

Review

Advanced Microsamples: Current Applications and Considerations for Mass Spectrometry-Based Metabolic Phenotyping Pipelines

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Abstract: Microsamples (collections usually less than 50 μ L) have been introduced in pre-clinical, clinical, and research settings to overcome obstacles in sampling via traditional venipuncture. However, venipuncture remains the sampling gold standard for metabolic phenotyping of blood. This presents several challenges in metabolic phenotyping workflows: accessibility for individuals in rural and remote underserved areas (due to the need for trained personnel), the unamenable nature to frequent sampling protocols in longitudinal research (for its invasive nature), and sample collection difficulty in the young and elderly. Furthermore, venous sample stability may be compromised when temperate conditions necessary for cold-chain transport are beyond control. Alternatively, research utilising microsamples extends phenotyping possibilities to inborn errors of metabolism, therapeutic drug monitoring, nutrition, as well as sport and anti-doping. Although the application of microsamples in metabolic phenotyping exists, it is still in its infancy, with whole blood being overwhelmingly the primary biofluid collected through the collection method of dried blood spots. Research into metabolic phenotyping of microsamples is limited; however, with advances in commercially available microsampling devices, common barriers such as volumetric inaccuracies and the 'haematocrit effect' in dried blood spot microsampling can be overcome. In this review, we provide an overview of the common uses and workflows for microsampling in metabolic phenotyping research. We discuss the advancements in technologies, highlighting key considerations and remaining knowledge gaps for employment of microsamples in metabolic phenotyping research. This review supports the translation of research from the 'bench to the community'.

Keywords: Microsampling; sample miniaturisation; dried blood spot (DBS); dried plasma spot (DPS); metabolic phenotyping; gas chromatography-mass spectrometry (GC-MS); liquid chromatography-mass spectrometry (LC-MS)

1. Introduction

Healthcare has undergone a paradigm shift in recent years, evolving from a reactive disease care focus, to one that is predictive, preventive, personalised, and participatory (P4 medicine) [1]. This shift has relied on advances in systems medicine platforms such as metabolic phenotyping, whereby sensitive detection and measurement of circulating metabolites provides researchers with detailed descriptors of the metabolic perturbations that occur in response to stimuli such as disease, thereby offering mechanistic insight into pathological processes [2, 3]. Furthermore, through the biochemical classification of an individual's physiological or pathological state, such approaches have facilitated stratification of populations, assisting in disease diagnosis, prognosis, response to therapeutic interventions, and the identification of disease risk factors at the population level [2, 3].

Gas chromatography- and liquid chromatography-mass spectrometry (GC-, LC-MS) techniques are routinely employed in metabolic phenotyping. Of the two techniques, LC-MS is the most beneficial for its ability to be set up for discovery and targeted pathway analysis. Through use of high resolution, accurate MS, the individual identification and quantification of metabolites can be performed [3, 4]. Additionally, triple quadrupole MS can be used for targeted applications [3, 4]. MS is performed following preparation of samples using different extraction solvents which are selected on the basis of their ability to perform optimal extraction of metabolites of interest based on their molecular properties.

When considering applications of metabolic phenotyping, the gold standard for measurement is venous whole blood which is commonly taken from the anti-cubital fossa, and then separated into either plasma or serum [5]. This entails time-consuming protocols for a venipuncture blood collection to occur in an approved clinical space; scheduling an appointment in advance with a trained phlebotomist, and depending on the nature of research, may require a patient to abstain from food for >8 hrs and travel to a laboratory for blood collection. Furthermore, lengthy post-collection protocols that venipuncture necessitates include sample processing via centrifugation, splitting (into smaller aliquots), and freezing at -80°C prior to cold-chain transport, which further add to the burden of sample collection and economic costs that impact budgets [6, 7]. In the laboratory, if the sample is intended for metabolic phenotyping technologies, whole blood from the collection then needs to be separated into either serum or plasma and frozen to ensure long term integrity of the sample. Venipuncture presents two main issues; reduced study sizes, and the inability to perform frequent sampling [8]. As such, venipuncture may limit the scope for metabolic phenotyping research, particularly in research areas which include remote or rural populations, patients with limited mobility or that are immunocompromised. This diminishes opportunities for research teams and population-based studies [9-11].

The invasive nature of venipuncture and its resultant frontend sample yields an excess of 400 – 2,000 times volumes required for metabolic phenotyping analyses (for typical phlebotomy collections of 2 – 10 mL) [2]. However, metabolic phenotyping assays that employ LC-MS are amenable to low sample volumes, for example LC-MS assays routinely require < 25 µL to perform metabolic phenotyping for both discovery profiling analysis and targeted pathway analysis [2, 12-20]. Such methods have been applied to metabolic phenotyping experiments that have assessed outcomes of health and disease including inflammation [17], cardiometabolic diseases [21], dementia [17], and nutrition [22]; and are commonly targeted for many clinical and human physiology studies. This move towards sample miniaturisation in the space of metabolic phenotyping has led research teams to begin evaluation of microsamples over traditional venipuncture for sample collection [23].

To advance the field of metabolic phenotyping, there is a growing demand for biospecimen collections to move beyond laboratory collections and into the community (i.e., patient self-sampling). The use of microsamples, particularly in the form of dried blood spots (DBSs) began back in the 1960s where Guthrie and Susi spotted neonates blood onto filter paper for screening of genetic conditions [24]. Since the DBS conception, other applications in research and routine laboratories have commenced, including therapeutic drug monitoring [25], routine illicit drug and alcohol monitoring [26], sport and anti-doping [27], nutrition [28], and detection of inborn errors of metabolism [29]. Microsamples facilitate self-sampling and are ideal candidates for biospecimen collections as small volumes (typically <50 µL) can be obtained using a range of specific devices and collection protocols [30]. Common collection technologies include traditional dried samples such as DBSs, and advanced microsampling devices (commercially available devices optimised to collect a variety of biological media including whole blood [31, 32], whole blood separated into plasma [33] or serum [34], and urine [35]). Most of these devices require a small amount of capillary blood from a minor skin incision such as a finger prick [28], usually from a fingertip; however other sites have been utilised such as the arm [36].

The employment of microsamples in metabolic phenotyping workflows has the potential to provide researchers with a widely applicable, less-invasive sampling workflow, that can be easily implemented in challenging environments to improve accessibility [9, 11, 30, 37]. This can include those in underserved rural and remote areas without access to cold storage and trained personnel. Microsampling also allows for safe sampling on those who are immunocompromised, facilitates amenable blood collections for elderly or infant participants, and improves access for individuals where travelling presents a barrier to their participation [9, 11] (Figure 1). Furthermore, the use of microsampling devices could ease sampling constraints, allowing researchers greater frequency of sampling and increased overall population sample size in epidemiology and clinical studies, thereby improving statistical sample power [10, 38] (Figure 2).

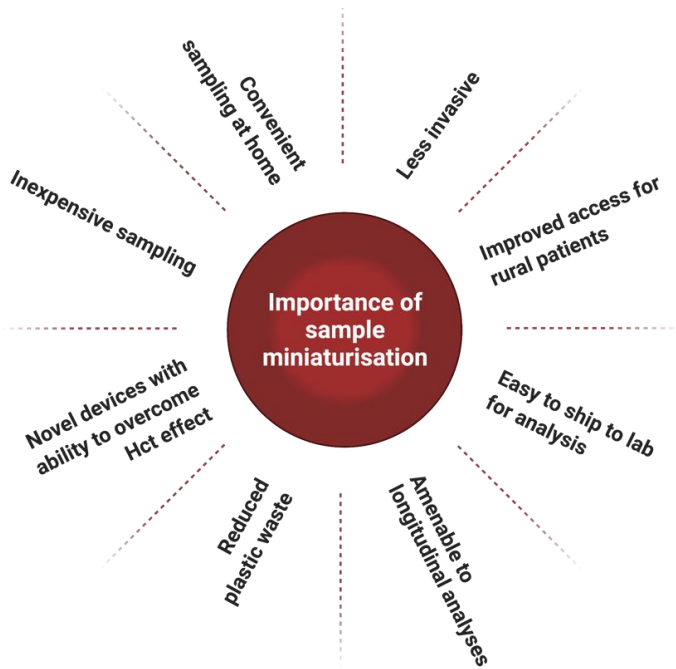


Figure 1. The importance of sample miniaturisation.

