# Chapter 2
# Collection and Preparation of Clinical Samples for Metabolomics

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**Abstract** A wide range of biofluids (urine, serum, plasma, saliva, etc.) as well as cellular and tissue samples can be collected and investigated in clinical metabolomic studies. The choice of sample is dependent on the clinical question being investigated with biofluids typically studied to identify biomarkers, whereas tissues and primary/immortalised cells are typically studied to investigate mechanisms associated with pathophysiological processes. Methods applied to collect samples, quench metabolism and extract samples differ between sample types from simple collect, dilute and analyse methods for urine to complex washing, quenching and biphasic extraction methods for tissues. The range of sample collection and extraction methods are discussed with sample-specific considerations highlighted. Finally, methods for imaging of cells and tissues and for *in vivo* metabolomic analysis will also be introduced.

**Keywords** Serum • Plasma • Urine • Human cells • Human tissues • Metabolic quenching • Extraction • Imaging

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## Abbreviations

| | |
|---|---|
| ATCC | American Type Culture Centre |
| BMI | Body mass index |
| BSTFA | N,O-Bis(trimethylsilyl)trifluoroacetamide |
| CHO | Chinese hamster ovary |
| CSF | Cerebrospinal fluid |
| DESI-MS | Desorption electrospray ionisation-mass spectrometry |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| ESI | Electrospray ionisation |
| GC-MS | Gas chromatography-mass spectrometry |
| HILIC | Hydrophilic interaction chromatography |
| HS-SPME | Headspace solid-phase microextraction |
| IPA | Isopropyl alcohol |
| LC | Liquid chromatography |
| LC-MS | Liquid chromatography-mass spectrometry |
| LLE | Liquid-liquid extraction |
| MALDI-MS | Matrix-assisted laser desorption/ionisation and mass spectrometry |
| MSTFA | N-Methyl-N-(trimethylsilyl) trifluoroacetamide |
| MTBE | Methyl tert-butyl ether |
| NMR | Nuclear magnetic resonance |
| PBS | Phosphate-buffered saline |
| RNA | Ribonucleic acid |
| SIMS | Secondary ion mass spectrometry |
| SOP | Standard operating procedure |
| SPE | Solid-phase extraction |
| SPME | Solid-phase microextraction |
| TCA | Tricarboxylic acid |

## 2.1 Introduction

The choice, collection and preparation of biological samples in clinical metabolomic studies can have a significant impact on the metabolomic data acquired, the quality of the analytical data collected and the conclusions derived from the study. Therefore, careful consideration of which sample type to collect, how to collect the sample to provide a metabolic snapshot of the sample at the time of sampling and how to extract samples is required. In this chapter, we will discuss the different types of samples that can be collected and analysed, highlight appropriate methods for collection and sample extraction and discuss current advantages and limitations. These discussions will be focused on untargeted metabolomic studies to investigate hundreds or thousands of metabolites in a single sample. Sample imaging and in vivo real-time analysis will also be introduced.

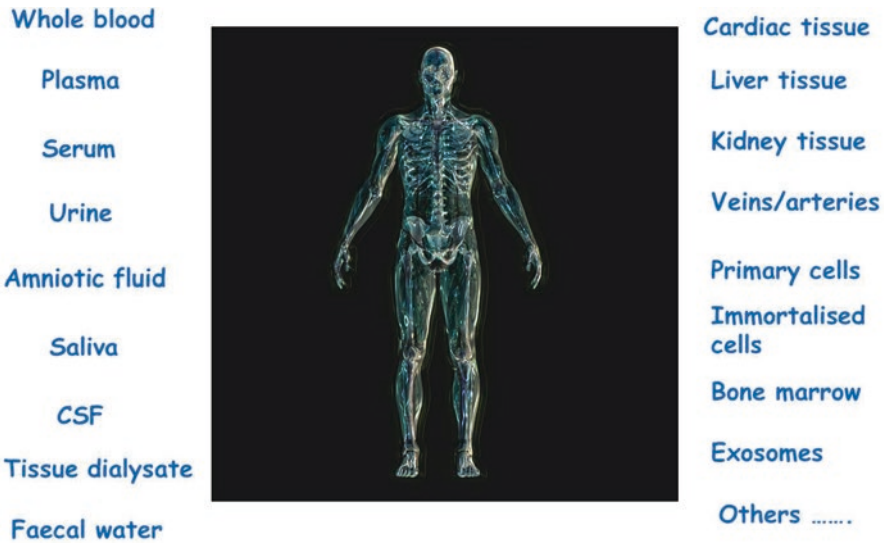


**Fig. 2.1** A summary of the different biofluids, cells and tissues studied in clinical metabolomics

### *2.1.1 Sample Types Investigated in Clinical Studies and Associated Considerations*

The range of samples that can be collected in a clinical study is wide ranging and can consist of biofluids, primary cells and tissues. Figure 2.1 provides an overview of different sample types accessible and applied in clinical metabolomic research, either currently or those that can be expected to be investigated in the future.

The sample type chosen can be defined by the clinical question being investigated. As examples:

(a) Biomarkers applied in the clinic are primarily assayed in biofluids, and therefore discovery studies to identify a single biomarker or a biomarker panel are typically also applied to biofluid samples.
(b) Studies to understand pathophysiological mechanisms are typically interested in a specific organ in the human body. To enable research to investigate the 'site of action' of a specific metabolic perturbation, then the organ should be sampled and studied.

