an ideal analytical technique for measuring extracts from Mitra. Due to the method being used primarily for the analysis of metal ions, simple extraction methods from Mitra can be developed to suit such analysis.

GC-MS

Less popular than LC-MS, is GC-MS, which has many of the same benefits and similarities as the liquid technique. However, GC-MS is limited to analytes that are volatile and stable enough to partition into a gas phase. This means that the technique is restricted to small volatile molecules, although measurement of fatty acids and steroids is possible.

HPLC-Other

Although the mass spectrometer is an ideal analyzer for LC (Liquid Chromatography) eluates, there are many other types of analyzers that interface with HPLC (High Performance Liquid Chromatography). However, many of these are not specific, so compound identification is limited to retention time and measurement of a physicochemical property of the molecule, such as UV absorption.

Immunoassay

Ligand binding is another successful and popular technique when working with aqueous extracts from Mitra. The reason for this is that the very process of the ligand binding experiment, if heterogeneric in nature, can purify the extract enough such that no sample preparation post extraction is necessary. Like LC-MS, analysis of a wide range of compounds is possible and large molecules such as immunoglobulins can be measured without the need for sample pre-treatment. The main disadvantage to immunoassay is that it is often less quantitative than LC-MS. Moreover, there is an upper limit of multiplexing unless specialized equipment is used. Furthermore, if closed immunoassay analyzers are used, where dilution and separation of analytes are conducted within the instrument, issues with sensitivity can be observed. The reason is that these analyzers are calibrated to receive a neat sample. A Mitra extract will be more dilute. Nevertheless, there are examples where successful research methods have been reported in the literature where analytes from Mitra extracts have been analyzed on such closed systems (1).

qPCR and Whole Genomic Sequencing

Measuring oligonucleotides is possible from Mitra extracts, but due to the nature of microsamples, minimum input quantities need to be assessed on a case-by-case basis. This is dependent on the choice of instrument and whether PCR is employed prior to sequencing.

Biochemistry Analyzers

These analyzers are important for many central laboratories. By and large, analyzing compounds using these analyzers is more challenging from microsample extracts. The reason for this is twofold. First, these systems are closed systems designed to receive a neat biofluid, yet extracts from Mitra will be diluted (10x-20x). With closed immunoassays, reaching desired detection limits can be a challenge, but not impossible. Second, those intending to conduct research on extracts of dried blood need to consider blood hemolysis. Blood rehydrated from Mitra tips is completely hemolyzed. However, many of the measurements taken by biochemistry analyzers are often spectrophotometric in nature, following an enzymatic reaction, where a colorless substrate is converted to a pigmented product. Hemolysis, in many instances, leads to too much background interference. That said, it is always good practice to check what the requirements are to run assays on this instrument. For example, immunoturbidimetry used on such analyzers can work for some analytes from Mitra extracts (2).

What biological matrices are compatible with Mitra in research studies?

Urine

Mitra is an ideal vehicle for analysis of components from urine. In many cases analytes from urine are very stable in their dried state (3). It has been observed that when using filter paper, however, urine spreads out on the paper too thinly. This necessitates cutting out large disks in attempt to obtain enough urine volume for sufficient analytical sensitivity. Due to the volumetric nature of Mitra collection, up to 30 µL of urine can be collected on the VAMS tip of a Mitra device. This results in a 30 µL sample collected in a more practical format for downstream processing compared to working with large filter paper disks. However, there are other considerations when measuring analytes in microsamples from dried urine extractions. One is that when urine is evacuated, the wet matrix is not at a constant concentration. Using another analyte to act as a control in an area ratio to help with quantitation is advisable. Another consideration is that some analytes released into urine are dependent on the time of day taken, so collecting just one urine microsample may not give the full picture. In this case, collecting several timepoints across a range may give a better picture of average analyte concentration. Alternatively, sampling from a pooled collection may be another solution.

Oral Fluid

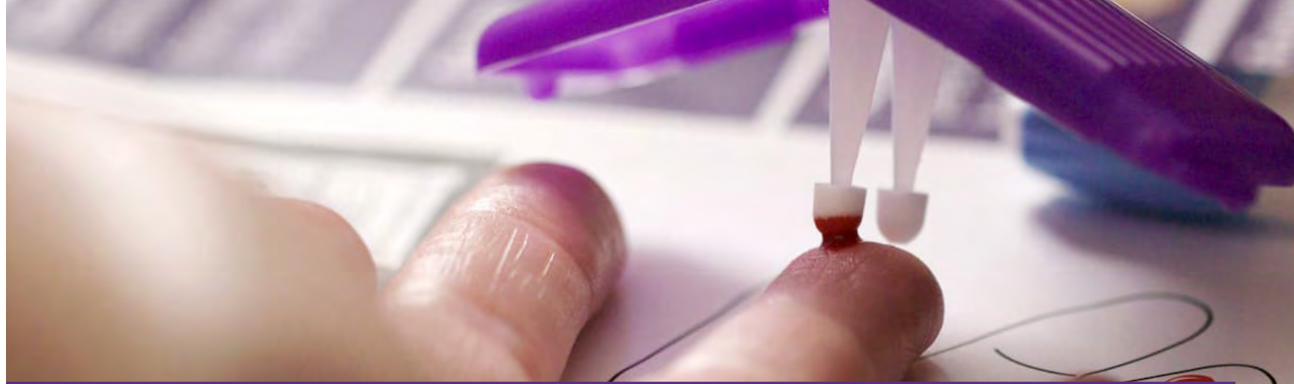
Another key biofluid that has been discussed in the literature is oral fluid, or saliva. As with urine, quantitation may be problematic due to differing oral fluid concentrations. Saliva is, however, a very convenient and non-invasive biofluid. There is a broad range of analytes which can be measured from saliva, from monitoring drugs to measuring hormones and antibodies. There are a number of publications using Mitra for measuring drugs from oral fluid extracts. Recently, researchers at the University of Bologna published on measuring antidepressant drugs in both blood and oral fluid from Mitra (4).

Plasma or Serum

Mitra is both an accurate and precise means of collecting biofluids, which lends itself to the collection of plasma or serum from tubes. This is especially useful when a sample needs to be sent to a specialized laboratory without costly cold chain shipping. Also, when proteinaceous biofluids are dried on Mitra and then the tips are exposed to an organic water miscible solvent like Methanol, the proteins will not dissolve or reconstitute into the methanol from the Mitra devices but small molecules will elute off. This “on-tip protein precipitation” technique negates the need for a centrifuge as used in more traditional protein precipitation procedures, making Mitra ideal for resource-limited environments. Furthermore, this would simplify automation of a standard protein precipitation procedure.