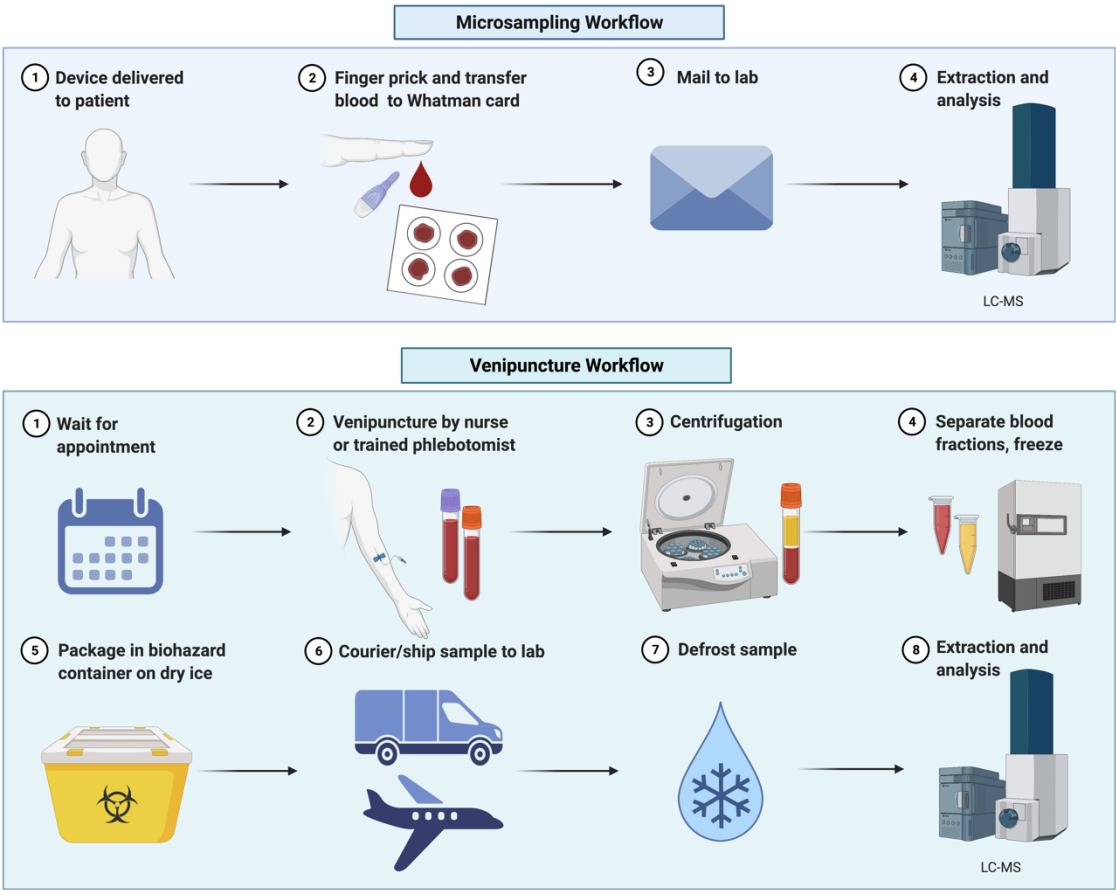


Figure 2. Metabolic phenotyping pipeline comparison for microsampling and venipuncture.

2. Approaches to microsample collections

2.1. Traditional dried samples: Dry blood spots (DBSs)

Historically, DBSs have been the flagbearer for microsampling. The concept for preservation of dried human biological samples as a spot is attributed to Ivar Christian Bang in 1913 [39, 40]. Typically, these microsamples can be collected via a skin prick with a lancet or created by transferring from a phlebotomy tube with a micro-pipette [30, 41]. Currently, such samples are collected on a specialized filter paper which can contain anywhere from 15–50 μ L of blood, and take approximately three to four hours to completely dry at room temperature [39]. This can make DBSs amenable to collections where cold-chain shipping protocols may not be feasible. However, it should be noted that below-zero temperatures (-20°C , -80°C) are still recommended for the long-term storage of DBSs [39] [30, 41].

DBS samples have been evaluated in MS-based metabolic phenotyping and have been used in applications of cancer [42, 43], air-pollution [44], drug discovery [45], acidemia [46], and pyruvate kinase deficiency [47]. Despite this wide range of applications of DBS samples, there still remains concerns around their volumetric accuracy, particularly in metabolic phenotyping where standardisation of sample volume is required. For example, the uneven spread of collected blood across the spot can introduce metabolite variation due to differences in viscosity/haematocrit. This has been observed in DBS studies using liquid scintillation analysis where sub-punches from an individual sample were taken and the number of metabolites measured varied vastly from sample to sample [48]. DBS collections are remarkably susceptible to variation based on an individual's haematocrit (hct; the fraction of blood made up of red blood cells). Varying haematocrit levels are of particular concern due to its propensity to undergo rapid changes in the body. This is a widely known phenomenon in DBS microsampling and is commonly referred to as the '*haematocrit effect*' [49], where external factors such as dehydration, polycythaemia, anaemia, over hydration, kidney failure, or chronic inflammatory conditions can cause plasma volume perturbations. Additionally, pregnancy may also cause slightly decreased hct due to an increase in blood volume [30, 50]. For reference, the normal range of hct is 36% - 48% for women and 42% - 52% for men [51]. Put simply, increases in hct beyond the normal range effect blood viscosity and therefore reduce the spread of the blood spotted on the carrier material, whereas decreases in hct (i.e., reduced viscosity) can create greater spread [52]. As such, the sample's spotted area has a linear, inverse relationship to hct [49, 50].

In MS based studies, normalisation techniques have been employed in order to address the '*haematocrit effect*' [53]. However, it remains one of the most prevalent challenges faced for translating the wide use of microsamples in metabolic phenotyping workflows. To date, studies have successfully performed DBS haematocrit normalisation through potassium content [54], using near infrared (NIR) spectroscopy [55], haemoglobin measurement using non-contact diffuse reflectance spectroscopy [56], and using wax barriers on DBSs [57]. Despite the development of these normalisation techniques for DBSs, they are yet to be widely adopted in metabolic phenotyping workflows, with many literature examples not implementing a normalisation step in their protocol descriptions [43, 44, 46, 47, 55, 58–60]. Only two studies have openly reported hct normalisation of their DBS samples as part of their metabolic phenotyping workflow. The first was by Koulman et al. who analysed infant heel-prick DBS samples, utilising a volumetric and hct-independent LC-MS method [61]. This was performed by relatively expressing an extracted lipid intensity of a given DBS sub-punch to its summed intensity. Interestingly, this method revealed that lipid profiles in DBSs showed comparable or better precision to plasma and whole blood samples which the authors propose was attributed to the halting of the oxidative process in dried samples compared to traditional venous whole blood and plasma samples [61]. Another study successfully employed an automated haematology analyser for analysis of hct in serum to normalise steroid concentrations obtained from traditional

DBSs [36]. This correction by Salamin et al. used the following equation: Corrected concentration = (DBS concentration)/(1-hct) [36].