However, in some studies, the preferred sample type cannot be collected, and a different 'surrogate' sample type has to be investigated. For example, the collection of 2000 kidney biopsies from healthy and diseased subjects to study metabolic mechanisms in kidney diseases is not feasible in relation to the expense of sample collection (e.g. surgery would be required for all subjects) and in performing an ethically acceptable study (e.g. the collection of kidney biopsies from healthy subjects is not ethically acceptable). However, the collection of urine or plasma samples could be performed as biofluid samples can be collected in an ethically acceptable

manner, and costs for collection would be significantly lower including for large-scale studies. Consideration as to whether the biofluid can identify metabolic changes related to the tissue of interest should also be undertaken. In this example, the analysis of urine, which is a by-product of the pathophysiological operation of the kidney, is a suitable 'surrogate' sample to determine metabolic or physiological changes in the kidney.

### 2.1.2 Sampling Considerations

The collection of samples requires a number of objectives to be met. The primary objective should be to ensure that the sample collected is qualitatively and quantitatively representative of the sample before it was collected. When this objective is met, then the data acquired from the sample is biologically representative of the question being asked, and any results can be viewed as valid. With this in mind, samples can be separated into two general classes: (i) metabolically active samples and (ii) metabolically inactive samples. Figure 2.2 shows the differences in relation to sample collection and extraction for these two classes of samples as defined for a mammalian cell culture. The extracellular metabolome can be typically viewed as a metabolically inactive sample, and the intracellular metabolome is viewed as the metabolically active sample.

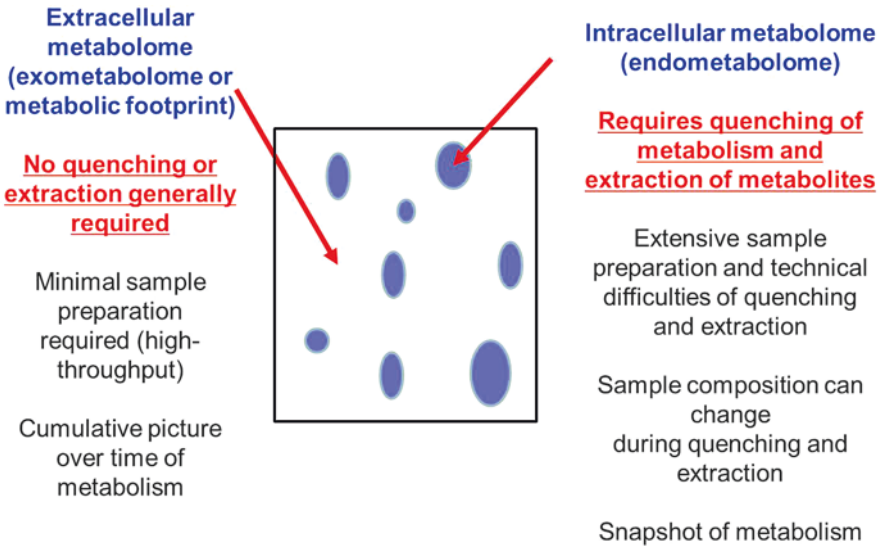


**Fig. 2.2** A comparison of sampling, metabolic quenching and extraction required for intracellular and extracellular metabolome samples. The example here is shown for the culture of mammalian cells

All cells and tissues can be viewed as metabolically active, and therefore quenching of metabolism is required and will be discussed later in this chapter. Biofluids can be defined as metabolically inactive as they are extracellular, but care should be taken. Urine is metabolically inactive, but serum/plasma, CSF and saliva could be viewed as metabolically active, and care should be taken. As a minimum we recommend that biofluids are stored on ice while being processed and then stored following processing for long periods at −80 °C.

Importantly, tissues require metabolic quenching and extraction. Metabolic quenching of tissues typically involves rapidly washing in a saline or phosphate-buffered solution and rapid quenching by placing in liquid nitrogen [1]. Most tissues are collected in an operating theatre with the exception of the muscle and skin (as a punch biopsy), faeces and the placenta, which is expelled after pregnancy. Health and safety guidelines do not allow liquid nitrogen to be placed in an operating theatre, and so tissues have to be transported to a different location, preferably on ice. Therefore, rapid quenching of tissue in this environment is not always feasible, and this should be considered when the requirement to study metabolic pathways with a high metabolic flux is required (e.g. the glycolytic and TCA metabolic pathways in cancerous tissue).

## 2.2 Biofluids

### *2.2.1 Urine*

Urine is one of the most widely studied biofluids in metabolomic research, primarily due to its ease of collection, as discussed later. The urinary metabolome has been used to investigate the metabolic consequences of disease for the entire body due to its being a major excretory route of water-soluble metabolites and xenobiotics [2]. The presence of both endogenous and exogenous metabolites means that urine metabolomics may be implemented for disease biomarker discovery [3], drug discovery and characterisation [4], determination of nutritional status [5] and effects of environmental toxicants [6]. To date over 3100 metabolites have been characterised in human urine, and this is considered the minimum number of metabolites present as new sample preparation and analytical techniques are likely to uncover other metabolites present at lower concentrations [7].

#### 2.2.1.1 Sample Collection

When collecting urine samples the timing of collection can make an appreciable qualitative and quantitative difference in the urinary metabolome [8, 9]. Typically there are three types of urine sample that can be collected, first morning void, spot urine and a 24 h urine collection [10]. Generally first morning voids are a preferred sample type, following an overnight fast of several hours thus reducing the effect of the last

meal or medication [11, 12]. Spot urine samples are taken at a time point during the day and are particularly common when some form of intervention such as dietary or pharmaceutical has been administered [10]. However, it is well known that a number of metabolites are excreted in a diurnal rhythm or cosine rhythm [8, 9]. Ideally, a urine sample would comprise of a pooled sample of all voids within a 24 and h period thus reducing the impact of any circadian variation in the sample, defined as a 24 h urine and representing a complete 24 h circadian cycle. Spot urine samples at specific time points that are uniform between all subjects such as first morning void also help to counter this problem when a 24 h sample collection is not feasible.