Whole Blood

Whole blood is the most popular, yet complicated biofluid to work with, although there are many examples of successfully validated methods reported in the literature across a wide range of analytes. One important observation when comparing whole blood data to plasma or serum values, is that there is often a negative bias observed. The reason for this is that there are many analytes which are present only in the plasma portion of the blood and not in the cellular fraction, which makes up around 45% of the blood volume, although this can range from 25 – 65% in some extreme cases. These negative biases need to be considered and compensated for, by measuring hemaglobin values from the same extract to act as predicate hematocrit (HCT) values, or by setting new reference ranges to the original matrix (5).



Considerations on matrix effects, process efficiency, extraction recovery, and ion suppression

The key to developing a robust extraction from Mitra microsamples is knowing where potential losses can be observed in the process of recovering an analyte from the matrix. Optimization is further refined by minimizing any interferences from co-extractives that can affect the analytical result. It must be noted that these tools assume no compound degradation so, if stability issues are suspected, separate experiments must be conducted. This will be addressed later in this guide. The tools in the toolbox allow us to understand where losses are seen due to systemic reasons or due to incomplete extraction (6).

Matrix effect (ME)

Those accustomed to working with biological matrices are all too aware of issues associated with the matrix. In its most basic form, the matrix can affect the result by releasing co-extractives, interfering with the assay performance, and competing with the analyte within the instrument / system. This often leads to a suppression of the signal or, in some cases, enhancement. To test for this, take a known concentration of your compound in the eluent and call this sample ‘A’. Then extract a tip, which has a blank matrix pre-dried on it, using your current extraction method. After extraction, spike in the analyte at an equimolar concentration to ‘A’. Call this one sample ‘B’. The matrix effect is a ratio of B/A x 100. Normally, this number would be less than 100% if, for example, the matrix components are competing for the same response from the detector. It could be at 100% if there is no matrix effect, which is the ideal situation. If greater than 100%, the matrix is enhancing the signal.

Process efficiency (PE)

To compare the efficiency of the entire analytical measurement (from extraction to final analysis), a third sample type is prepared to complement samples A and B used in the previous experiment. We will call this sample C. Sample C is where the analyte is spiked into the matrix before sampling and dried on Mitra. Extraction is the same as for sample B (blank matrix extract, post extraction spiked analytes). To obtain a process efficiency value, you take the ratio of C/A x100. Ideally the value

should be 100% or as close to 100% as possible. If the value is less than 100%, this indicates that the entire process is not as efficient as it could be. If above 100%, its indicative of signal enhancement in the MS.

Ionization suppression (IS)

This is simply the inverse of the matrix effect and is calculated as 100-ME and shows by which % the peak has been suppressed. A negative number indicates enhancement.

Extraction recovery (ER)

The key to a good extraction is to achieve over 85% extraction recovery. The way to test for this is to take the ratio of C/B x100 where one is measuring the ratio of pre-spiked dried Matrix (C) over post spiked sample extract from a dried blank blood Mitra sample (B). Because both samples are from the same matrix, any matrix effect is normalized across the two samples. If the value is less than one, this indicates that not all of the sample has been recovered off Mitra. It also could mean sample degradation, which we cover later in this guide.

Extraction recovery when working with dried whole blood

Dried blood is a complicated matrix to work with when considering optimizing extraction efficiency. The main issue is that not all analytes will extract at the same efficiency with the same method. Details on how to overcome this with different extraction approaches will be covered in the next section. To optimize good extraction recovery, there are two main considerations. The first is the effect of the % of hematocrit (HCT) on extraction recovery. The second is the age of the dried sample.

Effect of extraction recovery because of differing % HCT

By and large, most people will have a % HCT at around 40-45%, but this can be as low as 25% or as high at 65%. As a good rule of thumb, the higher % HCT, the more difficult it is to extract analytes off the sample. Moreover, the lower the concentration of the analyte, the more this effect is observed. This is because HCT can be perceived as an overly complicated solid phase extraction sorbent. And so, the higher the % HCT, the more there is for analytes to interact with. As a result, without a good extraction methodology, negative biases with respect to increased % HCT can be observed. The good news is that, for most cases, these biases can be solved by utilizing the right extraction methodology.

Effect of extraction recovery related to the age of the blood on the tip (Temporal Bias)

A phenomenon with dried blood is that the age of the blood influences the extraction recovery. The older the blood, the more difficult it is to extract analytes. This is known as temporal bias. Therefore, focusing on optimizing extraction recovery from tips that have been aged for at least 24 hours, up to 7 days in some cases, is advisable. Most of the time, good recoveries can be achieved by using an optimized extraction method that will allow for good recoveries whatever the age of the blood. We do recommend, though not always practical, to optimize the method based on the actual upper limit of time expected for extraction from field samples. For example, if the sample will be stored for 3 months prior to extraction it is advisable to develop an extraction method using a 3 month old Mitra microsample. It must be noted, however, that a negative bias based on the age of the sampled dried tip is also evidence of compound instability. Sometimes it is difficult to tease out which phenomenon is causing the temporal bias effect (stability vs. extractability). However, there are a couple of approaches which can help (see green box below).

Putting this all together

Due to the possible negative biases associated with HCT, in terms of both its percent and its age, it is important to work under conditions which are most challenging. It is recommended that you work initially with the highest HCT you will expect to see in the population you're studying. Also, work with a low concentration of your analytes such as a low QC or 5 x LLOQ. Finally, work with collected Mitra microsamples that have been aged for at least 24 hours.

Approaches for differentiating between analyte stability and temporal bias

The first approach, aimed at LC-MS users, is to look at your analytical results over time. Are you seeing other peaks appearing on the chromatogram with respect to the drop in analytical peak area? To see this, you will need to measure in full scan mode. Furthermore, does your MS recognize this as a related known degradant?

Some analyzers will not be able to give such comprehensive data (as seen for LC-MS) and only show reduction in peak areas / analytical values over time. However, it is possible to differentiate stability vs. extractability indirectly. The way to do this is to measure extraction efficiency across a wide range of HCT. If you see increased negative bias in the data with increasing % HCT, it indicates that temporal bias is at fault. If the same reduction in bias is seen across the HCT range, this points to degradation (7).