Another biological factor that affects DBS sample quality includes the nature of the analyte(s) of interest because blood cells can cause variations in the amount of analyte that is extractable from the surface of the DBS card itself [62]. The reason for this is that analyte partitioning can occur between plasma and blood cells which significantly influences the concentrations of analytes in plasma or whole blood samples taken from a DBS, although this is most commonly seen in monoclonal antibodies for pharmacokinetic studies [25, 63]. Additionally, prominent sources of variation in DBS homogeneity have been attributed to the paper substrate used [49]; inconsistencies in the storage, packaging, and transport of samples [42, 43]; and contact of DBSs with other surfaces [6].

The circulating blood metabolome is a tightly controlled homeostatic system, where preanalytical variation (paper substrate, storage, packaging, transport) can unavoidably lead to inaccurate and possibly misleading results [64]. This is a current limitation in pre-analytical workflows for DBS microsamples, particularly when considering the inherently heterogeneous nature of biological samples where physiological conditions (i.e., hct) already contribute dynamic changes [65]. As such, efforts seeking to enhance accuracy, sensitivity, and specificity during the analytical phase are in vain if the technical aspects that underpin microsample workflows are not reproducible. Thus, a lack of reproducibility and accuracy is detrimental to achieving outcomes of P4 medicine using DBS samples in metabolic phenotyping pipelines [1].

2.2. Improving microsample collection: Are advanced devices the future for metabolic phenotyping?

Advancements in microsampling devices have allowed for improvement in blood collections by removing some of the inconsistencies experienced in DBS collections whilst maintaining the convenience of microsampling (Table 1). These devices can be classified into three broad classes: advanced dried samples; passive separation devices; and whole-biofluid collectors, which will be discussed in the context of metabolic phenotyping below (Section 3).

Advanced dried sample devices collect samples as a fluid and produce a dried sample either with a polymer tip (volumetric absorptive microsampling “VAMS” – Neoteryx; Torrance, California, United States) or carriers akin to DBS cards such as the hemaPEN (Trajan; Melbourne, Victoria, Australia), HemaXis DB10 (DBS System SA; Gland, Switzerland), Capitainer qDBS and B-Vanadate (Capitainer AB; Solna, Sweden), TASSO-M20 (HemoLink; Seattle, Washington, United States), and HemaSpot HD and HF (Spot on Sciences; San Francisco, California, United States) [27, 28, 36, 66-73]. These technologies improve upon standard DBS cards by providing accurate/volumetric aspiration and thus hematocrit-independent sample collection over a wide range of microsample sizes (2.74 μ L – 30 μ L).

Passive separation devices allow for the *in-situ* separation of whole blood into its sub-components e.g., serum or plasma, which can then store the resultant product in liquid form: TASSO+ (HemoLink; Seattle, Washington, United States), as dried serum: HemaSpot SE (Spot on Sciences; San Francisco, California, United States), or dried plasma: Tellimmune Plasma Separation Cards (Novilytic; West Lafayette, Indiana, United States). Passive separation devices represent an expanding area commercially, with many dried plasma spot (DPS) devices currently in development including the DPS (Capitainer AB; Solna, Sweden), Book-type DPS (Q2 Solutions; Morrisville, North Carolina, United States), and the Hemaxis DX (DBS System SA; Gland, Switzerland) [74-76]. It is not known how these devices will perform in metabolic phenotyping. However, as they produce samples akin to those obtained from venous whole blood separations which are commonly used in metabolic phenotyping, they warrant further investigation in the field.

Whole biofluid collectors are advanced devices with the ability to collect and produce samples as liquid samples without the need for cellulose material. These devices can collect sample volumes as small as 23 μ L: the MSW2 (Shimadzu; Kyoto, Japan); and extend to 100 μ L: the TAP (Yourbio Health; Medford, Massachusetts, United States); and up to

600 μ L: the TASSO+ and TASSO-SST (HemoLink; Seattle, Washington, United States) [10, 77, 78].

All three broad classes of advanced microsampling devices are well positioned for direct implementation in metabolic phenotyping workflows in clinical and epidemiology studies. For example, they are already commercially available and therefore have advanced manufacturing consistency; they have been designed to counter specific challenges in DBS and traditional microsampling workflows including the '*haematocrit effect*' [30, 71] and have already achieved translation to non-metabolic phenotyping analytical chemistry protocols [27, 79]. Despite these benefits, translation of microsampling devices to metabolic phenotyping research is yet to be widely adopted by the field as extensive studies investigating comparability to venipuncture and metabolite stability are lacking [80]. This is important as validated microsampling methods that leverage the advances in device design have the potential to enhance metabolic phenotyping studies in clinical and epidemiology settings, facilitating greater sampling frequency and sample size, and thereby providing valuable gains in statistical power [80, 81].

Table 1. Microsampling devices.

1

Sampling Technology	Device (Manufacturer)	Reps (Model); Volume	Biofluids	Description	Refs
Traditional dried samples	Whatmann (Cytiva; Marlborough, Massachusetts, United States)	5 (903 protein Saver Card); 75 – 80 µL	Urine, WB	Traditional paper-based carrier for collection of dried samples	[82, 83]
	PerkinElmer (PerkinElmer; Waltham, Massachusetts, United States)	5 (226 Spot Saver Card); Up to 70 µL	WB	Traditional paper-based carrier for collection of dried samples	[84]
	Ahlstrom-Munksjö (Ahlstrom-Munksjö; Helsinki, Finland)	5 (BioSample Card); Up to 70 µL	WB	Traditional paper-based carrier for collection of dried samples	[85]
Advanced dried samples	TASSO (HemoLink; Seattle, Washington, United States)	4 (M20); 20 µL	WB	M20 device collect four dried whole blood samples. The sample pod can be removed from the button and sent to lab.	[36, 77, 86]
	Capitainer (Capitainer AB; Solna, Sweden)	2 (qDBS); 10 µL 2 (B Vanadate); 10 µL	WB	The qDBS collects two fixed volume DBSs. A drop of blood is applied to the two microchannels within the device. After filling, the film at the capillary outlet dissolves, emptying the blood on the pre-punched Ahlstrom filter paper discs via capillary action. Successful sampling is indicated by the exit area of the device turning dark. Discs are then dried at room temperature for a minimum of 2 hours. Then, the DBSs can be removed with tweezers for analysis. B Vanadate operates under the same mechanism with additional phospholipase D inhibitor, sodium metavanadate (NaVO ₃), in the DBS disc to allow for accurate phosphatidylethanol testing.	[87, 88]
	Volumetric Absorptive Microsampling (VAMS) Mitra (Neoteryx;	2 (Cartridge); 10, 20, or 30 µL	Urine, WB (incl. serum and plasma)	A porous absorbent white tip attached to a plastic handler. Tip consists of hydrophilic polymer and permits haematocrit-independent collection of desired fluid by wicking up an accurate volume via capillary action. Different sample volumes can be collected depending on	[89-91]