Collection of urine samples is predominantly easy in the general population. Subjects are asked to provide a urine sample into a pot and return the sample. Urine samples can be collected in the home and transported to a clinic, which is appropriate for 24 h urine samples, or spot urines can be collected at the clinic. No highly trained staff is required for the collection of urine samples. For immobile subjects urine samples can be collected with a catheter, and for babies urine can be collected with absorbent pads in nappies.

#### 2.2.1.2 Storage and Stability of Urine Samples

One concern with metabolomics is the time taken to collect all samples, extract samples and then collect a metabolic profile for each sample, as in most studies extended periods of sample storage is required, thus storage must have minimal/no effect on the metabolome of the sample being studied [13, 14]. Several studies utilising LC-MS, GC-MS and NMR have investigated the effect of different storage temperatures and number of freeze thaw cycles [13, 15–17]. It has been demonstrated that urine samples rapidly degrade when stored at room temperature even for short periods of time with glycolytic metabolites showing signs of degradation or metabolism [16]. For long-term storage, −20 °C has been demonstrated to have no effect on the urinary metabolome after storage for 6 months. However, −80 °C is recommended as no longer-term stability studies of length greater than 6 months have yet to be carried out applying untargeted metabolomics [2, 13, 16, 17]. Thus, it is optimal to freeze urine samples as soon as possible following collection to minimise the time spent at room temperature.

Analytical run times in large-scale clinical metabolomic studies can operate from hours to days in length during which time urine samples are kept in the autosamplers of LC-MS, GC-MS and NMR analysers at 4 °C. During this time, it is important that metabolic profiles at the end of the run are still representative of those in samples analysed at the start of the run. Studies suggest that 48 h at 4 °C does not significantly alter the urinary metabolome, as such it is recommended that only 48 h worth of samples should be stored at any one time in an autosampler [13, 17]. During the metabolomic workflow from sample collection to analysis, it is likely that samples will be frozen and thawed a number of times due to the time taken to collect and prepare samples. The impact of multiple freeze thaw cycles has been investigated using LC-MS, and it is reported that up to nine freeze thaw cycles have

no significant impact upon the urinary metabolome [13, 17]. However, it is prudent to limit the number of freeze thaw cycles to as few as reasonable possible [18].

A further complication to urine collection and storage is bacterial contamination and metabolism with a contribution to urinary metabolites by bacteria. While the urine itself is sterile in a healthy individual, bacterial contamination can be introduced via the urethra during urination. As such it is recommended to collect a midstream urine sample to minimise this risk [19]. Furthermore, a number of antibacterial additives such as sodium azide and sodium fluoride have been demonstrated to reduce metabolic variation as a result of bacterial contamination and metabolism [18, 20]. Storage of samples at −80 °C is also known to prevent any metabolism of urinary metabolites by contaminating bacteria with the added benefit of not adding any chemicals to the sample itself, which can be beneficial for GC-MS, NMR spectroscopy and LC-MS studies [18].

#### 2.2.1.3 Sample Preparation

Sample preparation for urinary metabolomics is an often overlooked piece of the experimental workflow yet plays a vital role in the quality, reliability and coverage of the urinary metabolome [14, 21–23]. To date the most widespread preparation methods for LC-MS or NMR are either the injection of neat urine following centrifugation or injection of diluted urine following centrifugation [10, 19, 23]. Both neat and dilute urine preparations have benefits for global metabolomics. Neat is unmodified and therefore contains the unadulterated metabolome; however, when analysed by LC-MS, the sensitivity to low-abundance metabolites suffers as a result of high-abundance co-eluting peaks and subsequent ion suppression. The high salt content of urine encourages the formation of a range of adducts within the electrospray source, in addition to fouling the LC column and the ESI source [24–26]. The dilution of urine prior to analysis reduces this ion suppression and may potentially allow detection of some less abundant metabolites, which were previously hidden by a high-abundance co-eluting metabolite. However, it has been shown that many low-abundance peaks are diluted to below the limit of detection using a ‘dilute-and-shoot’ method [24, 27, 28].

The use of solid-phase extraction (SPE) sample preparation methods, which incorporate a sample cleanup and a sample concentration step, is becoming more popular [28–30]. These methods allow unwanted urinary salts, matrix effects and proteins to be removed and with sample pre-concentration low-abundance metabolites become more easily detected [23]. Importantly, the use of SPE has been shown to have a similar repeatability to ‘dilute-and-shoot’ sample preparation suggesting that a more comprehensive coverage of the metabolome can be achieved without compromising on data quality [28, 29]. The use of such an extensive sample cleanup may also have the added benefit of reducing source and chromatographic column fouling and extending column life.

In GC-MS analysis, it is typical to lyophilise the urine samples prior to a chemical derivatisation step in order to improve derivatisation efficiency and thus sensitivity

[12, 31]. In these cases urine is typically derivatised using either BSTFA or MSTFA, although samples requiring storage are more stable following derivatisation with MSTFA [12]. Recently, a number of studies have begun to utilise liquid-liquid extraction and SPE as a sample cleanup step without the need for a derivatisation stage [32]. Furthermore, the use of headspace solid-phase microextraction (HS-SPME) has shown promise as a method of increasing sensitivity to volatile organic compounds in urine. Here urine is heated, and volatiles diffuse into the gas phase and become trapped on the SPME fibre before being thermally released into the GC-MS instrument [33, 34]. SPME has the benefit of not requiring any solvent and being a quick technique while incorporating a sample concentration step, which has benefits in terms of costs and environmental impact [33–35]. The high concentration of urea in urine can impact significantly on the quality of GC-MS data. The enzymatic removal of urea with urease is commonly applied to allow the detection of low-abundance metabolites, which co-elute with the broad urea peak [36]. However, some studies have reported a detrimental effect of urease treatment [37].

