Chapter 13

NMR Spectroscopy of Cell Culture, Tissues, and Other Biofluids

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13.1 General Introduction

Cellular materials are amenable to NMR spectroscopic investigation, and can reveal a wealth of information relating to intracellular and extracellular metabolome composition via metabolic profiling. In this chapter, we consider aspects of the sampling and analysis of biological materials from sources of varying structural complexity including cells, tissues, and whole organisms. On account of our own expertise, we focus predominantly on animal samples; specific aspects relating to plants are well covered in other texts.[1-3] While whole organisms and tissue commonly provide an ensemble of cell types, cultured cells can offer a unique perspective on various individual cell types and their corresponding suite of specialised functions. Cells in culture can also be analysed via their metabolic footprint in the culture media, offering a complementary set of measurements to those obtained that describe the intracellular metabolome. In this chapter, we also consider the sampling and analysis of specialised body fluids that can provide specific organ-, function-, and system-level information, and those that have been explored in metabolic profiling studies to date are discussed (specifically faeces, cerebrospinal fluid, milk, seminal fluid, and bile). In doing so, we highlight the need to accommodate their unique physical and biochemical properties (e.g. composition, dynamism, etc.) in order to generate high-quality NMR spectra, and direct the reader to exemplars in the scientific literature.

13.2 Sampling, Extraction and Analysis of Cellular Material

13.2.1 Introduction

Metabolomics is focused on characterising biochemical phenotypic traits and the variation these display over time, space and between individual entities. Therefore, the main aim of biosampling for metabolomics applications is to halt (quench) cellular metabolism as quickly as possible, in a way that keeps the chemical milieu of the sample intact and sufficiently representative. Metabolite pools (e.g. nucleotides) can turn over rapidly, with some responding at the (sub)second-timescale[4]. There is consensus that rapid quenching of metabolic processes is critical to ensure subsequent metabolic profiles best reflect the sample at the chosen sampling timepoint. Consequently, collection of samples for the purposes of metabolic profiling analysis typically involves methods

that are aimed at minimising the effects of the sampling process itself (e.g. continuation of metabolism on the bench). Another major consideration is the sufficient isolation of the sample from its immediate environment, which is particularly important when the composition of the extracellular matrix is dominated by one or more high concentration components, something that is common in cell or bacterial culture (e.g. high glucose concentration and low intra- to extracellular volume ratio). Failure to do so (e.g. with insufficient washing) can affect the quality and validity of resulting NMR spectra, with minor metabolites signals in multiple regions difficult/impossible to deconvolute from those of more abundant species.

Clearly, there are numerous factors that need be considered prior to sampling (e.g. growth conditions such as culture medium/serum used); these play an important role for the metabolite profiles of bacterial or tissue culture samples, and thus should be controlled for within a set of experiments. [5-7] It is also known that cellular metabolite concentrations depend on cell density and confluence of the cells. [8] The experimental system itself places demands on the analytical workflow; cell type and whether cells are adherent or cultured in suspension played an important role in determining which preparative methods maybe the most suitable. Other variables are cell wash cycles, solvent selection, addition of chelation or buffering compounds, use of filtration for suspension culture, and use of scraping devices for sampling of monolayer cultures.

Note: The analysis of *intact* tissues by magic angle spinning (MAS) NMR spectroscopy provides a unique approach to characterisation of various aspects of composition, cellular metabolism, compartmentation, and provides valuable insight on the tissue as an ensemble. Such analyses are also particularly well-suited to the provision of rapid histopathological information based on metabolic signatures; NMR spectroscopic platforms are now being introduced into surgical settings to augment existing assessment techniques and are elsewhere in this book.

The preparation of NMR-ready samples of whole organisms, intact tissue specimens, and cell culture materials follow the same pipeline or workflow. This can largely be divided into three main sets of tasks that precede spectroscopic analysis (see Figure 13.1):

1. Sampling and separation of the cellular material from its surrounding environment

2. Adequate quenching/cessation of metabolic processes to best preserve the metabolome status at the time of sampling
3. Extraction of metabolites according to target criteria for metabolome coverage

[Figure 13.1 near here]

Depending on the specific workflow and sample type, these steps may be integrated, and it is therefore useful to consider them in a holistic manner when approaching an analysis.

13.2.2 Sampling Cellular Material

At present, NMR spectroscopic methods for the routine analysis of single cells is not feasible in the context of metabolic profiling studies, and therefore material from multiple cells are combined to provide sufficient sample. The relative insensitivity of NMR spectroscopy compared with other spectroscopic and spectrometric techniques is well known, and while the analysis of very dilute samples is possible by the acquisition of a large number of transients, this obviously reduces sample throughput and increases per-sample resource cost. As with other biofluids described in this book, high-throughput analysis of samples with natural-abundance isotopes is restricted to ^{1}H for profiling applications, additional experiments using ^{13}C, ^{15}N and ^{19}F are possible and typically used for metabolite identification. Despite the requirement for sufficient material to overcome the inherent instrumental insensitivity, general sample requirements are not prohibitive for most applications; for tissues samples, a typical sample of around 100mg is ideal, with a lower limit of around 20mg; for mammalian cells in culture, adequate material for both intracellular extracts and extracellular media can be obtained from confluent T75-size flasks, although six-well plate cultures can be used (target of $5x10^5$ - $5x10^6$ cells), and some researchers prefer using larger cultures where feasible (particularly for microbial culture).

Notwithstanding the need to select a source that can provide sufficient and representative material at a given sampling time, the analyst must first decide on the type of sample that is most appropriate. Whole-organism analysis is commonly employed in areas of research where

subsampling would be prohibitively time-consuming, result in dramatic and confounding changes to the samples, or where the organism is small or structurally simple.[9-12] Ideally, the organism must be taken from its environment (natural or laboratory), and metabolism should be stopped before it responds to the sampling procedure to exclude confounding variation. Similarly, the cells of the organism should remain intact during this procedure as not to interfere with subsequent extraction step(s), back at the laboratory bench. Some sampling locations may present specific challenges for adequate sampling that are not present in the laboratory (where all instrumentation and facilities are near at hand) or in surgical/clinical scenarios (where the sample source can commonly be requested to attend a particular site); such sampling locations may be difficult to access, or associated with physical factors that limit what is practicable (e.g. weather, travel time to laboratory). For these reasons, this approach to sampling is employed commonly in the field of environmental metabolomics research, and measures put in place to ensure sufficiently rapid and effective isolation, storage and transport.

Whole organism sampling in a laboratory environment may also include cells in culture (i.e. single-celled organisms), which require isolation from their growth media and transfer from the culture vessel for further processing and analysis. For those suspended in culture media, this can be easily achieved by filtration or centrifugation, which separates the supernatant, and can be efficiently integrated into the laboratory workflow; using a filtration and syringe method, Bort *et al.* recently demonstrated reduced quenching and extraction time for suspension culture.[13] Adherent cells in culture present an obvious additional requirement in the sampling procedure in that they require detachment from the culture vessel surface as well as separation from the growth media. This is achieved by gentle scraping with a plastic implement across the vessel surface, which dislodges the cells and allows them to be removed; this step is typically integrated with quenching solvent addition (see below). Removal and/or sampling of culture media can be achieved by using simple suction apparatus, leaving the adhered cells attached to the culture flask. If required for subsequent analysis, media is typically centrifuged immediately after sampling to remove particulate matter and frozen for storage using by placement on dry ice or into a liquid nitrogen bath. Optionally, and dependent on the constraints of the experimental design/aim, samples may

be washed with salt buffer prior to quenching to remove residual media components, and thus reduce the potential conflation of intracellular and extracellular metabolic signatures. Ice-cold (4 C) saline / PBS/ Ringer's buffer/ methanol are often used as wash solution. While removal of the growth media may reduce the influence of the sample matrix on the derived profiles of harvested cells, there is the potential for intracellular metabolite pools to be altered during this process, due to the additional time required to perform the procedure, as a consequence of rapid cellular responses to altered culture environment or due to leakage. Trypsinisation is an alternative method for harvesting cell samples from adherent culture, and the effect of trypsin as an agent to detach adherent cells from their growth container has previously been assessed in relation to the metabolome using various platforms.[14-16] In brief, there are several limitations to this approach: i) it requires the addition of serum-containing media and introduces the requirement for further wash steps that may confound analysis of the metabolome profile; ii) the additional time required (~3 mins) for the samples to detach while in altered media conditions may allow confounding metabolic changes to occur; iii) the application may directly cause metabolic responses.