If you are finding that despite trying everything, temporal bias is still observed, it is recommended to age your calibrators and QCs to the expected age of the samples. In this way, the calibrators and QCs match the extraction efficiency of the sample. This works especially well when the temporal bias levels off after an observed amount of time.

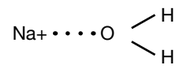
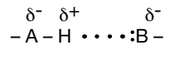
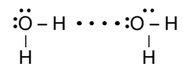
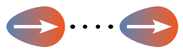
Extraction methods

Identifying what type of extraction method is best: Considerations on LogP

Hydrophobicity can be measured using a term known as LogP, which is defined as the log base 10 value of the partitioning coefficient of the two immiscible liquids. In the specific case where one of the solvents is water and the other is octanol, the LogP is a measure of hydrophobicity for the analyte being partitioned. To obtain a LogP value of an analyte, the concentration of said analyte is measured from both layers, having previously gone through a liquid-liquid extraction to allow for partitioning. The more hydrophobic analytes are, the greater their concentration is in the octanol layer. This then leads to LogP values of > 0. Conversely, very polar molecules return negative LogP values. Keep in mind that the more polar something is, the more it partitions into the water layer, and the lower / more negative the LogP value is.

We can exploit the LogP of analytes to help us choose the right extraction conditions. If the molecule has a positive LogP value, then it is worth attempting organic extraction methods first. If the molecule is polar in nature, then aqueous extractions may be more suited to maximize good extraction recovery. However, it is worth trying both organic and aqueous methods as there are many examples where hydrophobic analytes extract better in aqueous extractants or vice versa.

Figure 6 - Types of intermolecular interaction and their relative strengths*

Non-bonding (Intermolecular)				
Ion-dipole		Ion charge – dipole charge	40-600	
H bond		Polar bond to H – dipole charge (high EN of N, O, F)	10–40	
Dipole-dipole		Dipole charges	5–25	I – Cl • • • • I – Cl
Ion-induced dipole		Ion charge – polarizable e⁻ cloud	3–15	Fe^{2+} • • • • O_2
Dipole-induced dipole		Dipole charge – polarizable e⁻ cloud	2–10	H – Cl • • • • Cl – Cl
Dispersion (London)		Polarizable e⁻ clouds	0.05–40	F – F • • • • F – F

Notes on intermolecular forces: like interacts with like!

A good ‘rule of thumb’ in chemistry is to remember that like interacts with like. We sometimes refer to these types of interactions as Van der Waals forces. However, Van der Waals forces (sometimes called London dispersion forces) are infinitesimally weak relative to other intermolecular interactions (Figure 6). It is the absence of other intermolecular interactions, such as ionic, hydrogen bonding, dipole dipole, and pi bonding, which defines a hydrophobic molecule as predominantly interacting via Van der Waals forces.

Further, exclusion of hydrophobic molecules from the physical space of hydrophilic molecules occurs because the stronger interactions displace the weaker interactions. This leaves the molecules with strong intermolecular interactions (hydrophilic) physically together, and the molecules with weak intermolecular interactions physically together (hydrophobic). The presence of and the extent of moieties on an analyte conveys other intermolecular interactions. This leads to the overall polarity of a molecule, where ionic and hydrogen bonding are the strongest of these interactions and so molecules with such groups, display the highest degree of polarity.

Organic extraction methods

Organic extraction methods are by far the easiest to conduct, so it is certainly worth trying these first, especially if you have never worked with microsamples before. The idea behind the organic extraction method, is to expose the dried tips to a water miscible solvent, such as Methanol. Under these circumstances, any proteins present in the sample, such as albumin, will precipitate on the Mitra microsampling devices and small organic soluble components will extract off. If this is successful, then it is the perfect extraction method. The reason for this is that the Mitra devices in this scenario are actually part of the sample preparation process, rather than just the source of the matrix.

In some cases, no further sample preparation is required. It simply depends on the analytical methodology. For example, if this approach is to be used for LC-MS, then it is important to check that the injection solvent is compatible with the mobile phase. For example, if HILIC is being used, then MeOH can deleteriously affect the results. Moreover, if the analysis requires ligand binding, then MeOH can affect the results by deactivating the reagents. Under these circumstances, it is advisable to dry the sample and reconstitute it into a compatible buffer / solvent system.

An Example of an Organic Extraction Method

In the example below, it is suggested you use Mitra devices with 10 µL VAMS tips. However, if you are using Mitra devices with larger volume VAMS tips, it is recommended that you change the volume with respect to the change in tip size. Smaller volumes are possible, but it is important to have the devices fully submerged in the solvent, otherwise losses can be observed.

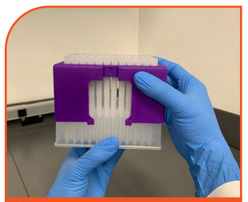
Place a 96-AutoRack™ containing dried Mitra samples onto a 2 mL, round bottom 8mm 96-well collection plate containing 200 µL of methanol in each well (containing internal standard) and follow the steps below.

STEP 1

Sonicate collection plate and 96-Autorack assembly for 15 minutes and/or

STEP 2

Shake collection plate and 96-Autorack assembly on Vibramax 100 (Heidolph) for 1 hour @ 1200 rpm

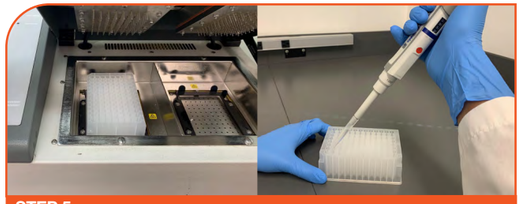


STEP 3

Remove 96-Autorack containing Mitra devices from the collection plate and discard

STEP 4

At this stage it is possible to analyze the sample, but further processing, such as centrifugation, may be necessary.



STEP 5

Further processing stages:

a. Dry down under nitrogen stream to dryness:
- Reconstitute sample with a solvent suitable for dissolution of analytes. (Consider keeping the organic percentage similar to initial mobile phase conditions for LC (Liquid Chromatography) analysis methods).

b. Diluting to keep similar organic percentage as initial mobile phase:
- Reconstitute sample with a solvent suitable for dissolution of analytes. (Consider keeping the organic percentage similar to initial mobile phase conditions for LC analysis methods).