#### 2.2.1.4 Pre-analysis Normalisation

The solute concentration of urine is known to vary by up to 15 times both between and within individuals providing separate samples [38]. This is due to a number of contributing factors such as fluid intake and health status [38, 39]. Consequently, without a normalisation method to correct for this variation in urine concentration between samples, metabolite variation may be mistakenly attributed to the case study, when in fact they are a result of differences in urine concentration between individuals. Recently a number of studies have shown that equalising urine concentration during the sample preparation process greatly reduces the effect on the biological results of varying urine concentration. This is achieved by measuring the urine concentration using methods such as osmolality or specific gravity and diluting samples down to the lowest concentration [40, 41].

### *2.2.2 Serum and Plasma*

Blood serum and plasma are the second most frequently applied biofluid in metabolomic studies after urine. Blood provides a snapshot of metabolism that integrates many tissues in the human body through its interactions with these tissues and so provides a metabolic picture of global metabolism though with a lower level of specificity in comparison to urine. The choice of serum or plasma is an important but as of yet not fully answered question, and different research groups use serum or plasma. A number of studies have investigated the qualitative and quantitative differences between serum and plasma, though only small differences have been identified, which do not providea a clear choice for either of the biofluids [42–44].

Indeed serum and plasma have been applied in a range of different applications related to cancer [45], endocrinology [46], inflammatory diseases [47] and diseases of the cardiovascular system [48].

#### 2.2.2.1 Sample Collection

Unlike for the collection of urine and saliva, the collection of blood requires trained staff (phlebotomists) to collect and process blood in suitable volumes, and therefore collection is routinely applied in the clinic. The collection of dried blood spots is one exception that does not require trained staff. Sample collection can be performed by each subject, and samples can be transported at room temperature to the clinic by the subjects or via postal services (e.g. see reference [49]).

The main difference between serum and plasma is the presence or absence of clotting. For serum, whole blood is collected into tubes and is allowed to clot for a specified time and temperature before centrifugation to pellet the clot and cells and provides the liquid serum containing all metabolites and proteins not removed in the clotting process. Large differences in the time and temperature of clotting have been shown to influence the metabolite composition of serum, and standardised protocols should be applied [50, 51]. The authors of this chapter recommend allowing samples to clot at 4 °C to reduce any metabolic activity, which in the human body operates optimally at 37 °C. Plasma is the liquid volume of whole blood, and the collection of plasma does not involve a clotting process. Instead, whole blood is mixed with an anticoagulant to inhibit clotting followed by centrifugation to separate the liquid plasma from red and white blood cells. Typical anticoagulants include lithium heparin, EDTA and citrate. We recommend the use of lithium heparin, which is a high molecular mass biochemical unlike citrate and EDTA, which have similar molecular masses to metabolites, and indeed citrate is an endogenous metabolite.

For the collection of serum or plasma, whole blood is collected into different types of tubes following venepuncture [52]. The tubes allow for the collection of serum or plasma, with serum tubes containing no additive or a gel to aid clotting, and plasma tubes are coated with an anticoagulant to inhibit clotting. Tubes are inverted several times to allow mixing of anticoagulants with whole blood. The tubes are centrifuged, and the liquid fraction (serum or plasma) is transferred to separate tubes for storage [53].

#### 2.2.2.2 Storage and Stability

It is recommended that serum or plasma is stored as 0.5 or 1.0 mL aliquots at −80 °C. Both biofluids contain proteins and enzymes, for example, released from cells and tissues in the human body before sampling, which can provide metabolic activity. Processing of whole blood should be performed ideally at 4 °C with plasma and serum aliquots being thawed on ice before metabolite extraction. As discussed

for urine samples, the time-extracted samples placed in an autosampler at 4 °C should also be considered, and the authors recommend a maximum time of 48 h in an autosampler.

#### 2.2.2.3 Sample Preparation

Serum and plasma are a composite of water, metabolites and higher molecular mass biochemicals including proteins, RNA and DNA. Metabolites range from small ionic species like sodium and ammonium ions, through water-soluble metabolites to lipids, and the method of sample preparation is dependent on the metabolites to be investigated. All protocols have the objective to remove higher molecular mass biochemicals and extract metabolites into a suitable solvent system. The most frequently applied method to extract metabolites is liquid-liquid extraction (LLE) [54–56]. Here an organic solvent is added in excess to serum or plasma, which acts to precipitate the higher molecular mass biochemicals while allowing all or a subset of metabolites to remain in solution. A number of different solvents have been reported including methanol, acetonitrile, isopropyl alcohol (IPA) [57] and acetone [58], and the use of different temperatures and extraction times have been investigated [54, 59]. It is the choice of the researcher to define an appropriate solvent and temperature for their biological question and metabolites of interest; for example, the use of IPA for extraction of lipids is highly reported [57]. Following precipitation for a defined period of time, the samples are centrifuged to pellet the precipitate, and the supernatants are aliquoted to a different tube for further processing or analysis.

Whether the drying of samples is required should be considered in relation to the solvents applied. For GC-MS applications, sample drying is typically required prior to derivatisation with MSTFA. However, for LC-MS applications, sample drying can be avoided if an appropriate solvent is used for sample dilution. For example, for HILIC applications, which require injection of the sample in an organic-rich solvent, the samples can be injected directly or after dilution on to the chromatographic column without drying.