In the majority of cases, the vasculature in and around excised tissue samples will contain blood. In order to minimise the influence of conflating the blood metabolome profile with that of the target sample tissue, it is common to ensure that tissues are washed. Allwood *et al.* (2012) suggest that washing with cold saline solution will remove extra- and intra-tissue blood prior to freezing and prevent cross-contamination of tissue types.[17] To avoid significant post-operative / post collection changes in the tissue metabolome, it is recommended that samples be cooled with a cryogen as soon as is practicable; the reduction of the sample temperature (e.g. -196 C for liq. N_2) results in cessation of metabolic processes within a few seconds. Rapid freezing of tissues can also be routinely achieved by full immersion in a cryogen, typically liquid nitrogen (N_2 (liq.) in a process known as 'snap-freezing'. Haukaas *et al.* (2016) examined the effect of freezing delay on metabolic profiles of excised tumor tissue, indicating that delay in snap freezing samples lead to NMR-observable changes, and suggesting a delays to freezing be minimized (less than 30 mins).[18] While keeping temperatures as low as possible whenever feasible, this is not always possible,

although it would appear there is consensus that samples should be maintained below −20 C during sample harvesting.[19]

5.2.3 Quenching Metabolism

The immediate cryogenic cooling of snap-freezing has clear benefits for sampling of culture media and tissues. It is therefore common for quenching steps to be directly integrated in the sampling procedure. For adherent cells, a quenching solution is applied (cold methanol/ cold aqueous methanol is often used) as soon as the wash solution/ media has been removed and cells are scraped off whilst remaining in contact with the quenching solution.

The problem of quenching is arguably bigger in microbial systems. As most microbes grow in broth or media of some sort, metabolite carry-over from these media is a potential issue - quenching also requires the separation of the intact cells from the growth medium. The pool sizes of the intracellular metabolites are at a maximum comparable to those of the extracellular matrix or medium (only for eukaryotes like yeast). For all bacteria, the absolute abundance of metabolite outside the cells by far outweigh the abundance inside the cells. Methanol quenching, during which cells are first rapidly cooled in -40 C methanol has been proposed [20] and this method with minor modifications became the *de facto* standard for some time.[21] Several studies reported cellular leakage induced by methanol quenching, particularly for Gram-negative bacteria.[22-24] Rapid filtration, in which cells are transferred onto a filter paper and then put directly into the extraction solvent[23] have been suggested as an alternative, but is relatively slow (~30s) in comparison to direct addition; this is particularly relevant in light of the high metabolic turn-over rates in exponentially growing bacteria. Another alternative approach in which cells are grown on a filter paper on top of an agar plate provides the potential for rapid quenching, but is limited by the fundamental alteration this setup makes to the physiological state of the bacteria (c.f. liquid culture).[25] Quenching, in the field of microbial metabolomics therefore remains an unresolved problem, although established protocols exist for some Gram-positive organisms.[26,27] More generally, membrane integrity during the quenching process should be considered, particularly if the supernatant from the quenching process is subsequently excluded from the analysis (e.g.

suspension culture); bacterial cells and mammalian cells may differ in their susceptibilities to membrane/ intracellular metabolite leakage in organic solvent due to the presence of cell wall in bacterial cells. For example, Canelas *et al.* (2008) demonstrated that applying pure methanol as quenching solution at below -40 C can prevent metabolite leakage in yeast cells.[28]

13.2.4. Extraction of Intracellular Metabolites

Cellular materials require preparation to facilitate the acquisition of solution-state NMR spectra spectra that are useful for metabolic profiling. In order to provide representative profile data, there is often a need to extract samples to separate metabolites and enable the acquisition of high-quality spectra that do not suffer from interference from the original sample matrix. There are a variety of physical and chemical processes that can be used for extraction, but care must be taken to select a method that is appropriate for the target criteria; extraction of samples is accompanied with variable metabolite selectivity, loss, and potential for metabolite alteration. Additionally, these methods may be used in combination to select or maximise metabolite recovery. In short: the choice of extraction method(s) is important as it impacts on the subset of metabolites that are recovered from a sample – and therefore potentially observable – in subsequent NMR spectroscopic analyses. Extraction of sampled tissues enables analysis of solution-state samples, which conveys several advantages at the expense of ensemble information analysis, and increased preparative effort (c.f. MAS NMR spectroscopy). In particular, analysis of tissue extracts enables broad separation of metabolites according to physicochemical properties (typically according to lipophilicity) and permits optimised analysis of these metabolome subsets. Analysis of multiple extract types (e.g. aqueous and organic fractions) can collectively improve the overall metabolite coverage available, and facilitate the use of optimised methods in subsequent profiling by NMR spectroscopy.

13.2.4.1 Physical Extraction

In order to achieve an efficient and rapid recovery of metabolites during extraction, pre-treatment by mechanical disruption can be used. This results in bulk sample homogenisation and cellular fracture, releasing intracellular metabolites, permitting partitioning into extraction solvents and

subsequent separation from the insoluble sample matrix components. The simplest method of achieving physical sample disruption is for frozen tissues to undergo manual grinding using a handheld/automated pestle and mortar[29] or mincer. Alternative methods are the use of a homogenizer, ball mill[30] or other type of bead beater[11 31]. Other methods for aiding cellular disruption includs cycles of freeze-thaw, [32-34] ultrasonication[35] or acoustic cavitation[33].

13.2.4.2 Chemical Extraction

Two main types of chemical extraction are commonly used in the preparation of cellular material for NMR spectroscopic analysis, discussed in turn below. (1) Perchloric acid extraction involves the addition of ice-cold perchloric acid (PCA; $HClO_4$) to harvested cells, followed by vortex-mixing to yield a precipitate of macromolecules including proteins and lipids, which can be separated by high speed centrifugation. [36] The acidic supernatant is removed and neutralised by titration with potassium hydroxide, yielding potassium perchlorate ($KClO_4$), which can be removed as a precipitate by further centrifugation on account of its low solubility. The neutralised supernatant is then either prepared directly or lyophilised and stored for subsequent reconstitution in NMR buffer for analysis. The macromolecular pellet can be neutralised and further extracted using organic solvents to yield a sample containing the precipitated lipids, which are amenable to NMR analysis. PCA extraction is limited by several factors including the potential for metabolite oxidation, the necessity of substantial pH change that may hydrolyse acid-labile species and the need to neutralise the supernatant/precipitate. (2) Dual-phase solvent extraction involves the sequential addition of aliquots of ice-cold methanol:water and chloroform to harvested cells, followed by vortex-mixing and then placement on ice to precipitate proteins (note: glass tubes should be used to prevent contamination from sample tube components).[37] Separation of phases is achieved by high-speed centrifugation, yielding a biphasic sample (upper methanolic aqueous phase; lower organic chloroform phase) with the insoluble precipitate at the interface. Both phases can be carefully removed to separate vials and dried by lyophilisation, speedvac, or under inert gas, prior to reconstitution in a suitable solvent for NMR spectroscopic analysis. Recovery can be improved by multiple extractions of the residual precipitate, and pooling of the separated fractions. The precipitate can be used for protein estimation if required (e.g. by use of the bicinchoninic acid

(BCA) protein assay[38]). This method does require several steps, but has the advantage of efficiently generating a complementary pair of aqueous and organic samples that can be analysed separately. Individual researchers have explored multiple variations of these two main extraction techniques, with specific steps of modifications able to preferentially select or preserve metabolites of interest.[39] For suspended cells, Sellick et al. (2011) have provided a very detailed protocol for preparation compatible with multiple analytical platforms.[40] Additionally, a variety of other solvents/compositions/methods have been used for the extraction of cellular metabolites; some key exemplars are summarised in Table 1.