STEP 6

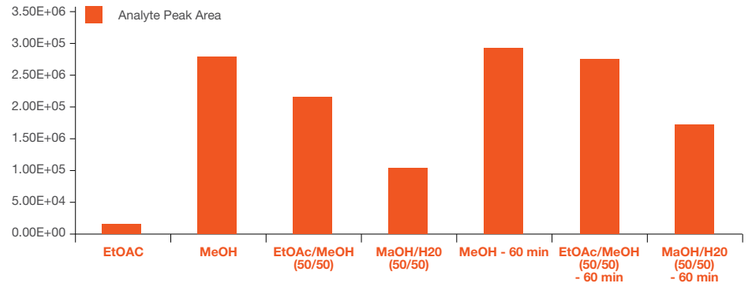
Analyze extract

Optimizing organic extraction methods

There are a number of approaches for optimizing extractions, and the first is through mechanical means. This involves using different combinations of sonication and vortexing, in terms of both duration and intensity. It must be noted that sometimes it is possible to extract under too harsh conditions, where co-extractives may elute off. Therefore, a good balance between extraction recovery and minimizing elution of co-extractives is sometimes required. The ultimate in mechanical extraction optimization is by employing Impacted Assisted Extraction, as first published by Altasciences (8). This approach employs the use of tissue homogenization equipment, where the VAMS tip is detached from the Mitra device body and vigorously vortexed in the presence of a ball bearing in the well of an enclosed 96 well plate. Unlike tissue homogenization, the tip remains intact, but it is thought that the mechanical ‘compressing and relaxing’ of the tip helps to release the components off the Mitra devices. While this is an extremely effective approach, specialized equipment is required.

A second approach that can be used in combination with mechanical optimization is to conduct chemical optimization. The first approach is to screen the most appropriate solvent system. For example, Figure 7 shows the difference of peak area when screening different solvent systems for optimizing the extraction of acetaminophen from blood.

Figure 7 - Solvent optimization for extraction of acetaminophen from Mitra samples



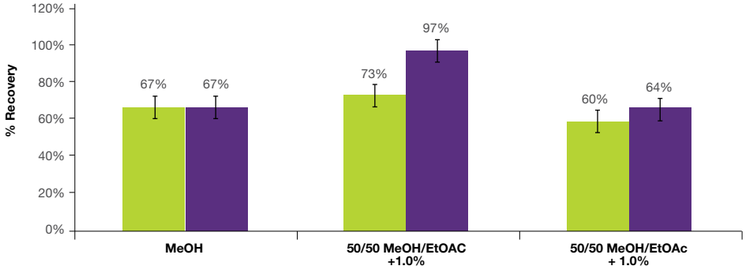
A good 'rule of thumb' is that the higher the LogP of the analyte, the stronger the extraction solvent required such as Ethyl Acetate (EtOAc). However, it is advisable to use mixes with water miscible solvents, otherwise poor recoveries can be observed.

Another effective approach is to change the pH of the extractant. Our findings suggest that the dried matrix has both ionic and hydrophobic properties which create non-specific binding to of many analytes of interest. Therefore, extraction efficiency can be enhanced by careful selection of solvent (as above) and adjustment of the pH in order to reduce the hydrophobic and ionic interactions between the analyte and the dried matrix. For example, take the drug naproxen (Figure 8). Due to the presence of a carboxylic acid group, the pK_a of naproxen is 4.15, and so at pH > 4.15 the molecule will be deprotonated and bear a negative charge. This means that if there are positive charges on the matrix, then naproxen will retain on the matrix ionically, resulting in poor extraction recoveries. Extraction efficiency can be improved by adjusting the pH of the extractant to pH < 4.15 in order to neutralize the naproxen and eliminate the non-specific ionic retention to the matrix. While this increases the hydrophobicity of the molecule, selecting a sufficiently strong organic solvent as an extractant will promote elution of the sample from the matrix.

Figure 8 - Structures of Naproxen (pK_a 4.15, left) and Diclofenac (pK_a 4.15, right)

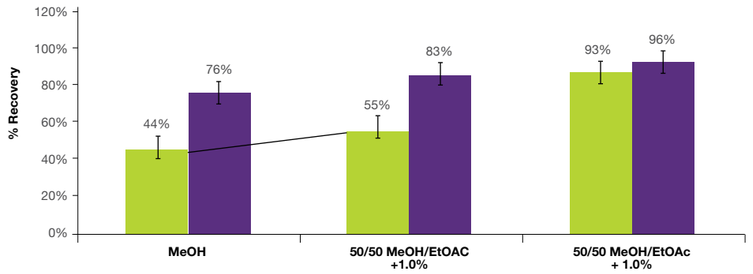
It can be seen in Figure 9 that the addition of 1% formic acid significantly improves the extraction efficiency of naproxen by neutralizing it. Moreover, the addition of EtOAc helps to break any Van der Waals forces, which again, help with extraction. However, and unsurprisingly, the addition of 1% ammonium hydroxide does not help, which likely indicates no change to the charge state of naproxen and therefore no improvement to the extraction.

Figure 9 - Optimization of acidic drug extractions using acidic pH modifier



The second drug examined in the experiment is diclofenac (Figure 8), which has a very similar acidic pK_a (4.15) to naproxen. However, because of the presence of the nitrogen, addition of the formic acid would act to protonate this basic moiety. It would again be charged, but this time with a +ve charge. Therefore, if the matrix has both positive and negative changes, this risks the molecule interacting ionically (with the opposite charge) and preventing elution. To elute this molecule from such a matrix, elution in the presence of a salt may act to compete with the ionic sites on the matrix.

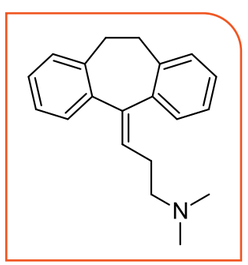
Figure 10 - Optimization of basic drug extractions using basic modifiers

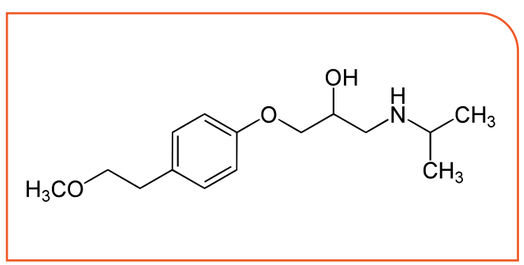


The same optimization can also be seen with basic moieties. Figure 10 shows the optimization of two basic drugs, amitriptyline and metoprolol (Figure 11). Both show improvements in extraction efficiency when base (1% ammonium formate) is used in the extractant. This is because both nitrogen groups on the drug molecules are basic in nature. So at pH = 7, they will carry positive charges and will interact with any negative groups in the matrix. An example of such moieties are phosphate groups from the phospholipid bilayers found on cell membranes. The basic modifier neutralizes these drugs by deprotonation using the ammonium salt. In so doing, strong ionic associations to the matrix are lost, allowing partitioning into the organic extraction solution. Finally, the addition of acid to the solution has a negligible effect and, where a small improvement in extraction efficiency is seen, it is more likely to be from the addition of the ethyl acetate to the solution, which helps break any Van der Waals forces.