The presence of lipids, predominantly glycerophospholipids, can impact on the quality of data and the number of unique metabolites detected in untargeted studies [60, 61]. Selected extraction procedures or further sample manipulation steps can be applied to remove single or multiple lipid classes from extracted samples prior to analysis. The use of biphasic extraction methods to move lipids into an organic solvent and water-soluble metabolites into a water/methanol solvent, with each solvent immiscible in the other, provides the ability to analyse only the lipid fraction or the water-soluble fraction [58]. The use of complementary LC-MS assays can benefit from this approach, for example, the use of HILIC methods to analyse the water-soluble metabolites and the use of a $C_{18}$ reversed-phase method for analysis of lipids. SPE can also be applied, typically the sorbent is a $C_{18}$ phase which absorbs lipids while allowing water-soluble metabolites to be eluted and analysed [61, 62].

### 2.2.3 *Other Biofluids*

#### 2.2.3.1 Saliva

Saliva has for the most part been an overlooked biological fluid for metabolomic analysis yet is thought to accurately reflect the plasma metabolome. With this in mind, saliva sampling could make a desirable surrogate for plasma as it does not require specialist collection and is easy to collect and is non-invasive [63, 64].

Consideration of the type of saliva sample either stimulated, whereby saliva production is stimulated with citric acid, or non-stimulated (resting) is required. A comparison of the two has shown that TCA cycle and amino acid profiles are disrupted and found at lower concentrations in stimulated saliva samples as a result of dilution [65]. As such the majority of studies into saliva metabolomics choose to use unstimulated samples, usually following a period of fasting and delayed oral hygiene to prevent contamination [66–68].

Saliva samples are typically collected and then frozen while awaiting analysis. Storage at −20 °C for up to 3 weeks has been shown to have no detrimental effect on the salivary metabolome [65]. Prior to any sample preparation, saliva samples are centrifuged to remove cellular and food debris [63].

The majority of salivary metabolomic studies have been performed using NMR, whereby samples are buffered in 0.2 mol $L^{-1}$ phosphate buffer and diluted with $D_2O$ [69, 70]. In LC-MS-based analysis, saliva has been hydrolysed using both NaOH and HCl in order to hydrolyse and release metabolites from proteins and cells present in the sample [63]. Hydrolysis via an ultrasonic probe has also been investigated as an enhanced hydrolysis method [63]. The use of any hydrolysis step has been demonstrated to significantly increase the number of metabolites detected; furthermore, a sample pre-concentration step is often required due to the low abundance of salivary metabolites [71].

#### 2.2.3.2 CSF

Cerebrospinal fluid (CSF) is a fluid that fills the spinal column and brain ventricles and plays a role in fluid regulation and nutrient transport in the central nervous system [72]. Metabolomic analysis of CSF offers great promise for understanding neurological disorders such as Alzheimer's disease and amyotrophic lateral sclerosis [73].

CSF samples are collected via ahealthcare professional using a lumbar puncture. In a non-traumatic collection, the fluid should be clear and void of blood. Following collection, samples require centrifugation to remove any cell debris and then frozen at −20 °C for long-term storage. For short-term storage, the CSF metabolome is known to be stable for up to 2 days at 4 °C but known to be unstable at temperatures exceeding 5 °C [72, 74].

To date CSF samples have mainly been analysed using basic dilution sample preparation. However, lyophilisation and pre-concentration have been used in NMR to improve coverage of the metabolome at the loss of some volatile organic metabolites

[74]. For GC-MS analysis, samples may be derivatised using either BSTFA or MSTFA [75]. So far, very little work has been completed to develop new sample preparation methods for CSF metabolomics; given the sparse nature of these methods, it may be of interest to develop new methods utilising SPE or SPME in order to increase the ability to detect lower-abundance metabolites.

#### 2.2.3.3 Sweat and Breast Milk

In addition to the biofluids already discussed, a number of others are used although much less frequently. Sweat composition is known to be modified in several disease states and as such is becoming a more popular matrix for metabolomic analysis [78]. Sweat collection is stimulated using a sweat inducer, which is applied to the skin, heats the area and collects the sweat [76, 77]. Few studies have analysed sweat but those that have used a basic sample dilution or neat sample following centrifugation for LC-MS or NMR analysis [76, 78]. Additionally, a sample cleanup method to remove the high salt content of sweat samples and allow a pre-concentration step using SPE has been developed [78].

Human breast milk has also begun to become a more popular biological fluid for metabolomic analysis particularly in the field of nutritional metabolomics for infants [79]. Care should be taken to record when the patient last fed to reduce the impact of the diet on the breast milk metabolome, and samples are cooled to as cold as possible to minimise any degradation [81]. Here for both LC-MS and NMR, a number of methods have been reported for extracting the polar and non-polar metabolites [80, 81].

## 2.3 Primary and Immortalised Cells

Metabolomic studies in human biological samples have mainly focused on the analysis of biofluids for clinical applications such as disease diagnosis or prognosis [82]. Metabolic profiling of a whole organism does not provide information about specific cell types under different conditions, which may be important for the development of drugs targeting specific cell phenotypes. In this regard, metabolomic studies of cell lines can be used to complement the information provided by whole organism metabolic phenotyping [83]. Metabolomic studies of mammalian cells are easier to perform and to interpret because there are no confounding factors to consider such as genotype, gender, age, BMI or alcohol intake, which are present in other clinical metabolomic applications [83, 84]. Another advantage of performing metabolomic experiments on cell lines is the relatively easy correlation with other ‘omics’ approaches such as transcriptomics or proteomics, thus enabling the construction of biological networks and pathway interactions at the system levels [85]. Cell culture metabolomics has been used in different areas including drug discovery and foodomics [83, 86, 87]. It has been explored recently for metabolic tracer and flux analysis [88, 89] and as a tool in basic biology to understand molecular mechanisms such as metabolic reprogramming in cancer cells and the Warburg

effect [90, 91]. Different cell lines from different organs, including the first human cancer cell lines, have been collected in cell culture biobanks and can be easily accessed from biological resource centres such as the American Type Culture Centre (ATCC, www.atcc.org). Cell culture systems are divided into primarily cell culture, cell lines and cell strains, which can grow in suspension or adherently [92].