[Table 13.1 near here]

Requires references: 13,14,20,35,37,39-53

Irrespective of the protocol used, we stress here that the need to work quickly and ensure that washing is conducted in a manner that does not disrupt the integrity of the cellular material composition is paramount. Additionally, we suggest that researchers record in detail the time between the excision of material and subsequent freezing, run order, and autosampler residence time, to allow *post-hoc* assessment of any time-related changes to the observed metabolic profile associated with this step (and potential to adjust analyses accordingly). [54]

While we have considered the needs of NMR spectroscopy, it is common practice for multiple analytical platforms to be used in parallel, which places additional – and potentially conflicting – requirements on the methods used for sampling/harvesting/preparation of biological materials. To this end, Beltran *et al.* (2012) addressed the issue of the compatibility of sample preparation in NMR spectroscopy and LCMS. [55] The authors highlighting the complications in LCMS analyses that can arise from the use of deuterated solvents, and the influence of pH on the ionization of analytes in the MS source as a consequence of extraction solvent choice (e.g. acid). They evaluated 12 different solvent extraction protocols / conditions for the analysis of liver tissue. In summary, they found that hydrogen/deuterium exchange did not affect LCMS profiles, and solvent choice dominated the extraction efficiency of the protocols over other factors (e.g. temperature).

They concluded that where NMR spectroscopic and LCMS analyses of the same sample are anticipated, the use of methanolic aqueous extractions are preferred.

13.2.5 NMR Spectroscopy of Cellular Materials

Samples are typically prepared by dilution or in buffer containing an internal reference at a concentration that is in the same range (or greater) than that expected for metabolites present, and used as both a chemical shift reference as well as for metabolite quantification/estimation. TSP-d_4 (3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid or DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) are most commonly used for aqueous samples. For samples with a considerable protein/lipoprotein content where the internal standard chemical shift and peakshape may be affected due to protein binding, it is common for spectra to be referenced to the anomeric proton resonance of α-glucose (if present) as it has a chemical shift that is largely unaffected by the presence of these species; glucose is commonly abundant in various media.[56] The synthetic compound 4,4-Dimethyl-4-silapentane-1-ammonium trifluoroacetate (DSA) has been suggested as an alternative, but has not found widespread use at the time of writing.[57] Lyophilised samples are commonly re-dissolved in D_2O or D_2O-based buffer as this minimised the requirement for substantial water suppression during NMR acquisition. For aqueous samples, the pH may be adjusted using standard methods (typically addition of ~5uL volumes of 1 M HCl or 1M NaOH, or their deuterated equivalents). Cell culture media analysis, commonly only requires addition of a deuterated solvent to facilitate spectrometer lock, and centrifugation to remove any suspended debris, prior to transfer to the analysis vessel. Lipid extracts that have been dried are commonly resuspended in $CDCl_3$ containing tetramethylsilane (TMS) as an internal chemical shift reference.

Prepared extracted cellular materials are amenable to the whole gamut of NMR experiments, as detailed elsewhere in this book, but may present instrumental challenges when at low concentration (e.g. extracts of limited tissue mass or from a small number of cells). In the context of metabolic profiling, the most commonly-used experiments are those that produce a one-dimensional profile spectrum either directly (1D) or from the calculation of a projection/skyline spectrum (e.g. 1D JRES). Specific challenges for extracted cellular materials include the need to

reduce the influence of the solvent signal(s), and also broad resonances arising from macromolecular components. These are discussed in turn below. For samples comprised of, or prepared in, aqueous media, there is a need to incorporate water suppression into ^{1}H NMR pulse sequences; failure to do so results in unusable spectra on account of the very large signal for this resonance. As mentioned above, one route to minimising this is to lyophilise the sample and reconstitute in D_2O or D_2O-based buffer, although this results in the loss of highly volatile compounds such as acetone. The most common approach to water resonance suppression, a presaturation pulse with a narrow bandwidth is applied during the recycle delay (typically 3-4s), centred at the water resonance (~4.7 ppm relative to TSP-d_4 at 0.0 ppm in aqueous samples), although other techniques such as WET sequence[58] or WATERGATE excitation sculpting can be implemented[59,60]. Similarly, presence of other protonated solvents in sample will present the need to employ solvent suppression at each of the resonances that result. 1D pulse sequences based on an increment of the nuclear Overhauser effect experiment that improve the efficiency of the water suppression through volume selection (informally referred to as a NOESYpresat experiment)[61] are commonplace.

NMR spectroscopic analysis of extracts that contain macromolecular components (e.g. proteins) at an appreciable concentration (e.g. cell culture media containing fetal calf serum or extracts where adequate removal has not been possible), yield spectra containing broad peaks that affect wide chemical shift regions, which can confound subsequent data analysis. These resonances result from the short time taken for T_2 spin-spin relaxation to occur in these slow-tumbling macromolecules, a phenomenon which can be minimised using spin-echo spectral editing techniques; pulse sequences based on the Carr-Purcell-Meiboom-Gill (CPMG sequence) are common, with the spin-echo parameters adjusted to provide adequate removal of the unwanted signals according to sample type.[62] J-resolved spectra are also used routinely, and result in a reduced complexity and overlap on account of the removal of coupling constant from the spectral projection of the profile.[63] As described above, the preparative steps for cell extracts include quenching of metabolism and/or removal of proteins and therefore samples are typically stable at room temperature for short periods. To reduce the potential for degradation to occur while in the

sample vessel, cooled sample holders (e.g. Bruker SampleJet) are recommended, particularly for samples that will reside in an instrument autosampler for an extended period.

13.3 Cellular Material Profiling Applications

NMR-based metabolomics has been shown to be a versatile tool for the study of whole organisms; these include metabolic changes associated with bacterial community evolution in various contexts,[64-66] response to environmental stress, [67,68] microbial classification,[69] general bacterial physiology, [70,71]and others. Multicellular organisms have been well studied, including the characterization of biochemical diversity of betaines in earthworms,[72] contribution to the taxonomic description of cryptic species [73], investigation into the effects of heavy metal pollution on the invertebrate communities. [74,75] and heat stress in Drosphila[76,77]. Blaise and co-workers have investigated the utility of NMR-based metabolomics with *C. elegans*[78] [79] with other studies focused on the metabolic changes associated with ageing and physiology[80-82].

Earthworm sampling provides a useful exemplar with which to illustrate the challenges of in-field sampling and subsequent extraction and analysis and will be discussed below. On field trips for environmental research, in order to quench metabolism, earthworms are often snap-frozen in liquid nitrogen on-site, as this is a relatively portable setup, and provides a balance with adequate cessation of metabolism. In the majority of cases and/or for larger samples, on-site subsampling would be unfeasible and likely result in considerable metabolic changes. Frozen samples of whole organisms are then mechanically ground in liquid nitrogen and subsequently extracted with a suitable solvent[73]. For earthworms, evidence suggests that even these extracts require a further 'stabilisation' step prior to resuspension in aqueous solvents prior to analysis; Liebeke and Bundy (2011) found that otherwise chemically-stable metabolites are converted by the residual enzymatic activity within the extracts[9].