A final optimization that can be conducted for organic extractions, is to pre-hydrate the VAMS tip before extraction into an organic solvent. Typically, the dried tips are exposed to water for a few seconds, removed from the water, and then allowed to absorb the water into the tip material. Finally, the partially hydrated tips are exposed to the organic extractant. The benefits of this are that the extractant can sometimes penetrate the dried blood more effectively and, thus, improve recoveries. However, this method can risk losses due to extraction into the initial aqueous solution.

Figure 11 - Structures of amitriptyline (p*K*a = 9.7, left) and metoprolol (p*K*a = 9.4, right)

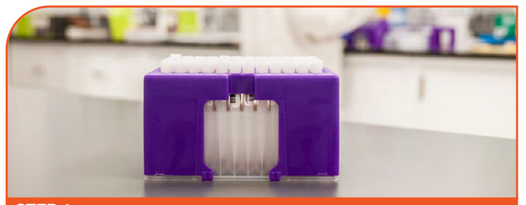




Aqueous extraction methods

For many analytes, organic extractions are highly successful, but there are occasions where it is more sensible or appropriate to extract in an aqueous-based solvent. For example, if the detector is only compatible with aqueous solutions, such as immunoassay, then an aqueous extraction is preferable to drying down an organic solvent and reconstituting it in aqueous. Another reason is that organic solvents may damage or alter an analyte in a certain way, such as happens with solvent-induced protein denaturation. There are also occasions where analytes are found only within the cells, such as cellular DNA, or observed with certain classes of drugs (e.g., calcineurin inhibiters). Under these circumstances, the fully hemolyzed solution will help to release the molecule for analysis. Finally, some analytes can be bound to proteins, so aqueous extraction followed by either protein precipitation or liquid-liquid extraction, can act to break such associations, thus releasing the analyte. An example of this would be extraction of steroid hormones for endocrinal research.

An Example of an Aqueous Extraction Method – Liquid-Liquid Extraction



STEP 1

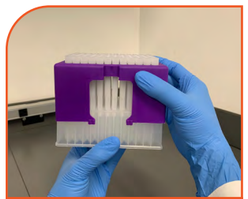
Place 96-AutoRack™ containing dried Mitra samples onto a 2 mL, round bottom 8 mm 96-well collection plate containing 200 µL of water in each well (containing internal standard-optional). Add 1 % formic acid (for neutrals and acids) or 0.25 M ammonium hydroxide (for neutrals or bases) to neutralize.

STEP 2

Sonicate collection plate and 96-Autroack assembly for 15 minutes.

STEP 3

Shake collection plate and 96-Autorack assembly on Vibramax 100 (Heidolph) for 1 hour @ 1200 rpm.

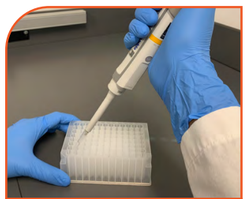


STEP 4

Remove 96-Autroack containing Mitra devices from the collection plate and discard.

STEP 5

If particulate is present, centrifuge the extraction plate at 1500 g for 5 minutes.



STEP 6

Remove supernatant from extraction plate and transfer to a secondary plate (optional)*.

STEP 7

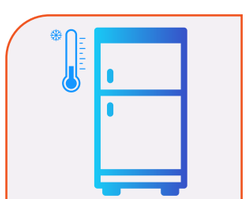
Add 1 mL organic solvent to each well (e.g. EtOAc, DCM, MTBE).

STEP 8

Mix 10 minutes using platform mixer.

STEP 9

Centrifuge the extraction plate at 1800 g for 10 minutes at room temperature.



STEP 10

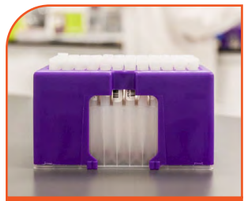
Freeze / transfer the supernatant.

STEP 11

See Organic Extraction protocol for sample solvent step.

An Example of an Aqueous Extraction Method - Protein Precipitation

An alternative precipitant would be trichloroacetic acid added to the water extract in lieu of Methanol and $ZnSO_4$. This extract, however, can damage some stationary phases on chromatography columns, so it's wise to check before you use this precipitant.



STEP 1

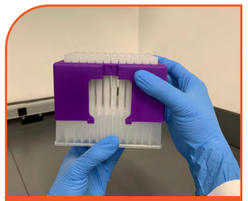
Place a Mitra 96-Autorack™ containing dried Mitra samples onto a 2 mL, round bottom 8mm 96-well collection plate containing 100 µL of water (containing internal standard-optional).

STEP 2

Sonicate collection plate and 96-Autorack assembly for 15 minutes.

STEP 3

Shake collection plate and 96-Autorack assembly on Vibramax 100 (Heidolph) for 1 hour @ 1200 rpm.



STEP 4

Remove 96-Autorack containing MItra devices from the collection plate and discard.

STEP 5

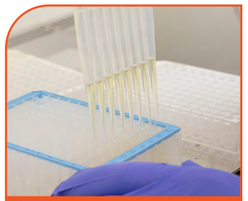
Add 100 µL of 8 % ZnSO4 to collection plate and mix for 5 minutes.

STEP 6

Add 100 µL of methanol with internal standard (optional) to collection plate and mix for 5 minutes.

STEP 7

Centrifuge at 1800 g for 10 minutes at room temperature (can transfer extract to centrifuge tubes, if desired).



STEP 8

Online SPE is recommended after centrifugation for further clean up, if needed.

STEP 9

Analyze the extract.