### 2.3.1 Considerations

Metabolomic experiments in cell lines face different challenges in sample preparation in comparison with the analysis of body fluids. Some of these issues include variability of growth medium formulation, influence of number of passages, metabolic quenching and metabolite extraction, which are time-consuming and might lead to metabolite degradation and leakage from the cell before extraction [86, 93].

Some of the above-mentioned issues in cell metabolomics can be solved by appropriate experimental design and the development of standard operating procedures (SOPs) for metabolite extraction. Experimental design is important whichever sample is to be studied [94, 95]. For example, it is also recommended to randomise both sample extraction and sample analysis accordingly. Cell cultures grown in different flasks or wells, and subsequently treated in a similar way (i.e. drug treatment), are considered as biological replicates [83]. Different aliquots sampled from the same flask or well after a defined treatment can be considered as technical (and not biological) replicates. For cell culture metabolomics, six biological replicates (i.e. from 6-well plate cultures) are recommended.

Another concern in cell line metabolomics, especially when comparing different cell types, relies on variations in growth media formulation. It is recommended to use the same media (and batch of media) for all cell lines to reduce variability in metabolic profiles. However, the use of sub-optimal media can also affect the metabolic phonotype because cells might not achieve the same growth conditions [93]. Mammalian cell culture media, for example, are complex mixtures containing several buffers, amino acids and other variable components such as foetal bovine serum that can cause significant ion suppression effects in LC-MS. When analysing intracellular metabolites, the growth media must be efficiently washed from the cell pellet to avoid contamination with exogenous components [94]. On the other hand, when analysing extracellular metabolites, particular attention should be taken to method development and evaluation, to avoid leakage of intracellular metabolites into the extracellular medium during sampling [95].

### 2.3.2 Sampling and Extraction

Metabolic profiles should represent the physiological status of the cells at the time of sampling. Therefore, metabolic quenching constitutes the key step to minimise changes in metabolite levels, to improve the reproducibility and to avoid

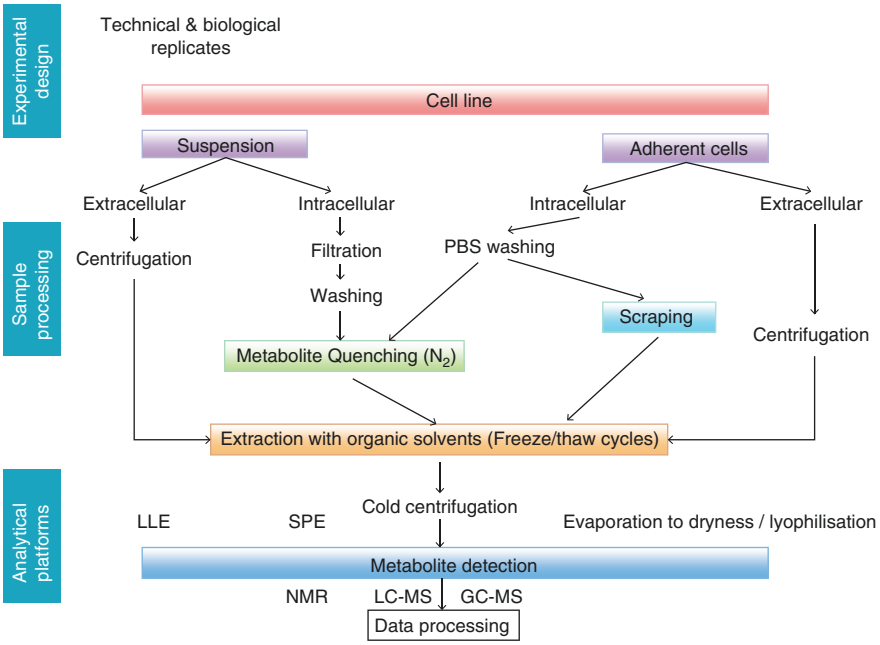


**Fig. 2.3** Schematic methodological workflow proposed for metabolic quenching and metabolite extraction from mammalian cell cultures

misleading results [93, 96]. Therefore, metabolite extraction from cell lines must be performed as quickly as possible to avoid enzymatic reactions that can change the qualitative and quantitative composition of the sample. A schematic representation of the methodological steps in metabolite extraction from cell lines is presented in Fig. 2.3.

The analysis of the extracellular metabolome, also known as the exometabolome, metabolic footprint or spent culture media, can provide significant advantages as defined in Fig. 2.2. Sampling is relatively simple with separation of media from cells in suspension achieved either by cold centrifugation or by the use of low molecular mass cut-off filters [97]. It is recommended to perform these procedures rapidly in order to avoid metabolic activity and quantitative changes to the metabolic profile. Cold centrifugation usually takes 5–15 min to complete, and in some cases, a washing step is needed to remove salts interfering with the mass spectrometry analysis [93]. Filtration is quicker, but it is more expensive, and the membrane can get easily blocked [98]. A robust fast filtration sampling method has recently been reported called MxP® FastQuench, followed by lipid/polar extraction for cells in suspension. This method suggests an efficient metabolite recovery and the potential to be extendable to all mammalian cell types [99]. The collection of the extracellular metabolome in adherent cells is easier because the media can be collected by pipetting, followed by a cold centrifugation step to eliminate cell debris. Metabolite extraction is then performed by adding the extraction solvent. After centrifugation,

the supernatant is analysed directly, or evaporated for further reconstitution for the preferred analytical procedure [100].