NMR spectroscopy of tissue extract has found widespread use across multiple areas of bioscience, notably in the assessment of toxicological responses, In several case, researchers have sampled and integrated metabolite profiles obtained from multiple tissues to provide a more holistic view; for example, Ling et al. (2014)[83] used a combination of 500 MHz ^{1}H NMR spectra

(one-dimensional and 2D J-resolved) to generate metabolic profiles of extracted tissues to delineate the effect of naphthalene exposure across multiple organs in a mouse model. A summary of the resulting multivariate analysis is shown in Figure 13.2. This highlights one key aspect of NMR-based metabolome profiles – it is relatively simple to employ a common protocol across multiple tissue types, and subsequently generate directly compatible datasets that report on metabolites in a consistent manner (see Fig. 13.2).

[Figure 13.2 near here]

Metabolome analysis of mammalian cell cultures is also now routine in many laboratories, with particular utility being found in preclinical mechanistic research; the diversity and volume of research published cannot easily be summarised, although Penet *et al.* (2012)[84] provide an excellent synopsis in relation to the cancer metabolome. Cancer cell lines have arguably received the greatest attention on account of the need to improve translational medicines, the diversity of cancer subtypes, and the common dysregulation of core metabolic processes (e.g. Warburg effect) as a hallmark of cancer. As an example, Cuperlovic-Culf *et al.* (2011) used NMR-based metabolic profiling in combination with RNA expression analysis to delineate the differences in breast cancer subtypes *in vitro*. [85] (Figure 13.3)

[Figure 13.3 near here]

It is common for parallel analyses of both intracellular extracts and extracellular supernatants to be combined to provide an efficient model of metabolite flux including uptake of media components, and export of metabolites from the cells. Media can be serially sampled at a relatively frequency, and provides an efficient and non-invasive/non-destructive means of monitoring culture conditions, particularly changes in high abundance components (e.g. glucose, lactate, amino acids).[86] Adherent cell extracts cannot be serially sampled in the same way, and therefore longitudinal sampling require parallel plates/flasks to be maintained, which has additional resource cost; increasing the plate well density necessarily reduces the number of cells at harvest, and therefore

places a limit on throughput. Advances in isotopic enrichment and DNP may offer a potential route towards much higher throughput/lower material requirements; Dumez *et al.* (2015) have demonstrated application of this technique to cell extracts, reporting the acquisition of high-quality 2D NMR spectra in a fraction the time (see Figure 13.4).

(a) Conventional HMBC spectrum, recorded in 13 h 42 min at 500 MHz with a cryogenic probe, on a partially enriched extract (*ca.* 57 million extracted cells) dissolved in 700 µL D_2O. (b) Hyperpolarized single-scan spectrum. The cell extract was dissolved in 200 µL of a mixture of H_2O/D_2O/glycerol-d_8 (2 : 3 : 5) with 25 mM TEMPOL and polarized for 30 min at 1.2 K and 6.7 T, and finally dissolved with 5 mL D_2O. A fraction of 700 µL of the hyperpolarized sample was injected in a 500 MHz spectrometer equipped with a cryogenic probe where the spectrum was recorded in a single scan. (c) Same as (b), but with a natural abundance extract (*ca.* 113 million cells) obtained from the same SKBR3 cell line.

[Figure 13.4 near here]

13.4 Other Biofluids

13.4.1 Introduction

As detailed elsewhere in this book, the most commonly used sample types in metabolic profiling of animals are urine and blood (typically serum or plasma preparations); these fluids are convenient to collect, and report on metabolic phenotypes at the system-level. A vast array of other distinct, and physiologically-specialised biofluids exist, each performing different biological roles, and under coordinated regulation within organisms (Fig. 13.5). Importantly, the small-molecule composition of each fluid encodes a different set of metabolic information, relating to its source, distribution, longevity, function, and a host of other interacting factors. Similar to the analysis of specific types of cellular materials discussed earlier in this chapter, the ability to sample and characterise more exotic fluids can provide additional windows on cellular, tissue, organ, and systemic function that

report more specifically than blood plasma or urine. Furthermore, the parallel profiling of multiple specialised fluids can provide a more comprehensive view of the determinants of the overall systemic function. As noted by Nicholson *et al.* (2012), in most cases, only a small number of biofluids or tissues can be readily accessed, with each providing an *'island of information'*; the long-term challenge is to develop appropriate methods for systems-level integration of metabolic information obtained from individual sample types. [87]

[Figure 13.5 near here]

The nature of the NMR spectroscopic analytical platforms used to conduct metabolic profiling experiments is such that almost any biofluid is amenable to characterisation in some meaningful way. In general, differences in analysis lie in a) the preparative steps used to ensure high-quality spectra/data can be obtained (i.e. by limiting the effects of the sample matrix) and b) in the range of commonly-observed metabolites. Here, we focus on fluids other than urine and blood that have been explored using NMR spectroscopy-based metabolic profiling; we consider in turn cerebrospinal fluid, faeces, milk, seminal fluid, synovial fluid and bile – five sample types that are either easily or routinely collected in clinical and preclinical study scenarios, and for which NMR-based spectroscopic analysis for the purposes of metabonomics analysis has been investigated. More recondite fluids (e.g. bronchiolar alveolar lavage fluid, amniotic fluid ascites, cervical mucus, pus, organ dialysates, tears, etc.), are discussed only briefly as their exploration by metabolic profiling is still in its infancy and literature is scarce.

13.4.2 Faeces

Faeces is the solid waste matter that is passed from the digestive tract, containing undigested food, metabolic by-products, mucus, bile, bilirubin, microorganisms and water.[88] Due to the direct relevance it has to both individual nutritional practices, and interface with the gastrointestinal (GI) tract itself, metabolic profiling of faecal samples has been used in a wide variety of research related to diet and GI disease.[89] Additionally, because the mammalian gut is populated by a diverse, and highly dynamic set of microorganisms, that are intricately involved in a number of

biological processes (e.g. digestion, co-metabolism, enterohepatic recirculation, and immune regulation), metabolic profiles obtained from faecal matter are of particular interest to those wishing to understand mammalian-microbial interactions (especially in conjunction with microbiome analyses). Faeces can be easily obtained in a non-invasive manner, and can therefore be routinely collected in clinical and preclinical settings.

As a bulk excretory material with a strong relationship with food intake, faecal matter can vary considerably in composition, and samples may be heterogeneous in nature. Metabolic profiling of faecal matter by NMR spectroscopy requires samples to be processed, and several studies of have been conducted to establish appropriate methods for sample extraction; approaches using very limited sample preparation have been attempted; Bezabeh *et al.* (2009) reported that application of ^{1}H NMR spectroscopy to unextracted / raw faecal material, resulted in spectra with relatively poor resolution.[90] In addition to the aqueous- and lipid- soluble components of faecal matter typically contains (semi-digested) dietary fibre as a consequence of ingesting plant-based material. Preparation of faecal samples typically includes the following steps to generate extracts that are both amenable to high-resolution NMR spectroscopic analysis and representative of their source material: i) homogenisation of the sample to improve representativeness of individual aliquots; ii) physical disruption of sample components using a tissuelyser to release trapped/partitioned metabolites; iii) removal of particulate matter by filtration or centrifugation to ensure high-quality spectra can be obtained; iv) selection of an extraction solvent appropriate; v) sufficient repetition of the extraction procedure. Methods for faecal sample preparation in metabolic profiling applications that address these considerations (including specific considerations for NMR spectroscopic analysis) have recently been reviewed in detail by Deda *et al.* (2015).[91]