	Torrance, California, United States)	4 (Clamshell)		tip size. Sample preparation can be performed manually by detaching the tip to transfer for extraction.	
		96 (Autorack)			
	hemaPEN (Trajan; Melbourne, Victoria, Australia)	4; 2.74 µL	WB	Haematocrit-independent sampling system for volumetric collection of capillary blood on pre-punched filter paper discs. Contains four EDTA-coated capillaries which fill by touching surface of blood drop of minimally 20 µL. Subsequently, clicking device into plastic base and inverting allows capillaries to transfer blood onto four integrated pre-punched filter paper discs. Device is available with both Whatman and Perkin Elmer discs. TGA approved.	[92]
	HemaXis (DBS System SA; Gland, Switzerland)	4 (DB10); 10 µL	WB	DB10 utilises a conventional Whatman or Perkin Elmer card combined with 4 volumetric capillary channels. Following fingerstick lancing, a blood drop is applied to the inlet of the capillary, collecting 10 µL of blood per channel. When filling of a channel is completed, blood can be observed at the outlet of the channel. This indicates sufficient application. The device is then manually closed to allow contact between the capillary outlet and the card, taking about 5 seconds for transfer of blood.	[93]
	HemaSpot (Spot on Sciences; San Francisco, California, United States)	Multiple (HD); 160 µL 8 (HF); 9.2 µL	WB	The HD is a large DBS that allows for multiple technical replicates upon performing a sub-punch; HF fan-shaped device has eight identical blades in protective plastic cassette. Two – three drops of blood must be applied to the centre of device to ensure equal distribution of blood (taking approximately one minute). Each blade holds 1/8th of the total sample volume (~9.2 µL). The device can be closed after filling and is then left to dry, facilitated by the integrated desiccant before it is ready for extraction.	[94, 95]
Passive separation devices	Capitainer (Capitainer AB; Solna, Sweden)	1 (DPS*); 11.6 µL	WB (separated into plasma)	The autonomous microfluidic DPS device collects plasma samples in under 6 minutes. It consists of a filtration membrane, a capillary metering channel, absorbent paper, and a drainage valve to remove excess plasma. A blood drop is applied to the filtration membrane, and the filtered plasma fills the capillary metering channel before it is absorbed by the DPS paper. Excess plasma is removed by the drainage valve.	[74]
	Book-Type Dried Plasma Spot Cards (Q2 Solutions; Morrisville, North Carolina, United States)	4 (DPS*); variable (8-14 mm spots)	WB (separated into plasma)	Device consists of two layers that filter erythrocytes from WB to produce plasma. Blood is applied to the card in the closed-book configuration. After approximately 3 minutes, the sample book can be opened to collect the DPSs by removing the paper substrate. Volume of the DPS obtained depends on applied WB volume (approximately 0.303 µL plasma per 1 µL of WB).	[75]
	HemaXis (DBS System SA; Gland, Switzerland)	1 (DX*); 2µL	WB (separated into plasma)	The DX operates in the same way, however, creates DPSs under the process of sedimentation once the device is closed. This is achieved through capillary pressure in a microfluidic system created with two polydimethylsiloxane sub-units.	[76]
	Telimmune Plasma Separation Cards (Novilytic;	1 (Uno); 2.5 µL (plasma)	WB (separated into plasma)	Telimmune (formerly Noviplex) Uno and Duo collect dried plasma through membrane filtration. Blood is applied to the top layer of the device. Respectively, 25 µL of blood and 60 µL of blood are required for application on the devices, an indicator control spot on the top	[33]

	West Lafayette, Indiana, United States)	2 (Duo); 3.8 µL (plasma)		layer changes colour once this amount is reached. A separation membrane beneath the top layer retains erythrocytes through size filtration. This allows plasma to flow through. The process takes approximately 3 mins, after which the top layer is peeled and discarded before drying the DPS for an additional 15 minutes. The DPS can then be removed using tweezers.	
	HemaSpot (Spot on Sciences; San Francisco, California, United States)	Multiple (SE); ~4 µL	WB (separated into serum)	SE device has a spiral-shaped design. It separates large whole blood components such as RBCs, platelets, and leukocytes from serum. Three-four blood drops applied to the centre allow the spiral-shaped membrane to separate different blood components through lateral flow. The serum components flow freely over the membrane, whereas the larger components are retained near centre of the spiral. After sample is dried for two minutes, the cartridge is closed and is ready for analysis. Sub-punches can be made in different parts of spiral.	[34]
Whole biofluid collectors	MSW2 (Shimadzu; Kyoto, Japan)	1 (Wing); 23 µL (WB) 5.6 µL (plasma)	WB (separated into plasma)	Blood is collected at the tip of the Wing after performing a fingerstick (or similar) with a lancing device. The device is filled once blood reaches the reservoir (23 µL). A specialised centrifuge rotor is required (the Windmill) which can hold 14 Wings for centrifugation. Typically, the sample is then spun down as 2,000 x G for 10 minutes. Following centrifugation two plasma zones are created and can be snapped off with hands (ie. does not require a specialised cutter) into a microtube for extraction.	[32]
	Touch Activated Phlebotomy (TAP) (Yourbio Health; Medford, Massachusetts, United States)	1 (TAP); 100 µL 1 (TAP II*); 250 µL	WB	Collects blood under vacuum pressure through microneedles, painlessly. Device sticks onto the skin (upper arm). Once button pushed, a ring of 30 small microneedles projects out of the device to micropuncture the skin. The needles immediately withdraw, creating a vacuum to induce collection of capillary blood. Blood flows through microfluidic channels into storage space, prefilled with lithium heparin anticoagulant. Subsequently, indicator window turns red when the reservoir is full (up to three minutes). Entire device can be sent to lab for collection and analysis of blood from device.	[96, 97]
	TASSO (HemoLink; Seattle, Washington, United States)	1 (TASSO+); 200 – 600 µL 1 (SST); 200 – 300 µL	WB (incl. serum and plasma)	Placed on upper arm. Collects WB by pressing the button, which results in a lancet puncturing the skin. Blood collected from capillaries under vacuum pressure into the sample pod (different formats): TASSO+ device collects whole blood for a variety of standard collection tubes (i.e., EDTA and lithium-heparin) and passive separation tubes (serum and plasma); SST device pod collects liquid WB, which can be spun into serum at lab.	[31, 98]

Note. Reps = number of technical replicates a given advanced microsampling device is able to produce; * = a device in development/ not currently commercially available; WB = whole blood.

2

3. Advanced microsamples: Current applications in metabolic phenotyping

Recently, the implementation of advanced microsampling devices in metabolic phenotyping workflows has been reported in literature, however collection and analytical protocols have not been extensively evaluated and optimised for the given devices available. These reported advanced microsampling devices cover a range of technologies for capillary blood collection including advanced dried samples, passive separation devices, and whole biofluid collectors (Table 2). However, by no means do they encompass all devices currently available, commercially or for research purposes. And although the literature presents a large variety of applications and pre-analytical sample preparation techniques, all studies primarily utilised LC-MS based platforms for analysis.

3.1. Advanced dried microsamples

Advanced dried microsamples represent the largest portion of published literature in the scope of metabolic phenotyping, for which metabolite coverage and metabolite stability have been the key focus. A variety of devices of this technology type have been used, with findings detailed below. Some studies additionally performed analysis of haematocrit to identify the ability of the devices to perform haematocrit-independent sample collection. Which are also detailed below, separately. A summary of applications of advanced dried microsamples in metabolic phenotyping has also been provided below.

3.1.1. Metabolite coverage and stability of different advanced dried sample devices

The **Capitainer B-Vanadate** (Capitainer AB; Solna, Sweden), was used for quantification of caffeine and paraxanthine using venous blood from a healthy, caffeine-abstinent female (N = 1) [73]. This research was limited to simulated blood collections using venous blood transferred to the microsampling device with a micropipette, and stability testing was performed in triplicate for each of the two time points. Caffeine and paraxanthine concentrations were examined as stable at 4 days (60°C) and at 3 months (room temperature or -20°C).