Intracellular metabolite profiling of cells in suspension can be achieved following metabolic quenching by heating or by adding ice-cold solvents. Here the suspension (a composite of media and cells) is sampled followed by metabolic quenching and typically subsequent separation of cells from the media and solvents applied during quenching. The addition of ice-cold 100 % methanol at −40 °C for metabolic quenching should be avoided because it might lead to metabolite leakage after membrane solubilisation [86, 101]. Cold isotonic saline quenching solutions at 4 °C have arisen to prevent cell membrane damage. For cells in suspension, a mixture of acetonitrile/water (1:1) was demonstrated as optimal for metabolite extraction in a detailed study with different conditions for Chinese hamster ovary (CHO) cells [102].

Analysis of intracellular metabolites from adherent cells requires some additional and critical steps. The medium must first be discarded either by pouring it from all samples simultaneously or by aspiration with a pipette or a vacuum pump. Samples are then washed two to three times with ice-cold phosphate-buffered saline (PBS) to remove any medium residue. The excess of PBS is then washed out also by aspiration from all the samples; this is a critical step because the PBS can generate ion suppression in mass spectrometry analysis [103]. The extraction solvent (stored in the freezer at –20 °C) is then added to the cells in the range of $1–2 \times 10^6$ cells $mL^{-1}$, and this volume is consistent for all the samples. Plates/flasks can be incubated at −80 °C for 10 min, and then the cells are scraped to detach them from the growth surface for a final centrifugation step where the supernatant contains the metabolites of interest [103].

Different solvent compositions can be used for metabolite extraction from cell cultures. A mixture of methanol/water (4:1) has been reported, but different compositions containing methanol/acetonitrile/water can favour the extraction of highly polar metabolites such as nucleotide triphosphates [104]. Some authors have described the use of combined quenching and extraction procedures. A quick method for metabolite extraction involving a one-step washing with water, followed by direct addition of liquid nitrogen and a 1min solvent extraction step using a methanol/chloroform mixture (9:1), has been reported [105]. A recently proposed method for the analysis of cell metabolism using LC-MS and isotope tracers has been reported. They suggest a polar extraction solvent-containing methanol/acetonitrile/water (5:3:2), followed by a cell scraping procedure to fully wipe debris from the growth surface [89]. Others have reported cell scraping into an extraction solvent as an optimal procedure to simultaneously quench and harvest adherent cells [106].

For adherent cells, a trypsinisation procedure is commonly used to detach cells from wells or flasks. However, it has been reported that this procedure might affect the metabolic profiling because extra steps of washing and centrifugation can result in metabolite lost. In addition, this enzymatic procedure is cell type dependent, so if the cells are exposed to trypsin for more than 2–3 min, then cell lysis can occur with subsequent intracellular metabolite leaking [107]. Supernatants from simultaneous quenching and extraction can be directly injected into the mass spectrometer.

However, depending on both the number of cells in the experiment and the extraction method used for metabolite isolation, the concentration of some metabolites can fall below the limit of detection of the analytical instrument. A sample concentration procedure can be performed by either evaporating to dryness or lyophilising the samples and subsequent reconstitution with an appropriate solvent for the analytical platform of choice.

### *2.3.3 Normalisation*

Normalisation of metabolomic data from cell line studies constitutes an important topic to consider when developing and evaluating a method. Cell counting can be a good practice, and it must be performed immediately prior to the incubation period. Separate experiments have to be performed exclusively to determine the number of cells. Alternatively, normalisation procedures including total protein content and total peak area have been used [108, 109]. DNA concentration has also been recently proposed as an efficient and robust method for normalising metabolomic data [110].

## 2.4 Tissues

A wide array of tissues have been studied applying metabolomic approaches including muscle [111], cardiac tissue [112], liver [113], cancerous lung tissue [114], placenta [115], arteries [116] and skin [117]. The selection of a tissue sample provides localised metabolic activity snapshots relevant to the tissue chosen in comparison to biofluids, which can reflect changes in multiple organs in the human body. The study of tissues is normally performed to investigate mechanistic differences related to pathophysiological processes.

### *2.4.1 Sample Collection, Storage and Stability*

Human tissues are metabolically active and therefore require rapid metabolic quenching when they have been collected. Typically, tissues are collected, then are rapidly washed in a phosphate-buffered aqueous solution or in saline to remove as much blood as possible and are then rapidly frozen in liquid nitrogen to quench metabolism [118]. Washing of tissues to remove as much blood as possible is an important step as the blood metabolome will be different to the tissue metabolome and therefore contaminates the tissue metabolome. As for other sample types, tissue should be separated into appropriate size aliquots and stored frozen at −80 °C. For untargeted or targeted metabolomic studies, typically 20–100 mg of tissue is required to provide good coverage of the tissue metabolome [115, 119]. One exception is faeces, where up to 2 g of material is typically extracted [120].

Human tissue samples are normally collected via an invasive procedure with the exception of a small number of tissues, which can be collected with minimally invasive or non-invasive techniques. The placenta is a large pregnancy-related tissue, which is naturally expelled from the body following delivery of the foetus and which can be sampled and studied [115]. However, metabolic activity can still be in operation during this process, and different levels of oxygenation and nutrient delivery before and during sampling should be considered; it has been shown, for example, that the metabolic composition of tissue early and late in the first trimester is different and related to the level of blood delivery of oxygen and nutrients [115]. Skin and tissue biopsies can be collected with minimally invasive techniques; faeces can be collected without an invasive technique, while most other tissues are collected in a clinic or operating theatre.

One important aspect for tissues is that they are not homogenous when compared to biofluids. If small samples are collected from a large tissue, then careful consideration must be taken to ensure the same area of the tissue is collected from different subjects. For example, the adult human liver weighs approximately 1.5 kg and has four lobes, which are metabolically different. Therefore, the collection of 50 mg of tissue has to be carefully considered to ensure the same area of the liver is being sampled. Small metabolic differences have been observed in different parts of a tissue. For example, differences between the centre and edge of placenta have been reported [115].