The complexity of this sample matrix has led to multiple strategies for obtaining adequately prepared samples; citing limitation of previous studies that did not fully account for the effects of the sample:solvent ratio or the resultant pH, Wu *et al.* (2010) compared different bulk sample preparation techniques (ultrasonic homogenisation and tissuelyser bead disruption), alongside optimising sample extraction parameters. [92] The authors' recommendation that for optimal spectral

signal-to-noise ratio, pH-related chemical shift consistency, and appropriateness for high-throughput / large scale analyses, that homogenisation be conducted using a tissuelyser, and the sample diluted in buffer to a final faeces concentration of 0.1 mg*ul^{-1} (weight of faeces-to-buffer volume ratio; W_f:V_b of 1:10). The authors noted that manual ultrasonication of samples can provide a similar extraction where a tissuelyser is not available, and that their use in combination also yields largely similar spectral profiles. Lamichhane *et al.* (2015) revisited this aspect of faecal sample to provide specific advice for the preparation of human samples.[93] Citing the clear inter-species differences in faecal metabolite composition described by Saric *et al.* (2008)[94], the authors concluded that a W_f:V_b of 1:2 was optimal. They also investigated the effects of freeze-thaw and sonication on subsequent metabolite profiles and found them to have a relatively minor effect on metabolite resonance signal-to-noise. Lyophilisation of faecal samples is commonly included as a preparative step, to minimise gross concentration differences resulting from contrasting water content in samples. It has been noted that some or all volatile compounds will be lost, including some considered integral to investigations of the diet, gut contents and action of the microbiome, such as short chain fatty acids (SCFAs) [92,94]

NMR spectroscopy experiments for obtaining metabolic profiles of prepared (homogenised and extracted) faecal samples are largely similar to those for urine (as detailed elsewhere in this book) and in standard protocols[95], although the influence of extreme dilution (either global dilution in the original sample) or in the preparative steps may require additional acquisition time to compensate for low metabolite concentrations (or pretreatment by lyophilisation as described above). As illustrated by the work of Wu *et al. (*2010), around 40 abundant metabolites can be readily assigned in typical ^{1}H NMR spectra of faecal extracts (Fig. 13.6), including amino acids, SCFAs, and bile acids. Jacobs *et al.* (2008) assessed ^{1}H NMR spectroscopy as an analytical platform for faeces metabolome profiling, with a focus on the effect of nutritional interventions (grape juice and wine consumption) on the gut microbial composition[96] and more recently, Bjerrum *et at.* (2015) investigated metabolite profiles of patients with chronic inflammatory bowel conditions (ulcerative colitis and Crohn's disease), compared to healthy controls.[97] In this latter study, the authors generated ^{1}H 1D CMPG spectra for stool samples collected from 113 individuals (48 ulcerative

colitis, 44 Crohn's disease, 21 healthy controls) and produced multivariate statistical models based on these. They noted the strong influence of surgical procedures and medicinal treatments. The use of metabolomics to interrogate the influence of the microbiota on health has been reviewed by Martin *et al.* (2012). [98]

[Figure 13.6 near here]

13.4.3 Cerebrospinal Fluid

Cerebrospinal fluid (CSF) is found in the subarachnoid space and ventricular system in and around the brain and spinal cord. It facilitates essential physical, chemical, and immunological functions, including protection from mechanical injury, maintenance of homeostasis, and as a route for exchange of substrates and waste products of brain biochemical processes. CSF is produced by structures in the brain (predominantly the choroid plexuses), and the volume experiences continual turnover (approximately every six hours, totalling a production of 400-600mL per day).[99] CSF is commonly sampled in a clinical context by lumbar puncture for diagnosis of neurological abnormalities, detection of infection, and determination of intercranial pressure.[100] Sweatman *et al.* (1993) conducted the first major high-field ^{1}H NMR-based study of CSF small molecule composition, using a variety of one- and two-dimensional experiments.[101] They successfully identified and assigned a total of 46 metabolites of endogenous origin. The authors also explored the use of lyophilisation and reconstitution of CSF, reporting an improvement in spectral characteristics, alongside the expected loss of volatiles such as acetone. Wevers *et al.* (1995) subsequently reported a standardised method of analysis and indicated the utility of CSF metabolic profiling as a diagnostic tool through the characterisation of clinical samples obtained from individuals with inborn errors of metabolism.[102] The authors reported that, as with other biofluids, the pH of the prepared NMR sample was important to ensure good concordance of NMR spectroscopic resonances; sample pH has been highlighted as a potential factor in the misinterpretation of metabolic profile data (and related chemometric models) by Cruz *et al.* (2014)[103], who reviewed the work of Kork *et al.* (2009, 2012)[104,105] and questioned the validity of the assignments made / models generated for classification of Alzheimer's disease patients and

severity on account of the substantial pH-dependent shifts observed for the resonances. Further assignment of resonances in ^{1}H NMR spectra of cerebrospinal fluid (CSF) was conducted by Lutz *et al.* (1998) and increased the tally of routinely assignable metabolites considerably, with a 1D ^{1}H NMR database created containing the assignments of ~150 metabolites.[106] To date, the Wishart research group have recently published the most comprehensive CSF analysis – using multiple analytical platforms including NMR - and database of CSF metabolome components is now available as a community resource (www.csfmetabolome.ca).[107,108]

Levine *et al.* (2000) explored the effect of sample handling as a preanalytical factor, comparing CSF before and after 72 hrs at room temperature.[109] They found significant changes in citrate, lactate, glutamine, creatine and creatinine resulted from this prolonged bench residence time, highlighting the need to ensure timely analysis to obtain more representative NMR spectroscopic profile data. A more extensive optimisation of sample pre-treatment was conducted by Paskevich *et al.* (2013), who determined that the optimal preparation of CSF used buffered, deuterated media.[110] One major consideration in the preparation and analysis of CSF by NMR spectroscopy is sample volume; while it is possible to obtain CSF samples from humans in the milliliter range, other species may yield far less (e.g. rats and mice can be sampled in the low microliter range). Consequently, NMR spectroscopic analyses may be tailored to accommodate a smaller prepared sample volume using narrow-bore students (e.g. <3mm diameter tubes and probes). CSF is naturally well buffered at pH 7.3, but to prevent minor differences in pH having a deleterious effect on efficient spectral alignment, preparation of CSF typically includes the addition of a buffer solution.[108,111] NMR spectral acquisition typically employs the same one-dimensional, spin-echo, and J-resolved pulse sequences as used for other biofluids; presaturation of the water resonance is required. The physiological role of CSF obviously makes it a candidate reporter for metabolic events related to brain and nervous system function, and this is born out in the dominance of publications relating to neurodegeneration, including multiple sclerosis[112] (reviewed by Lutz and Cozzonne, (2011))[113], cervical myelopathy and lumbar radiculopathy[114] and use of NMR spectroscopic profiles for neurological disease diagnosis. [115] and effect of interventions.[116] NMR-

based analyses related to inborn errors of metabolism[117] and vitamin deficiency [118] have also been reported.