The **VAMS Mitra** (Neoteryx; Torrance, California, United States) device has been employed on two published accounts in metabolic phenotyping for assessment of metabolite coverage and stability. In a study performed by Kok et al. the 10 µL VAMS Mitra (Neoteryx; Torrance, California, United States) device, was assessed using reversed-phase chromatography and hydrophilic interaction liquid chromatography on plasma taken from one individual [99]. From the two methodologies, 36 analytes were recovered (24 amino acids and 12 organic acids) and assessed for stability over 15 days with measures being repeated at 2 hrs, and 1, 4, 7, and 15 days. No stability issues were observed during the first four days when stored at room temperature. However, at 15 days, increases and decreases were observed in some amino acids (a decrease in 19.2% and 15.4%, respectively for methionine and tyrosine). These changes were seen more markedly in organic acids, highlighting that they are less stable than amino acids. Specifically, these were: malic acid (-21.8%), glutathione (-31.8%), uric acid (-22.9%), glyoxylic acid (+36.8%), pyruvic acid (+26.0%), 3-hydroxypropionic acid (+37.9), and succinic acid (+46.3%). These stability findings contrast known instability of organic acids and amino acids stored in liquid blood for the same time [99]. In another study, an untargeted methodology was used by Volani et al. to analyse an unknown number of pooled EDTA venous blood samples using 69 VAMS Mitra (Neoteryx) devices. The results of this study contrasting those of Kok et al. who demonstrated metabolites were stable up to 4 days when stored at room temperature [100]. Specifically, differences were observed in ~75% (77/103) putatively identified metabolites between 2 hours, 24 to 48 hours, and 4 days, when visualized with principal component analysis (PCA), and in fact up to 6 months (one-way ANOVA, $p < 0.005$). With 36% of those differences in metabolites manifesting as decreases over time (e.g., histidine, glutamine, and asparagine), and 31% of those metabolites increasing over time (e.g., glutamic acid, glyceric acid, and methionine) [100]. Reasons for metabolite increases were not postulated, but could potentially include conversion of one metabolite to another or a relative concentration effect.

In a study of 20 healthy participants, the **hemaPEN** (Trajan; Melbourne, Victoria, Australia) was used to collect blood samples for analysis of 13 metabolites influenced by exercise, at training intervals, including nine amino acids and four organic acids [72]. Nine metabolite concentrations had variation <15% upon storage at -20°C for five months.

However, concentration decreases were observed in 2-oxoglutaric acid (35.1%) and methionine (56.5%); and concentrations increased for creatine (17.6%), and taurine (15.7%) [72].

Venous-derived serum, cited as the World Anti-Doping Agency (WADA) gold standard for sample collection in steroid profiling, was compared to a variety of advanced dried microsamples by Salamin et al. [36]. The researchers analysed 11 free and 8 conjugated steroids following testosterone gel administration in 14 healthy, eumenorrheic women. Testosterone concentrations were first measured in traditional DBSs (created with venous blood) and were found to highly correlate with those in serum ($r > 0.84$ with Passing-Bablok regression analysis). Following these positive results, Salamin et al. also investigated the **TASSO-M20** (HemoLink; Seattle, Washington, United States) (placed on the upper arm) for steroid profiles using capillary blood from 14 healthy volunteers (7 females, 7 males) compared to traditional Whatman Protein Saver Card DBSs (Cytiva; Marlborough, Massachusetts, United States) (via fingerprick). Fingertip DBSs and TASSO-M20 (HemoLink; Seattle, Washington, United States) dried samples demonstrated strong correlation for all quantified steroids using a Spearman's correlation. These steroids included testosterone ($r = 0.97$, $p < 0.0001$), androstenedione ($r = 0.85$, $p < 0.0001$), DHEA ($r = 0.93$, $p < 0.0001$), 17 α -OH-progesterone ($r = 0.98$, $p < 0.0001$), progesterone ($r = 0.93$, $p < 0.01$), and cortisol ($r = 0.91$, $p < 0.0001$). The researchers caveat their capillary DBS findings with the likelihood of high capillary testosterone concentrations being attributed to the topical application of the testosterone gel, although use of advanced devices such as the TASSO-M20 (HemoLink; Seattle, Washington, United States) can overcome this pitfall due to its independence of sample collection site (i.e., can be applied to upper arm) [36, 77]. Furthermore, Salamin et al. highlights the potential of DBS storage of steroids for 1 and 3 weeks at room temperature with minimal risk of degradation (contrary to serum samples) [36]. This translation is important for future applications of microsamples in the context of anti-doping (i.e., WADA).[36, 77].

3.1.2. Analysis of haematocrit

Evaluation of hct across studies using advanced dried microsamples has shown promising results. Results by Velghe et al. demonstrated the ability of the **Capitainer B-Vanadate** (Capitainer AB; Solna, Sweden) device to eliminate hct bias over a range of 18.8 – 55.0 [73]. Linear regression of Capitainer samples revealed that caffeine and paraxanthine were not affected by hct when compared to whole blood. Interestingly, when repeating the analysis using a traditional DBS sub-punch (again, simulated with a micropipette from venous blood), linear regression revealed the presence of a negative hct bias. Here, there was reduced analyte concentrations witnessed in DBSs compared to whole blood [73]. Accurate and precise collection of samples collected from a single drop of blood with minimal influence by hematocrit are similarly demonstrated by the **hemaPEN** (Trajan; Melbourne, Victoria, Australia) [27, 72, 79]. This has been examined in the scope of analytical chemistry for caffeine and paraxanthine, where comparing hemaPEN (Trajan; Melbourne, Victoria, Australia) concentrations and those in whole blood revealed 6.90% and 5.40% difference in mean concentrations, respectively [27]. Although statistically significant, the researchers concluded this variation was negligible, when comparing hemaPEN (Trajan; Melbourne, Victoria, Australia) to conventional sub-punch DBS results, and sub-punch DBS results to whole blood. In therapeutic drug monitoring of fluoxetine and sertraline (and their metabolites), hemaPEN (Trajan; Melbourne, Victoria, Australia) samples revealed a range of 3-9% variation in analyte recovery for different values of hct [79]. Conversely, other advanced dried sample technologies purported (and marketed) to overcome the "*haematocrit effect*", such as the **VAMS Mitra** (Neoteryx; Torrance, California, United States), are yet to investigate hct in metabolic phenotyping workflows.

3.1.3. Summary of advanced dried microsample findings

Generally, metabolic phenotyping of advanced dried samples performed analysis within a few days of microsample collection, with infrequent longitudinal stability assessment, up to a maximum of six months [99]. This is in contrast to traditional DBSs which have been performed up to 21 years following hct correction (a factor that may not be necessary for future use of archived advanced samples that are hct-independent) [56]. Storage conditions typically employed those readily available in laboratory settings (room temperature, -20°C, -80°C), which may not reflect the reality of multi-site clinical studies where microsamples are likely to undergo temperature variations or may encounter

extreme climates (high heat and humidity) [101]. Pre-analytical workflows for these samples mainly relied on manufacturer protocols and demonstrated great variability in drying time, and analytical turnaround. Furthermore, 3/6 studies were limited to an $N = 1$ [36, 73, 99], with only one study reaching an $N = 20$ [72], which further research should seek to expand upon to increase statistical power and ensure reproducibility of results.

3.2. Passive separation devices

Passive separation devices with the ability to obtain blood fractions such as plasma and sera currently represent the area of greatest focus in microsampling technology innovation. Many plasma separation devices are yet to be commercially available, including the Capitainer DPS (Capitainer AB; Solna, Sweden), Book-Type DPS Card (Q2 Solutions; Morrisville, North Carolina, United States), and the HemaXis DX (DBS System SA; Gland, Switzerland), and thus have yet to be translated to metabolic phenotyping.