An Example of a Simple Aqueous Extraction Method - Ideal for Ligand Binding Assays

Sometimes it is helpful to extract in a buffer system such as PBS and BSA in the presence of a surfactant, which helps to stabilize proteins. Finally, when working with nucleic acids where there are protein associations, incubating the tips with proteinase K as part of the extractant helps to release the molecules of interest.

STEP 1

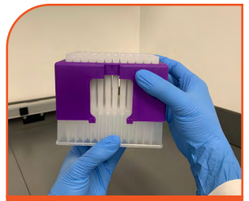
Place a Mitra 96-AutoRack™ containing dried Mitra devices onto a 2 mL, round bottom 8mm 96-well collection plate containing 200 µL of water in each well (containing internal standard-optional).

STEP 2

Sonicate collection plate and 96-Autorack assembly for 15 minutes.

STEP 3

Shake collection plate and 96-Autorack assembly on Vibramax 100 (Heidolph) for 1 hour @ 1200 rpm.



STEP 4

Remove 96-Autorack containing Mitra devices from the collection plate and discard.

STEP 5

If particulate is present, centrifuge the extraction plate at 1500 g for 5 minutes.

STEP 6

Remove supernatant from extraction plate and transfer to a secondary plate (optional).

STEP 7

Analyze the extract.

Initial experiments to help maximize successful validations for your research projects

This section outlines initial screening experiments to maximize the likelihood of developing a robust method. This involves two key experiments:

1. This is an initial screen to optimize extraction conditions aiming for >85% extraction recovery. This involves evaluating multiple extraction methods.
2. Hematocrit bias experiment (when conducting research with dried blood only): To check that the extraction conditions pass assay bias at extreme HCT values.

1. Screen best conditions for optimal extraction recovery

Extraction optimization should be performed under the sample conditions outlined below. These conditions are designed to stress test the extraction methods to hopefully improve the likelihood of an optimized method using the least number of VAMS® tips. For this, use the extraction methods and recommendations outlined in the previous section above, based on the nature of the analyte, matrix, and detector.

- Use analyte concentrations at 5x the LLOQ. The reason is that greater negative extraction biases can be seen at lower analyte concentrations with unoptimized methods.
- To test for initial temporal bias, 24 hours of drying is preferred.
- If your research involves testing dried blood, evaluate the upper and lower expected range of the hematocrit for your samples. If only one hematocrit value can be studied at this stage, then use the highest expected HCT value. Remember, high hematocrit levels can lead to negative extraction biases unless extraction is optimized under these conditions.

Prepare samples

This experimental design involves pre-spiked blood for your samples (N=4), post-spiked samples (analyte in the extraction solvent; N=3), and a double blank (no IS and no sample).

Prepare spiked matrix with your analyte at 5 x LLOQ or use a Low QC standard

- Sample and dry 4 Mitra samples (preferably overnight).
- Extract with IS and analyze.

Prepare non-spiked matrix

- Sample and dry 3 Mitra samples (preferably overnight).
- Extract with IS.
- After extracting the blank matrix tips, spike your analyte in the extractant at the calculated concentration of the expected extraction efficiency at 100%.

Prepare a double blank matrix sample

- Sample and dry a Mitra sample (preferably overnight) in the matrix you will be using (no sample, no IS).

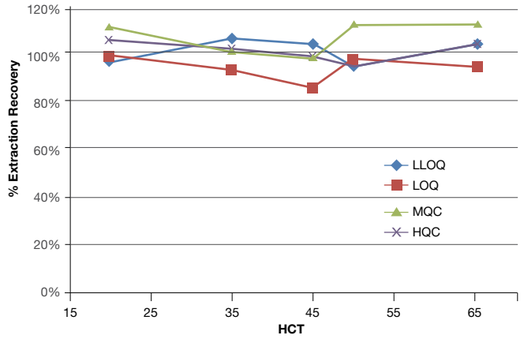
For each sample

- Check for any matrix effect by using the double blank sample.
- Calculate extraction recoveries for each sample for each extraction method until you have found one which gives you >85% extraction recovery.

2. Simple hematocrit bias evaluation experiment (for research using dried blood as a matrix)

Use the best extraction method from the optimization experiments above and perform a 5-point HCT bias evaluation. Use 5 blood tubes from the same subject with increasing HCT levels (20, 35, 45, 55, and 65%). To create such samples, first measure the hematocrit of the blood then centrifuge the blood to separate the cells from the plasma. After, take a subaliquot of the cellular fraction and add in by weight the desired fraction of plasma. Finally, analyze the HCT level of each sample to obtain a measured value. Once created, sample each blood with Mitra and dry tips at least overnight and ideally for 24 hours. You are aiming for flat bias curves as shown in Figure 12.

Figure 12 - Ideal HCT bias curves across a range of concentrations (Data Courtesy of Ji QC, Basdeo S, Discenza L, D'Arienzo C, Olah TV, and Arnold ME, Bristol-Myers Squibb Co).



With blood samples from each level of hematocrit to be studied, conduct the following:

Spike blood with analyte at 5x LLOQ

- Sample and dry 4 Mitra samples.
- Extract with IS and analyze.

Take non-spiked blood

- Sample and dry 3 Mitra samples.
- Extract with IS.
- After extraction, spike the analyte in extractant at the calculated concentration, if one assumes 100% extraction efficiency off a spiked tip.