13.4.4 Milk

Breast milk is a complex, water-based colloidal emulsion (a liquid phase dispersed in another liquid phase) produced by mammary glands. Dissolved in the colloid are numerous small molecule metabolites, proteins, minerals, salts and antibodies that provide a key source of nutrition and immunological protection for mammalian neonates. After weaning, humans commonly consume animal milk (whole and in preparations) throughout life as part of the everyday diet. Despite the widespread consumption of milk, relatively few studies have investigated the metabolic composition and/or dynamism of human breast milk using NMR spectroscopy. An overview of the state of development of NMR-based metabolomics of milk has previously been published.[119] As a complex colloid, milk places additional demands on the NMR sample preparation; one key consideration is the removal of casein micelles (phosphoproteins that comprise a large percentage of total milk protein) by precipitation or centrifugation to limit their influence on the small molecule NMR spectral profile, and as expected, CPMG acquisitions are preferred to suppress broad signals in most studies. Quantitatively, lactose is the most abundant metabolite, with other major components including galactose and citrate. Other individual metabolites are typically one or more order of magnitude lower in abundance. The first published example was conducted by Cesare-Marincola and co-workers, who used NMR spectroscopy in conjunction with GC-MS in a comparative metabonomics analysis of human breast milk and formula milk.[120] Given the volume of animal milk consumed, animal studies have sought to investigate biological markers relating to both quality of the milk and also the health status of the animal; for example, Sundekilde *et al.* (2011, 2013) identified metabolic profile features correlated with both breed and somatic cell count in bovine milk. [121,122] There is considerable interest in developing optimised metabolic profiling methods for human milk, particularly in relation to understanding neonate/child nutrition and health. [120,123-125] Wu et al. (2016) recently used NMR spectroscopic analysis to explore milk metabolome compositional changes during stages of lactation (early 9-24 days after delivery vs late 31-87 days); surveying a set of 36 metabolites, the authors noted that milk samples obtained in later

stages contained elevated lactose, choline and changes in the amino acid profile, alongside decreases in PC and GPC components. [126] The ability to rapidly collect, analyse and report metabolic composition by NMR spectroscopy in this way potentiates the ability to more closely monitor infant nutrition and augment other measures in neonatal care. In additional to evaluating the effects of relative storage temperature (-20 C vs -80 C) for various periods (up to seven days), these authors also compared methanolic precipitation with ultrafiltration, with the latter reported to more optimally remove lipid and proteins. Key metabonomics studies of milk are shown in Table 13.1.

[Table 13.1 near here]

Requires references: [120,125,127 128]

13.4.5 Seminal Fluid

Semen is a complex biofluid originating in the male gonads, containing seminal fluid (SF) plasma (collectively produced by the seminal vesicle, prostate gland, the bulbourethral glands, and other minor accessory structures) and spermatozoa (produced in the testes). The SF provides a protective medium in which spermatozoa can survive, containing an abundance of available sugars (particularly fructose) that meet the high energy requirements of these motile cells, and polyamines (e.g. spermine, spermidine) and citrate that afford a capacity to buffer pH and inorganic ion concentrations, respectively. In addition to those of low-molecular-weight, other SF components modulate the physical and chemical properties of semen, and include mucus, proteins, and proteolytic enzymes (including prostate-specific antigen, PSA; responsible for the liquefaction of coagulated semen).

The simplest preparation of seminal plasma from semen samples is centrifugation to remove cellular material. In this case, there is requirement to wait until liquefaction has occurred (~20 minutes at RT post-ejaculation), resulting from the action of PSA. Dilution with D_2O containing a NMR chemical shift reference compound (e.g. TSP-d_4) has been used to reduce viscosity[129] for an efficient sample preparation (can easily be combined with an additional lyophilisation step).

Tomlins *et al.* (1998) investigated pre-analytical / preparative aspects in detail using human semen samples, and described the time-related changes that occur in SF that could be observed by ^{1}H NMR at a field strength of 14.1 Tesla. Conversion of phosphorylcholine to choline, and uridine-5'-monophosphate to uridine were the main observable changes occurring in the experimental timescale (sampled between 2 min and 3 hr post-ejaculation). The authors also investigated the effect of immediate incubation of semen samples with the chelating agent ethylenediaminetetraacetic acid (EDTA), showing an inhibition of peptide hydrolysis by SF metalloenzymes, evidenced by the constancy of amino acid resonances up to 24 hrs after addition. Resonances characteristic of complexes of EDTA with Ca2+, Zn2+ and Mg2+ were observed, and their formation concomitant with reduced chelation by citrate. Lynch and coworkers (1994) subsequently investigated individually seminal fluid (SF, allowed to liquefy for 30 min post ejaculation), and its main component fluids: prostatic fluid (PF, collected by prostatic massage), and seminal vesicle fluid (SVF, collected during surgery). [129] The authors report that NMR spectra of prostatic fluid have very large contributions from citrate, spermine and *myo*-inositol, in contrast to SVF where GC and lactate are major contributors. These authors investigated the effect of vasal aplasia (occlusion of the *vas deferens*) on the fluid profiles, which were highly characteristic of the limited SVF contribution in these samples versus unaffected control. Given the direct interaction and role of semen in the male reproductive system, analyses of the component fluids have largely been focused on their application to fertility and chronic disease of the reproductive organs. For example, Hamamah *et al.* (1998) explored the differences in seminal metabolite profiles in relation to azoospermia arising from various conditions, reporting that key profile discriminants related to specific ratios of glycerol-phosphatidylcholine, choline, citrate and lactate, and suggesting a path towards more tailored interventions relating to male infertility that minimise invasive procedures. [130] More recently, Bonechi *et al.* (2015) conducted an analysis of human semen, and generated multivariate statistical models that could discriminate samples based on a number of sperm quality metrics, and also identified atypical samples as outliers suggesting a more role for such analyses in clinical fertility care. [131] Averna *et al.* (2005)[132] conducted a ^{1}H NMR spectroscopic investigation semen metabolic profiles in relation to of prostate cancer status (on account of the high proportion of prostatic fluid in overall semen content), reporting significant decreases in citrate concentrations

associated with tumorigenesis, and highlighting how NMR spectroscopy could facilitate rapid screening in this context.

13.4.6 Bile

Bile is continuously produced in the liver, temporarily stored in the gallbladder, and subsequently secreted into the upper small intestine (duodenum) in response to consumption of foodstuffs; bile contains amphipathic bile salts that help emulsify lipids in the gut contents to form droplets known as micelles, a process that greatly increases the available surface area that can be accessed by lipases. Biliary excretion is an important route for the efflux of higher molecular weight metabolites, such as xenobiotic conjugates. As a consequence, the metabolite profile of bile reports on specific aspects of liver function, and can also indicate how exogenous compounds are handled by the body. Although relatively plentiful, bile presents a particularly challenging biofluid to obtain, handle and use in the context of metabolome studies. In addition to the difficulty of collection (c.f. urine, plasma), one issue that affects NMR analyses in particular is the emulsifying properties of bile and the formation of micelles. The affinity of lipophilic compounds for the lipid core of the micelles means they become compartmentalised and result in very broad resonance in NMR spectra. Accordingly, depending on the focus on the analysis, preparation of bile for analysis by NMR may involve a simple dilute-and-shoot approach of the whole bile, or a more involved extraction procedure to preselect and/or concentrate specific metabolite classes to aid detection, permit resolution from other bile components, and eliminate/limit the effect of micelles in the sample. Bile can be obtained from the liver or gallbladder, each of which have a distinct but largely similar composition from the perspective of sample preparation and NMR analysis.

The first NMR analysis of hepatic bile at 18.8 T was conducted by Duarte *et al.* (2009). Example spectra are shown in Figure. 13.7. These authors were the first to characterise hepatic bile, and incorporated 800MHz ^{1}H NMR spectroscopy into a multi-platform analytical setup. [133] Using two-dimensional ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HSQC spectra, the authors were able to positively assign 40 compounds in spectra obtained from a whole bile sample (see Fig 13.8).

[Figure 13.7 near here]

[Figure 13.8 near here]

Metabolites identified included amino acids, organic acids, carbohydrates (including glucose, glycerol and *myo*-inositol), alongside choline and several related quaternary ammonium compounds (including phosphocholine, phosphatidylcholine, betaine, trimethylamine-*N*-oxide). It was noted that the observed chemical shifts differed from value available in community databases, attributed to differences in the bulk diamagnetic susceptibility in this biofluid. In addition to high abundance bile acids, the authors noted that the related glycine and taurine conjugates of these major bile acid metabolites could not be adequately resolved to allow positive identification. To date, multiple NMR- spectroscopic studies have been conducted to characterise bile composition[134-137] with applications focused on characterising biliary and hepatic diseases and pancreatic cancer[138]; reviewed by [139] For example, Gowda *et al.* (2009) used ^{1}H NMR profiling of bile obtained from the gallbladder of 44 individuals with a range of liver pathologies (17 controls, 11 hepatcellularcarcinoma (HCC), 7 cholangiocarcinoma, 9 non-malignant liver disease). [140] The authors prepared bile samples in both aqueous media (dilution with H_2O/D_2O) and non-aqueous media (DMSO) prior to analysis. They found significant changes in the bile acid profile - notably the ratio between major glycine- and taurine- conjugated bile acids were decreased in the non-malignant group, relative to control. HCC samples could also be differentiated from the non-malignant group based on the concentration of glycine-conjugated bile acids.