Telimmune plasma cards (formerly Noviplex plasma prep cards) are currently the only plasma separation technology that has been translated to metabolic phenotyping research [53]. This was performed by Cvetko et al. who compared the glycoprofiles of 10 participants. This study utilised self-sampling, to compare Telimmune plasma cards (Novilytic), DBSs, and the VAMS Mitra (Neoteryx) devices to plasma. Both Telimmune plasma cards (Novilytic) and the VAMS Mitra (Neoteryx) devices managed to adequately replicate venous-derived plasma glycoprofiles. Their comparability was calculated using relative deviance between the advanced microsampling devices and traditional DBSs, to plasma for each of the 39 glycan peaks. Interestingly, Telimmune plasma cards (Novilytic) had the least relative deviance to plasma (0.069), followed by VAMS Mitra (Neoteryx) (0.092), with most deviance to plasma being displayed in traditional DBS samples (0.674).

A smaller subset of 5 participants was utilised to perform self-sampling in hexaplicate to assess analytical reproducibility of different microsample platforms. These were calculated as average %CV (coefficient of variation) for all glycan peaks. Telimmune plasma cards (Novilytic) displayed the least variation (4.831%), followed by the VAMS Mitra (Neoteryx) (7.098%), with most variation being seen in DBSs (14.305%). Cvetko et al. demonstrate the ability of passive plasma separation devices like the Telimmune plasma cards (Novilytic) to show reproducibility with venous derived plasma, and analytical reproducibility when sampling is self-administered by participants, thus highlighting the great potential for use of this advanced microsampling technology in larger cohort studies [53]. Importantly, although acknowledged as a limitation, variation in sample volume and hct from self-sampling are factors that were not addressed in this research. Furthermore, haemolysis (from inappropriate capillary lancing techniques) and under-sampling by participants may interfere with mass-spectrometry based metabolic phenotyping pipelines, introducing erroneous results [53].

The only serum passive separation device in existence is the **HemaSpot SE** (Spot on Sciences; San Francisco, California, United States). Currently, applications are limited to manufacturer publications, highlighting a gap in translation of serum separating technologies to metabolic phenotyping [34].

3.3. Whole biofluid collectors

Presently, only one reported account of whole biofluid collection technology has been used in metabolic phenotyping [78]. This is for the **MSW2** (Shimadzu; Kyoto, Japan) by Hotta et al. who investigated the pharmacokinetic effects of administering a cocktail of antiepileptic drugs (carbamazepine, lamotrigine, and phenytoin) in 4 rats, and performed qualitative metabolite identification in rats ($N = 2$) using carbamazepine (100 mg/kg) as a model drug. In the pharmacokinetic study, blood samples were collected via the tail vein using a glass capillary to inoculate the device with blood before centrifuging to obtain the respective separated blood fragment 'chips'. Drug stability testing was then performed for a variety of storage conditions on the different chips. The stability data of the drugs revealed percent biases within the acceptable range ($\leq \pm 15.0\%$) for all conditions. This highlights the stability of these antiepileptic drug samples over wide range of storage conditions. These conditions included plasma at ambient temperature for 24 hours, whole blood at ambient temperature for 1 hour, and plasma at -20°C for 140 days (the total storage duration from sample collection to analysis). Additionally, freeze/thaw (-20°C /ambient temperature) was investigated for two cycles, as were processed plasma samples at 6°C for 42 hours.

For the latter metabolite identification study, blood samples were collected at 1, 2, 4, 8, and 24 h post-carbamazepine dose. The contents of two device chips at the same time points were pooled in a tube (to total 5.6 μ L of plasma). This plasma was then diluted 10-fold with blank rat plasma. Following which, LC-MS/MS analysis allowed for characterisation of seven metabolites of carbamazepine. This allowed for the development of a proposed metabolic pathway of carbamazepine in rat plasma. Hotta et al. concluded that the MSW2 (Shimadzu; Kyoto, Japan) is technically easy to use with minimal training, and their findings suggest whole biofluid collection technologies can be useful for assessment of metabolite safety and to perform metabolite identification [78].

Table 2. Comparison of microsamples in mass spectrometry-based analytical chemistry and metabolic phenotyping methodologies: Extraction and analytical methods.

Microsampling Technology	Extraction solvent	Metabolic phenotyping method	Title	Ref
Traditional dried samples (DBS)	MeOH	Targeted DI-HRMS for amino acids, ketones, lipids	Understanding acute metabolic decompensation in propionic and methylmalonic acidemias: A deep metabolic phenotyping approach	[46]
		Untargeted DI-HRMS		
Traditional dried samples (DBS)	MeOH	UPLC-MS/ MS TQ, BEH C18 column Targeted analysis of anticancer drug, cyclophosphamide, metabolites	Phenotyping study of cyclophosphamide 4-hydroxylation in Malay cancer patients	[43]
Traditional dried samples (DBS)	MeOH:H ₂ O (4:1, v/v) 2% formic acid	Untargeted UHPLC- qTOF-MS RP and HILIC separation	A feasibility study of metabolic phenotyping of dried blood spot specimens in rural Chinese women exposed to household air pollution	[44]
Traditional dried samples (DBS)	100 µL of MilliQ H ₂ O 250 µL of MeOH 500 µL of MTBE	Orbitrap – DIHRMS lipidomics	The development and validation of a fast and robust dried blood spot based lipid profiling method to study infant metabolism	[61]
Traditional dried samples (DBS, DPS)	MeOH	7890A GC System coupled to 5975 inert MSD with Triple-Axis Detector	Evaluation of dried blood spots as sample matrix for gas chromatography/mass spectrometry based metabolomic profiling	[58]
Traditional dried samples (DBS, DUS)	DBS: MeOH:H ₂ O (4:1, v/v) DUS: MeOH:H ₂ O (8:1, v/v)	UHPLC-MS RP, HILIC, lipid methods	Investigation of the 12-month stability of dried blood and urine spots applying untargeted UHPLC-MS metabolomic assays	[55]
Traditional dried samples (DBS)	ACN/MeOH with H ₂ O at a ratio of 2:1–4:1	LC-MS/MS Drug, lipid, acylcarnitine analyses	Drug, lipid, and acylcarnitine profiling using dried blood spot (DBS) technology in drug discovery	[45]
Traditional dried samples (DBS)	Not specified: “relatively uncomplicated sample extraction steps”	DIHRMS	Untargeted metabolic profiling in dried blood spots identifies disease fingerprint for pyruvate kinase deficiency	[47]
Traditional dried samples (DBS)	MeOH:ACN (1:1)	LCMS	Nontargeted mass spectrometry of dried blood spots for interrogation of the human circulating metabolome	[60]
Traditional dried samples (DBS)	25% (v/v) aqueous MeOH	UPLC-MS	Metabolite profiles from dried blood spots for metabonomic studies using UPLC combined with orthogonal acceleration ToF-MS: effects of different papers and sample storage stability	[59]
Traditional dried samples (DBS)	MeOH:H ₂ O (95:5, v/v)	UHPLC-MS/MS MRM of steroid hormones	Steroid profiling by UHPLC-MS/MS in dried blood spots collected from healthy women with and without testosterone gel administration	[36]
Advanced dried samples (HemaXis DB10, TASSO-M20)				