The metabolic stability of tissue samples has not been studied in detail applying untargeted metabolomic approaches to our knowledge. We recommend similar storage times and range for all samples collected for a study and matching of storage times between different biological classes. As for other sample types, we recommend storage of the tissue at −80 °C and storage in an autosampler at 4 °C following extraction for a maximum of 48 h.

### *2.4.2 Sample Preparation*

The preparation of tissues can be separated into two processes. Tissue is normally homogenised applying physical techniques including manual mortar and pestle [115] or ball grinding with stainless steel or silica particles [121, 122]. Normally, this process is performed with the tissue and extraction solvent combined as the physical process of homogenisation also results in cell lysis and extraction of metabolites from the tissue into the solvent. The solvent or solvents applied vary depending on the assay to be applied. Extraction methods are either monophasic (one miscible solvent system) or biphasic (two immiscible solvent layers). Monophasic extraction methods provide an extract, which typically provides greater coverage of the metabolome in a single solution. Biphasic extractions have the advantage that water-soluble metabolites can be separated from lipids through the use of two immiscible solvents. Although each solvent system contains fewer metabolites, when analysed separately and the data combined, then a greater

coverage of the tissue metabolome is observed because of fewer interferences in the assays applied [123]. Monophasic extractions typically apply a water/methanol or water/acetonitrile solvent system through a combination of water/chloroform/methanol as a single-miscible solvent system has been used [124]. The use of IPA with other solvents to selectively extract lipids has also been applied [123]. For biphasic extractions, water and methanol are typically applied with a non-polar solvent and chloroform [115], dichloromethane [119] and MTBE [125] have all been applied. The most common technique applied is the Folch extraction, developed in the 1950s, which extracts into a single-miscible solution of water/chloroform/methanol followed by an addition of further water to create phase separation [126]. More recently, the Matyash method has grown in frequency of application, especially for the analysis of lipids [127]. One experimental limitation is observed in biphasic extractions. The two immiscible solvents are separated by a layer composed of the cellular debris. The aliquoting of the top layer is relatively easy, but the aliquoting of the lower layer requires the puncturing of the debris layer with needle or pipette which can cause some of the debris to enter the lower phase and contaminate it. Chloroform and dichloromethane are the lower layers when applied with methanol and water as their densities are greater than water. The water/methanol solution is the lower layer when applied with MTBE as MTBE has a density less than water. Therefore, the choice of solvent can depend on whether water-soluble metabolites or lipids are to be investigated. If lipids are to be investigated only then MTBE/methanol/water is appropriate, as the lipids will be present in the upper layer, whereas if water-soluble metabolites are to be investigated, then a chloroform/methanol/water extraction method is appropriate to ensure the water/methanol solution is the top layer.

### *2.4.3 Pre-analysis Normalisation*

The normalisation of tissue mass extracted is an important aspect of tissue metabolomics. With only a 5–10 % difference in the masses of tissues to be extracted, then the same volume of extraction solution can be applied. However, with larger variations of tissue masses being extracted the volume of solvent should be normalised to the mass of tissue. For example, if you were extracting two tissues of mass 20 and 40 mg, then you would use 2× the volume of solvent for the 40 mg tissue compared to the 20 mg tissue. This ensures the ratio of tissue and solvent is identical for all samples as this ratio can influence the percentage recovery of metabolites [128].

## 2.5 Cell and Tissue Imaging

The sampling of cells and tissues typically involves the homogenisation of tissues and the lysis of cells during the extraction protocol. These processes remove relevant qualitative and quantitative information on the distribution of metabolites within a single cell or the collection of cells in a tissue. The subcellular location of

metabolites can provide further data in many clinical studies, which aid mechanistic interpretation. Imaging of intact cells and tissues can provide information on the spatial distribution of metabolites. A range of different imaging technologies can be applied dependent on the sample size and spatial resolution required.

Mass spectral imaging is frequently applied in metabolomics and includes MALDI-MS imaging [129], DESI-MS imaging [130] and SIMS imaging [131]. Thin tissue slices are prepared followed by analysis. Some controversy has been centred on whether paraffin-imbedded tissues, used commonly in pathology, could be applied for mass spectral imaging; recent work has shown the applicability of these sample types [132]. These imaging techniques raster the source across a tissue and collect a mass spectrum at each pixel of the sample. The mass spectral image is a composite of all the pixels and through computational analysis the distribution of different metabolites can be visualised. This approach will be discussed further in Chap. 12.

## 2.6 Studies Without the Need for Sampling

The importance of a suitable method for sample collection and preparation has been highlighted for the entire sample types discussed in this chapter. Cells and tissues require rapid metabolic quenching, which is not always feasible in the clinical environment. The time required for preparation and analysis of samples can be a number of hours, which is not ideal when data is being applied for clinical decision-making, especially during surgery. The ability to collect data *in vivo* and in real time during surgery and the use of these data for rapid clinical decision-making will move metabolomics into the operating theatre. The recent invention and translation of the intelligent knife (iKnife) is the most significant example of *in vivo* and real-time data collection being applied during surgery. Here the surgeon applies electrosurgical knives, which use an electrical current to rapidly heat tissue, cutting through it while minimising blood loss. This process vaporises the tissue and releases a smoke plume that can be sucked through a tube into a mass spectrometer placed in the operating theatre to provide real-time data for clinical decision-making [133]. For example, this technique can differentiate between tumour and healthy tissue allowing all of the tumours to be removed while not removing too much healthy tissues, both allowing a more positive clinical outcome [133]. Extensions into colonoscopy and other applications are expected in the next 5 years [134].

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