13.4.7 Less Commonly Reported Biofluids

As above, most biofluids that can be sampled have the potential to provide highly specific information about system, organ, and cellular function. Many are not routinely collected, but can be useful in relation to the diagnosis of particular pathologies, and NMR-based metabolome analysis offers a useful window to capture the small-molecule component of such investigations; NMR-based metabolomics has been used to diagnostic fluids such as amniotic fluid[141],

tears/meibomian[142], synovial fluid[143,144], cervical mucus[114], and pathological fluids such as ascites[145] among many others.

13.5 Summary and Future Developments

While the influence on the various physicochemical (e.g. time, temperature, mechanics) and biochemical (e.g. enzymatic action, sequestration) aspects of these procedures continue to provide researchers with an ongoing challenge to best extract metabolites across many chemical classes, the general workflows appear largely established. Converging on the ability to generate soluble fractions of the original sample that are free from confounding matrix, and prepared in a way so as to minimise the effects of sampling/handling on resultant metabolic profile data, predominantly one-dimensional ^{1}H NMR experiments can provide spectra that faithfully represent the small molecular composition at the time and site of sampling. Partly due to the lack of effective quenching and separation procedure, but mainly due to low sensitivity, the role of NMR spectroscopy for intracellular metabolite profiling has declined in recent years. However, for some applications that require detection of high concentration metabolites of different chemical classes such as osmolytes or storage metabolites, NMR remains a powerful and useful analytical platform, especially if concomitant structural elucidation is important.[146] Coupled with advances in the sensitivity of instrumentation/analyses (high field strengths, cryoprobe technology, improved pulse sequences), and more routine use of techniques such as DNP[147] and stable isotope labelling[148,149], it is likely that NMR will remain in the toolkit for metabolic profiling of cellular materials for the foreseeable future.

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References

1 H. K. Kim, Y. H. Choi and R. Verpoorte, *Nat Protoc*, 2010, **5**, 536–549.
2 J. Schripsema, *Phytochem Anal*, 2010, **21**, 14–21.
3 J. G. M. Pontes, A. J. M. Brasil, G. C. F. Cruz, R. N. de Souza and L. Tasic, *Anal. Methods*, 2017, **9**, 1078–1096.
4 D. E. Harrison and P. K. Maitra, *Biochemical Journal*, 1969, **112**, 647–656.
5 S. E. Franks, A. C. Kuesel, N. W. Lutz and W. E. Hull, *Anticancer Res.*, 1996, **16**, 1365–1374.
6 Z. Huang, W. Shao, J. Gu, X. Hu, Y. Shi, W. Xu, C. Huang and D. Lin, *Mol Biosyst*, 2015, **11**, 1832–1840.
7 S. F. Shedd, N. W. Lutz and W. E. Hull, *NMR Biomed*, 1993, **6**, 254–263.
8 J.-P. Galons, C. Job and R. J. Gillies, *Magn Reson Med*, 1995, **33**, 422–426.
9 M. Liebeke and J. G. Bundy, *Metabolomics*, 2011, **8**, 819–830.
10 M. D. C. Alvarez, J. A. Donarski, M. Elliott and A. J. Charlton, *Metabolomics*, 2010, **6**, 541–549.
11 F. M. Geier, E. J. Want, A. M. Leroi and J. G. Bundy, *Anal. Chem.*, 2011, **83**, 3730–3736.
12 N. S. Taylor, R. J. M. Weber, A. D. Southam, T. G. Payne, O. Hrydziuszko, T. N. Arvanitis and M. R. Viant, *Metabolomics*, 2008, **5**, 44–58.
13 J. A. Hernández Bort, V. Shanmukam, M. Pabst, M. Windwarder, L. Neumann, A. Alchalabi, G. Krebiehl, G. Koellensperger, S. Hann, D. Sonntag, F. Altmann, C. Heel and N. Borth, *J. Biotechnol.*, 2014, **182-183**, 97–103.
14 Q. Teng, W. Huang, T. W. Collette, D. R. Ekman and C. Tan, *Metabolomics*, 2008, **5**, 199–208.
15 K. Dettmer, N. Nürnberger, H. Kaspar, M. A. Gruber, M. F. Almstetter and P. J. Oefner, *Anal Bioanal Chem*, 2011, **399**, 1127–1139.
16 H. Bi, K. W. Krausz, S. K. Manna, F. Li, C. H. Johnson and F. J. Gonzalez, *Anal Bioanal Chem*, 2013, **405**, 5279–5289.
17 J. William Allwood, C. L. Winder, W. B. Dunn and R. Goodacre, in *Methodologies for Metabolomics*, eds. N. Lutz, J. Sweedler and R. Wevers, Cambridge University Press, Cambridge, 2012, pp. 79–118.
18 T. H. Haukaas, S. A. Moestue, R. Vettukattil, B. Sitter, S. Lamichhane, R. Segura, G. F. Giskeødegård and T. F. Bathen, *Frontiers in Oncology*, 2016, **6**, 63.
19 H. C. Lange, M. Eman, G. van Zuijlen, D. Visser, J. C. van Dam, J. Frank, M. J. T. de Mattos and J. J. Heijnen, *Biotechnology and Bioengineering*, 2001, **75**, 406–4113.
20 W. de Koning and K. V. Dam, *Anal. Biochem.*, 1992, **204**, 118–123.
21 M. R. Mashego, K. Rumbold, M. De Mey, E. Vandamme, W. Soetaert and J. J. Heijnen, *Biotechnol. Lett.*, 2007, **29**, 1–16.
22 C. Wittmann, J. O. Krömer, P. Kiefer, T. Binz and E. Heinzle, *Anal. Biochem.*, 2004, **327**, 135–139.
23 C. J. Bolten, P. Kiefer, F. Letisse, J.-C. Portais and C. Wittmann, *Anal. Chem.*, 2007, **79**, 3843–3849.
24 M. Oldiges, S. Lütz, S. Pflug, K. Schroer, N. Stein and C. Wiendahl, *Appl. Microbiol. Biotechnol.*, 2007, **76**, 495–511.
25 B. D. Bennett, J. Yuan, E. H. Kimball and J. D. Rabinowitz, *Nat Protoc*, 2008, **3**, 1299–1311.
26 H. Meyer, M. Liebeke and M. Lalk, *Anal. Biochem.*, 2010, **401**, 250–259.
27 H. Meyer, H. Weidmann and M. Lalk, *Microbial Cell Factories*, 2013, **12**, 69.
28 A. B. Canelas, C. Ras, A. ten Pierick, J. C. van Dam, J. J. Heijnen and W. M. van Gulik, *Metabolomics*, 2008, **4**, 226–239.
29 A. E. P. Heazell, M. Brown, W. B. Dunn, S. A. Worton, I. P. Crocker, P. N. Baker and D. B. Kell, *Placenta*, 2008, **29**, 691–698.
30 C. Denkert, J. Budczies, W. Weichert, G. Wohlgemuth, M. Scholz, T. Kind, S. Niesporek, A. Noske, A. Buckendahl, M. Dietel and O. Fiehn, *Mol. Cancer*, 2008, **7**, 72.
31 P. de Jong-Gubbels, P. Vanrolleghem, S. Heijnen, J. P. van Dijken and J. T. Pronk, *Yeast*, 1995, **11**, 407–418.
32 S. G. Villas-Bôas, J. Højer-Pedersen, M. Akesson, J. Smedsgaard and J. Nielsen, *Yeast*, 2005, **22**, 1155–1169.
33 Y. Kassama, Y. Xu, W. B. Dunn, N. Geukens, J. Anné and R. Goodacre, *Analyst*, 2010, **135**, 934–942.
34 C. L. Winder, W. B. Dunn, S. Schuler, D. Broadhurst, R. Jarvis, G. M. Stephens and R. Goodacre, *Anal. Chem.*, 2008, **80**, 2939–2948.
35 J. L. Sporty, M. M. Kabir, K. W. Turteltaub, T. Ognibene, S.-J. Lin and G. Bench, *J Sep Sci*, 2008, **31**, 3202–3211.
36 J. Henke, W. Willker, J. Engelmann and D. Leibfritz, *Anticancer Res.*, 1996, **16**, 1417–1427.
37 R. K. Tyagi, A. Azrad, H. Degani and Y. Salomon, *Magn Reson Med*, 1996, **35**, 194–200.
38 P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson and D. C. Klenk, *Anal. Biochem.*, 1985, **150**, 76–813.