Advanced dried samples (VAMS)	ACN:H ₂ O (60:40, v/v)	UHPLC-MS/MS for organic acids	Targeted metabolomics of whole blood using volumetric absorptive microsampling	[99]
		HILIC-MS/MS for amino acids		
Advanced dried samples (VAMS)	ACN:H ₂ O (70:30, v/v)	UHPLC-Q-ToF-MS HILIC Untargeted analysis	Pre-analytical evaluation of volumetric absorptive microsampling and integration in a mass spectrometry-based metabolomics workflow	[100]
Advanced dried samples (hemaPEN)	ACN:H ₂ O (60:40, v/v)	UHPLC-MS/MS Organic acid, amino acid methods	Blood microsampling to monitor metabolic profiles during physical exercise	[72]
Advanced dried samples (hemaPEN)	MeOH:H ₂ O (80:20, v/v) 0.01% formic acid	LC-MS/MS Targeted analysis of caffeine and paraxanthine	Evaluation of the performance and hematocrit independence of the hemaPEN as a volumetric dried blood spot collection device	[27]
Advanced dried samples (Capitainer B Vanadate)	MeOH:H ₂ O (80:20, v/v) 0.01% formic acid	UPLC-MS/MS Targeted analysis of caffeine and paraxanthine	Evaluation of the Capitainer-B microfluidic device as a new hematocrit-independent alternative for dried blood spot collection	[73]
Traditional dried samples (DBS)	H ₂ O	HILIC-UPLC-FLR Glycoprofiling	Comparison of self-sampling blood collection for N-glycosylation analysis	[53]
Advanced dried samples (VAMS)				
Passive separation devices (Telimmune, formerly Noviplex plasma prep cards)				
Whole biofluid collectors (MSW2)	5 µL IS: ACN/MeOH (70:30, v/v) + 100 µL MeOH	LC-MS/MS Targeted analysis of carbamazepine (and 7 of its metabolites), lamotrigine, phenytoin antiepileptic drugs	Quantitative and qualitative application of a novel capillary microsampling device, Microsampling Wing™ (MSW), using antiepileptic drugs in rats	[78]

4. Considerations for future application of advanced microsampling in metabolic phenotyping workflows

The use of advanced microsampling devices (of each technology type) demonstrated within the literature, have shown promise for bridging the gap in routine adoption of microsamples in metabolic phenotyping and within clinical research. However, there remain crucial shortcomings associated with microsample collections that need to be addressed. These include assessing the long-term storage and stability of advanced microsamples, pre-analytical reproducibility in microsample preparation, and analyte concentration variations between whole blood, plasma, and serum. This is needed before successful identification, validation, and quantification of novel biomarkers can occur. Indeed, if microsampling is going to be successful in population-based metabolic phenotyping research, patient self-variability/error is an additional area of contention which needs to be addressed. These are important considerations for future adoption of advanced microsampling devices in longitudinal metabolic phenotyping studies with regular participant follow-up.

4.1. Microsample collection and stability

DBSs have traditionally been used in a variety of applications due to the ability to provide a low volume, fast, and minimally invasive collection. In fact, multiple protocols and guidelines exist for collection of traditional dried microsamples (ie. DBSs), such as those for proper sampling of capillary blood [102], selection of filter paper [103], sample application to the carrier [103], and packaging and transport of samples [104] thus ensuring some level of sampling reproducibility in current applications of DBSs in the field of metabolic phenotyping. However, these sampling protocols do not apply to advanced microsamples which are limited to manufacturer SOPs. A research gap therefore exists in assessing optimal collection protocols that consider different drying times, temperatures, and storage conditions, which is inherently linked to microsample stability.

Assessment of long-term stability and optimal storage conditions of advanced devices is still necessary for best practice in metabolic phenotyping pipelines. Accurate detection of metabolite concentrations in biological samples naturally require sample stability, as fluctuations in environmental conditions, such as temperature and humidity, may have a deleterious effect on biological samples in the pre-analytical phase [6, 8]. For example, plasma and serum samples are known to be affected by storage conditions such as increased temperature and repeated freeze-thaw cycles [78]. Microsamples are no different, and thus considerations from the literature have included ensuring airtight and leak-proof packaging with desiccant to prevent deterioration from heat and moisture accumulation. As well as providing protocols for temperate conditions during transport (in multi-site studies), and timeframes for sample turnaround [100, 105].

Recent microsample applications have implemented the use of data loggers (EL-USB2+; Lascar Electronics, Erie, Pennsylvania, United States) to monitor the temperature, dew point and humidity of DBS samples during shipments. In one study, temperature and humidity were measured at 30 min intervals in DBS samples which travelled 11,600 miles in six days [6, 8]. Samples were observed to range from below freezing to over 25°C throughout the course of the journey. Although the compound analysed by Bowen et al. was not disclosed, the results of their in-house stability assessment (based on temperatures their DBSs encountered previously) were performed by setting the low QC level to -20°C and high QC level to 40°C. Taken together, these studies by Bowen et al. indicated that temperature extremes had no deleterious effect on the stability of a single compound in analytical replicates, with %CV $\leq 8\%$ on average, following ambient storage for 4 months, and at -20°C and 40°C storage for 48 hours [7, 8].

Another advantage of dried sample matrices is enhanced metabolite stability over a range of temperatures during storage and transportation. Most research concludes that analytes appear to be stable (for a variety of matrices) when stored in conditions with low temperature and humidity (~30%, akin to a lyophilised environment) [106]. Indeed, in a study by Strnadová et al., who examined the long-term stability of amino acids and

acylcarnitines, it was found that, of the analysed amino acids, valine was stable for up to 14 years in DBS samples stored at room temperature. However, other amino acids and acylcarnitines degraded more rapidly “per-year” [107]. In 2017, similar results were demonstrated in a lipidomic analysis of air-dried DBS cards stored with desiccants at different temperatures (4°C to 37°C) by Gao et al. [108]. Significant changes were noted for diacylglycerides in cards stored at 4°C and room temperature for up to two weeks, and in most lipids stored at 37°C. Also in 2017, Drolet et al. attributed increased variation in 350 DBS metabolites to storage temperatures up to 37°C in comparison to those at room temperature and -20°C [109]. The highest level of variability Drolet et al. observed was at 37°C on Day 14 of the storage conditions [109]. Future research must consider translating these findings to advanced microsampling devices, as this is yet to be performed beyond temperatures commonly encountered in a laboratory setting (-80°C, -20°C, 4°C, and room temperature).

A variety of procedures and environmental conditions have been investigated for their impact on metabolite stability of advanced microsamples. However, in the current literature, most research has not provided meaningful conclusions relevant to long-term storage outcomes for metabolites. For instance, ‘variation’ of metabolites (classes, panels, or even individually) is often reported without an associated direction (increase or decrease). Additionally, analytical reference points for assessing stability were often weak, and timepoints spanned relatively short periods (or were infrequent if conducted over a longer time). The interpretations of these stability data were inconsistent, mainly focusing on analyte recoveries and concentrations for classification of disease outcomes. Future research should seek to identify the reproducibility of purported metabolite stabilities based on storage duration and temperature. Furthermore, these should be over a greater period, with more frequent timepoints. This will allow for meaningful comparisons between studies, and address the limited research published on advanced microsampling device long-term stability.

4.2. Microsample preparation

Consistent microsample preparation including sub-punches (if using traditional DBSs), extraction, and automation is important for reproducible analyte extraction. Similarly, consistent enrichment is important, should detection limits need to be improved for quantification (preconcentration) and selective removal of interfering substances (sample clean-up) [110]. These processes are integral in metabolic phenotyping workflows as they determine molecular concentrations in complex biological matrices (including blood, urine, and body tissues) used to derive clinical and biological conclusions. As such, sample preparation optimisation (specific to the advanced microsample device in use, i.e., advanced dried sample, passive separation device, or whole biofluid collector) is vital for accurate interpretation of results.