Use a blank

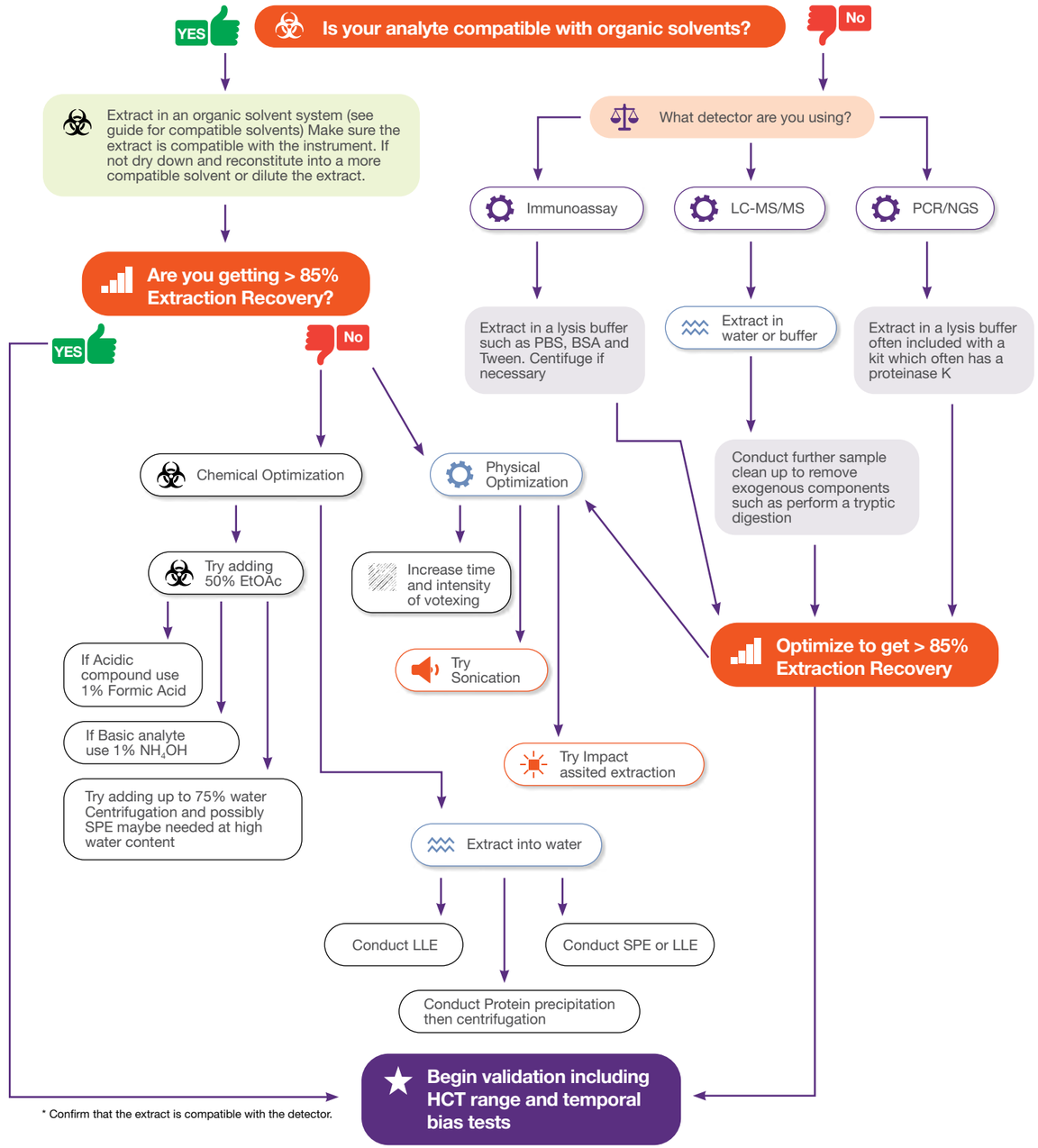
- Take non-spiked blood (double blank)
- Sample and dry 1 Mitra sample.
- Extract with extractant, no IS.

For each sample

- Normalize for any matrix effect by using the double blank sample.
- Calculate extraction recoveries for each HCT value. Plot on a bias plot (Figure 11).

Having completed these initial experiments, you will be ready to conduct an analytical validation based on guidance from your regulatory authority.

Decision Tree*



* Confirm that the extract is compatible with the detector.

Thanks for being a volumetric microsampling innovator!

We hope you have found this user guide helpful for your research projects. For further information on how to develop robust methods using dried matrix microsamples, there are several excellent resources in the literature (see references) that are worthwhile reading. Should you have questions about volumetric absorptive microsampling with Mitra devices and VAMS technology, please feel free to contact a microsampling specialist at Neoteryx for assistance or send your questions to support@neoteryx.com.

About Neoteryx

Neoteryx LLC, a medical device company in Southern California, delivers simple, quantitative and automatable microsampling solutions. Its Mitra® device facilitates remote specimen collection and transportation of blood and other biological fluids to improve human health, reduce laboratory costs and enable new models of care. Neoteryx's customers include scientific researchers, laboratories and health providers working to advance telemedicine, pharmaceutical development, biotechnology research and clinical diagnostics.

Mitra devices are intended as a specimen collector and for the storage and transport of biological fluids. They are CE-IVD self-certified in the UK and EU, a Class 1 IVD in Australia, Brazil and China, Class B in South Africa, and registered with health agencies in Canada, Thailand, and Ukraine. In the United States, Mitra devices are for Research Use Only (RUO). In some countries, Mitra devices may be used in clinical diagnostic laboratory systems after the laboratory has validated their complete system in compliance with relevant rules and regulations. Neoteryx operates a Quality Management System (QMS) that is based on FDA good manufacturing practices, 21 CFR 820 regulations, and ISO-13485.

Resources

Neoteryx offers microsampling content for further guidance and resources on its website. Please use the links below to visit our library of peer-reviewed publications, microsampling blog, educational videos, and other resources.

1. Publications Library
   https://www.neoteryx.com/analytes-detected-vams-microsampling
2. Microsampling Blog
   https://www.neoteryx.com/microsampling-blog
3. Microsampling Videos
   https://www.youtube.com/c/Neoteryx/videos
4. Collecting a Microsample
   https://www.neoteryx.com/how-to-properly-take-a-blood-sample-using-the-mitra-microsampler-vams
5. Calculate Costs for Clinical Trials
   https://calculator.neoteryx.com

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2. Add A1c app note
3. Enhanced urinary stability of peptide hormones and growth factors bydried urine microsampling
4. Whole blood and oral fluid microsampling for the monitoring of patients under treatment with antidepressant drugs
5. Quantification of testosterone, androstenedione and 17-hydroxyprogesterone in whole blood collected using Mitra microsampling devices
6. Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC–MS/MS
7. Official International Association for Therapeutic Drug Monitoring and Clinical Toxicology Guideline
8. Volumetric absorptive microsampling combined with impact-assisted extraction for hematocrit effect free assays
9. Tutorial: Volumetric absorptive microsampling (VAMS)
10. Microsampling: considerations for its use in pharmaceutical drug discovery and development

Glossary of Terms

Analyte: Small trace elements, small organic molecules (drugs), large biological molecules (proteins), and nucleic acids.

Assay: An investigative procedure in a laboratory for qualitatively assessing or quantitatively measuring the presence, amount, or functional activity of a target entity.

Autorack / 96-Autorack: This high-throughput rack made by Neoteryx allows for rapid processing of Mitra microsamples for method development, plate building, extractions. Autoracks are available empty or filled with 96 Mitra devices (10, 20, & 30 µL volumes).

Biochemistry Analyzers: Automated biochemistry analyzers are designed to measure the concentration of certain proteins, enzymes, electrolytes, metabolites, or drugs in specimen samples of urine, blood, serum, plasma, or other biological fluids. The machines typically include a tray where samples are loaded for analysis.

Biological fluid / bio-fluid: Also referred to as "bodily fluids," these are any bio-organic fluid produced by the human body, or other organism's body. Examples include blood, serum, plasma, urine, saliva, tears, breast milk, interstitial fluid, etc.

Capillary blood: Unlike venous blood, which flows through the major arteries and veins, capillary blood runs through the tiny blood vessels in the body known as capillaries. A sample of capillary blood is typically drawn by pricking the fingertip. Capillary and venous blood samples are similar, but capillary blood has higher hemoglobin and hematocrit values than venous blood.

Desiccant: A desiccant is a hygroscopic substance that is used in some packaging to maintain a state of dryness. Typically comprised of gel silica, desiccants are solids that are meant to absorb moisture/water.

Dried blood spot (DBS): Dried blood spot, or DBS, is a form of blood sampling where blood samples are drawn from a small finger-prick (or arm-prick), then blotted and dried on filter paper. The dried samples on "DBS cards" can be shipped to an analytical laboratory and analyzed using different methods, such as HPLC.

Extraction recovery: The recovery rate of a compound is the amount of that compound present in the extract (from a biological specimen sample), compared to the total amount of the compound found in both.

Extraction solvent (water miscible, etc.): Solvent extraction and partitioning is a method used to separate compounds, based on their relative solubilities in 2 different immiscible liquids (e.g., water and an organic solvent.)

GC-MS: Gas chromatography–mass spectrometry (GC-MS) is an analytical method combining gas-chromatography and mass spectrometry to identify different substances in a specimen sample.

Hematrocrit Effect/hematocrit bias: The blood hematocrit effect, or hematocit bias, can affect data reliability and quality. If blood spreads unevenly on a DBS card, for example, the area that's later punched for lab analysis might vary, because even punches of the same size may not contain the same amount of blood.

Hemolysis: The rapid destruction or rupture of red blood cells, which can cause the release of hemoglobin into the bloodstream.

HPLC: High-performance liquid chromatography (HPLC) is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture.

ICP-MS: Inductively coupled plasma mass spectrometry (ICP-MS) is a type of mass spectrometry that uses an inductively coupled plasma to ionize a specimen sample and create atomic and small polyatomic ions, which are then detected.

Immunoassay: A biochemical procedure for detecting/measuring specific proteins or other substances through their properties, such as antigens or antibodies.

Intermolecular forces / van der Waals: Intermolecular forces mediate interaction between molecules, including forces of attraction or repulsion between atoms and other types of neighboring particles (e.g., ions). Named after Dutch physicist Johannes Diderik van der Waals, a "van der Waals" force is a distance-dependent interaction between atoms or molecules.

Ion suppression / ionization suppression: Ion suppression in LC-MS and LC-MS/MS refers to reduced detector response, or signal:noise. Simply put, it is an adverse effect on detector response due to reduced ionization efficiency for analyte(s) of interest, due to the presence of species in the sample matrix that compete for ionization or inhibit efficient ionization.

LC-MS: Liquid chromatography (LC) is a technique widely used to separate compounds from a sample prior to analysis and is frequently coupled to mass spectrometry (MS).

LogP: The log base 10 value of the partitioning coefficient of two immiscible liquids, which is used to measure hydrophobicity.

Mass Spectrometry / Time of Flight Mass Spectrometry: Mass spectrometry (MS) is an analytical technique used to measure the mass-to-charge ratio of ions. The results usually are presented as a mass spectrum. Time-of-flight mass spectrometry (TOFMS) is a method of MS in which an ion's mass-to-charge ratio is determined by a TOF measurement.

Matrix / dried matrix (matrices): In a chemical analysis context, matrix refers to the components of a specimen sample other than the analyte of interest. In a biological context, matrices are bio-fluids such as blood, saliva, urine, etc. These bio-fluids can be sampled and dried, after which they are analyzed as dried matrices.

Matrix effect (ME): This is the effect on an analytical assay caused by all other components of the sample other than the specific analyte (compound) being analyzed.

Method validation: The process in lab studies of performing numerous assessments to verify that an analytical test system is suitable for its intended purpose and can provide beneficial and legitimate analytical data.

Microsampling / microsample: A very small amount of bio-fluid (e.g., blood) or tissue on which laboratory analysis is performed. A microsample generally refers to a sample of ≤50 µl.

Mitra device / Mitra microsampler: A small, portable device with a porous hydrophilic tip (an absorbent VAMS® tip) that enables the collection of accurate and precise bio-fluid volumes (i.e., blood).

Mitra cartridge: A plastic protective case that contains between 2-4 Mitra® devices designed for specimen collection and transport of bio-fluid microsamples. The cartridge protects samples during shipping and storage.

PCR/qPCR: Polymerase chain reaction, or PCR, is a test to detect genetic material from a specific organism, such as a virus. Quantitative PCR, or qPCR, is a technology used for measuring DNA using PCR.

Process efficiency: Process efficiency (PE) is expressed as the ratio of the mean peak area of an analyte fortified before extraction.

Temporal bias: Temporal bias occurs when one assumes the wrong sequence of events, and this can mislead one's reasoning about causality.

Volumetric absorptive microsampling: A microsampling technique used to obtain very small specimen samples of blood or other biological fluids for analysis as dried specimens. This approach involves the absorption of a liquid sample onto a porous substrate, allowing for precise fixed-volume sample collection.

VAMS technology / VAMS tips: The inventors of volumetric absorptive microsampling also developed a porous substrate in the form of a VAMS® tip, which is affixed to the end of a portable Mitra® device from Neoteryx, designed for easy remote specimen collection by both professionals and laypersons.

Venous blood / whole blood: Venous blood has passed through the capillaries of various tissues other than the lungs, and is found in the veins, the right chambers of the heart, and pulmonary arteries. It is typically drawn/collected from an arm vein and is dark red in color due to a lower content of oxygen.

neoteryx®

The promise of microsampling, delivered

The Mitra Microsampling User Guide

How to Extract and Process Biological Microsamples in a Research Laboratory

For more resources and information or to contact the Microsampling Specialists visit **www.neoteryx.com.**