In DBS microsample preparation workflows, a sub-punch (also referred to as a partial-punch) usually 3 mm in diameter, is taken. Theoretically, this is to reduce volumetric inaccuracies by using a fixed diameter punch, much like taking a fixed volume of plasma with a pipette. However, literature disagrees on the usefulness of this, largely due to sources of error related to the potential effect of hct and punch location bias when the whole spot is not analysed. Importantly, the size of DBS sub-punches can also artificially inflate values of metabolites compared to venous blood (when DBSs are simulated with venous blood as a result of inaccurate sample delivery) [48]. Accuracy and precision can be improved by including the fixed or individual hct values into the models that relate plasma and blood concentrations of analytes [49]. Following a sub-punch, the microsample is then ready for extraction. In metabolic phenotyping, extractions are normally performed using a variety of organic solvents to perform a protein precipitation prior to metabolic phenotyping [64, 65, 100, 111].

The move towards automating pre-analytical processes, like microsample preparation, has the potential to improve throughput and decrease manual error in metabolic phenotyping pipelines. In fact, this consideration has recently been adopted in the design of advanced microsampling devices such as the VAMS (Neoteryx; Torrance, California,

United States), which are also available in 96-well plate formats (VAMS 96-Autorack) (Neoteryx; Torrance, California, United States) [89]. Such designs have the potential for automation of extraction in advanced microsampling device metabolic phenotyping pipelines, however, this kind of use is yet to be reported on a large scale. Future applications of advanced microsampling devices in automated metabolic phenotyping workflows offer the potential to conduct large epidemiological studies. Derivatisation is often used in metabolic phenotyping pipelines to improve analyte volatility (i.e., in GC) and analytical sensitivity [112]. However, it may affect the detection and reproducibility of certain classes of metabolites and hamper the detection of compounds [58]. Currently, derivatisation has been automated for fatty-acid analysis of the phospholipid fraction of human plasma, and to date has been successfully applied to more than 28,000 samples of the InterAct project, measuring plasma-phospholipid profiles in over 12,000 diabetes cases and over 16,000 sub-cohort participants as part of a Cancer study [112]. Scales of this size have not yet been achieved in automated workflows for metabolic phenotyping of microsamples.

4.3. Determining an equivalent concentration factor

The type of blood collection performed to obtain a microsample (venous, arterial, or capillary), will influence the metabolite content of the sample [102, 103]. These differences mainly arise between capillary and venous samples (e.g., when comparing their sera or plasma). Differences are primarily due to capillary samples being derived from a dermal puncture which results in contamination of venous blood with arterial blood, interstitial fluid, and intracellular fluid [102]. One study comparing capillary and venipuncture samples found that spot size is the most significant factor when applying a correction formula to account for differences in metabolite concentrations [113]. The formula derived by the researchers was $Y = f(X, T, H)$. Which calculates the estimated standard value (Y) from DBS treatment (X), the treatment conditions such as temperature, humidity, drying time, shipment time, spot size (T), and donor characteristics such as health, age, and sex (H) reflecting potential interaction effects [113]. Use of newly developed microsampling devices which collect a fixed volume of blood may eliminate this consideration of spot size and punching [27].

Recently, correlations between DBS and dried serum spot (DSS) samples for analysis of 25-hydroxyvitamin D (25OHD) were performed using LC-MS/MS technology [114]. Karvaly and colleagues observed that the mean biases showed no correlation with the 25OHD levels between DBS and DSS matrices ($r \leq 0.0671$) [114]. In another study, DBS HbA1c (commonly used for diabetes monitoring) demonstrated over 95% correlation with standard venous samples, however results from DBS cards older than 7 days had to be adjusted, indicating that metabolite concentrations will vary between site of sample and matrices collected on [115]. Bridging studies should ideally be performed to validate that the concentrations obtained from capillary microsamples are equivalent to venous whole blood. Specifically, research is needed in the use of anticoagulants in venipuncture collections compared to advanced microsamples where anticoagulants are not included [106]. This is to overcome analytical and physiological issues associated with miniaturised volumes and complex collection matrices which demand method development to focus on robust extraction procedures and selection of sensitive analytical platforms.

4.4. Self-sampling

The bulk of literature in metabolic phenotyping of microsamples has utilised samples collected prior to the study (excess/remnant samples) or has utilised trained personnel to collect microsamples (primarily DBSs). Alternatively, pipelines that adopt self-sampling offer the potential for improved participant accessibility, and retention in research studies. Such patient-centric benefits are exemplified by the established use of blood glucose monitoring devices by diabetics [28]. However, routine adoption of self-sampling (with advanced microsampling devices) is currently limited to a few accounts, with only a single published application in metabolic phenotyping (to our knowledge) [53]. Currently, only Cvetko et al. has investigated the use of self-sampling for dried samples (DBS) (VAMS) (Neoteryx; Torrance, California, United States) and a passive plasma separation device

(Telimmune plasma cards) (Novilytic; West Lafayette, Indiana, United States) for metabolic phenotyping [53]. They reported issues in undersampling, hct variation, and haemolysis. Importantly, this study acknowledged its relatively small sample size ($N = 10$) who were “familiar with self-sampling procedures”, for which only 5 participants participated in sample collection reproducibility testing. Interestingly, the reproducibility testing required collection of hexaplicates for each of the three devices (i.e., 18 microsamples collected per participant in a single sitting) which potentially introduced repeated lancing pain. Furthermore, this account does not specify if these findings were device-specific [53]. Beyond metabolic phenotyping, advanced microsampling devices like the HemaSpot HF (Spot on Sciences; San Francisco, California, United States) have shown promising results in improving health-engagement in men with HIV-1 [28]. This study had a return rate of 75.5% with 418/554 enrolled participants returning a microsample. Of those, 80.6% (337/418 participants) returned kits that contained enough blood for testing. Of those who received a kit, interestingly 49 required a second kit due to losing the kit or difficulties collecting blood (11 of whom attributed this to problems with the lancet because of calloused fingertips). Kocher et al. concluded that home-collection with these devices for assessment of viral load could be utilised as a monitoring tool between clinical visits for patients who struggle with antiretroviral therapy adherence [28]. Since the COVID-19 pandemic, the general public are much more adapted to home-sampling, albeit with immunoassay kits, but the practice of self-sampling is now more accepted for health monitoring in the home. Advanced microsampling devices have the potential to leverage this familiarity of self-sampling for future research applications in the home-setting.

5. Conclusion

Microsamples (collections usually less than 50 μL) have demonstrated applicability in the field of metabolic phenotyping. This is evidenced in the literature by the use of both traditional dried microsamples as well as advanced microsample devices (advanced dried samples, passive separation devices, and whole biofluid collectors) for sample collections in mass spectrometry-based metabolic phenotyping. In particular the advance in microsampling technology has provided promising avenues to overcome some of the traditional challenges associated with microsampling, and paves the way for wider application in the field. Early adoption of microsampling in metabolic phenotyping literature indicates the vast potential for their integration into larger scale clinical/epidemiology applications. However, much evaluation and optimisation is still required from future studies to support the wide adoption of microsamples in replacing routine venipuncture for metabolic phenotyping workflows.

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Abbreviations

25OHD	25-hydroxyvitamin D
ACN	Acetonitrile
CV	Coefficient of variation
DBS	Dried blood spot

DPS	Dried plasma spot
DSS	Dried serum spot
DUS	Dried urine spot
EDTA	Ethylenediaminetetraacetic
GC-MS	Gas chromatography-mass spectrometry
hct	Haematocrit
LC-MS	Liquid chromatography-mass spectrometry
MeOH	Methanol
mL	Milliliter
QC	Quality control
TAP	Touch activated phlebotomy
UHPLC-MS	Ultra high performance liquid chromatography—mass spectrometry
VAMS	Volumetric absorptive microsampling
WB	Whole blood
µL	Microliter

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