39 C. A. Sellick, D. Knight, A. S. Croxford, A. R. Maqsood, G. M. Stephens, R. Goodacre and A. J. Dickson, *Metabolomics*, 2010, **6**, 427–438.
40 C. A. Sellick, R. Hansen, G. M. Stephens, R. Goodacre and A. J. Dickson, *Nat Protoc*, 2011, **6**, 1241–1249.
41 M. Volmer, J. Gettmann, S. Scholz, H. Büntemeyer and T. Noll, *BMC Proceedings*, 2011, **5**, P93.
42 M. Volmer, S. Northoff, S. Scholz, T. Thüte, H. Büntemeyer and T. Noll, *Biotechnol. Lett.*, 2011, **33**, 495–502.
43 B. Cao, J. Aa, G. Wang, X. Wu, L. Liu, M. Li, J. Shi, X. Wang, C. Zhao, T. Zheng, S. Guo and J. Duan, *Anal Bioanal Chem*, 2011, **400**, 2983–2993.
44 M. H. Shin, D. Y. Lee, K.-H. Liu, O. Fiehn and K. H. Kim, *Anal. Chem.*, 2010, **82**, 6660–6666.
45 J. C. Shryock, R. Rubio and R. M. Berne, *Anal. Biochem.*, 1986, **159**, 73–81.
46 C.-Y. Lin, H. Wu, R. S. Tjeerdema and M. R. Viant, *Metabolomics*, 2007, **3**, 55–67.
47 P. Lu, A. Rangan, S. Y. Chan, D. R. Appling, D. W. Hoffman and E. M. Marcotte, *Metab. Eng.*, 2007, **9**, 8–20.
48 B. Gonzalez, J. François and M. Renaud, *Yeast*, 1997, **13**, 1347–13513.
49 S. Dietmair, N. E. Timmins, P. P. Gray, L. K. Nielsen and J. O. Krömer, *Anal. Biochem.*, 2010, **404**, 155–164.
50 V. M. Boer, C. A. Crutchfield, P. H. Bradley, D. Botstein and J. D. Rabinowitz, *Molecular Biology of the Cell*, 2010, **21**, 198–211.
51 M. J. Sáez and R. Lagunas, *Mol. Cell. Biochem.*, 1976, **13**, 73–78.
52 E. G. BLIGH and W. J. DYER, *Can J Biochem Physiol*, 1959, **37**, 911–917.
53 J. FOLCH, M. LEES and G. H. SLOANE STANLEY, *J. Biol. Chem.*, 1957, **226**, 497–509.
54 P. Jonsson, A. Wuolikainen, E. Thysell, E. Chorell, P. Stattin, P. Wikström and H. Antti, *Metabolomics*, 2015, **11**, 1667–1678.
55 A. Beltran, M. Suarez, M. A. Rodríguez, M. Vinaixa, S. Samino, L. Arola, X. Correig and O. Yanes, *Anal. Chem.*, 2012, **84**, 5838–5844.
56 J. T. M. Pearce, T. J. Athersuch, T. M. D. Ebbels, J. C. Lindon, J. K. Nicholson and H. C. Keun, *Anal. Chem.*, 2008, **80**, 7158–7162.
57 J. S. Nowick, O. Khakshoor, M. Hashemzadeh and J. O. Brower, *Organic Letters*, 2003, **5**, 3511–3513.
58 S. H. Smallcombe, S. L. Patt and P. A. Keifer, *Journal of Magnetic Resonance, Series A*, 1995, **117**, 295–303.
59 M. Liu, X.-A. Mao, C. Ye, H. Huang, J. K. Nicholson and J. C. Lindon, *Journal of Magnetic Resonance*, 1998, **132**, 125–129.
60 M. Piotto, V. Saudek and V. Sklenár, *J. Biomol. NMR*, 1992, **2**, 661–6613.
61 D. Neuhaus, I. M. Ismail and C.-W. Chung, *Journal of Magnetic Resonance, Series A*, 1996, **118**, 256–263.
62 Y. Wang, M. E. Bollard, H. Keun, H. Antti, O. Beckonert, T. M. Ebbels, J. C. Lindon, E. Holmes, H. Tang and J. K. Nicholson, *Anal. Biochem.*, 2003, **323**, 26–32.
63 M. R. Viant, *Biochem. Biophys. Res. Commun.*, 2003, **310**, 943–948.
64 V. Behrends, R. P. Maharjan, B. Ryall, L. Feng, B. Liu, L. Wang, J. G. Bundy and T. Ferenci, *Mol Biosyst*, 2014, **10**, 2820–2822.
65 D. Lawrence, F. Fiegna, V. Behrends, J. G. Bundy, A. B. Phillimore, T. Bell and T. G. Barraclough, *PLoS Biol.*, 2012, **10**, e1001330.
66 V. Behrends, B. Ryall, J. E. A. Zlosnik, D. P. Speert, J. G. Bundy and H. D. Williams, *Environ. Microbiol.*, 2013, **15**, 398–408.
67 M. L. Workentine, J. J. Harrison, A. M. Weljie, V. A. Tran, P. U. Stenroos, V. Tremaroli, H. J. Vogel, H. Ceri and R. J. Turner, *Environ. Microbiol.*, 2010, **12**, 1565–1577.
68 V. Tremaroli, M. L. Workentine, A. M. Weljie, H. J. Vogel, H. Ceri, C. Viti, E. Tatti, P. Zhang, A. P. Hynes, R. J. Turner and D. Zannoni, *Appl. Environ. Microbiol.*, 2009, **75**, 719–728.
69 J. Allen, H. M. Davey, D. Broadhurst, J. K. Heald, J. J. Rowland, S. G. Oliver and D. B. Kell, *Nat. Biotechnol.*, 2003, **21**, 692–696.
70 N. Granucci, F. R. Pinu, T.-L. Han and S. G. Villas-Bôas, *Mol Biosyst*, 2015, **11**, 3297–3304.
71 V. Behrends, T. M. D. Ebbels, H. D. Williams and J. G. Bundy, *Appl. Environ. Microbiol.*, 2009, **75**, 2453–2463.
72 M. Liebeke and J. G. Bundy, *Biochem. Biophys. Res. Commun.*, 2013, **430**, 1306–1311.
73 M. Liebeke, M. W. Bruford, R. K. Donnelly, T. M. D. Ebbels, J. Hao, P. Kille, E. Lahive, R. M. Madison, A. J. Morgan, G. A. Pinto-Juma, D. J. Spurgeon, C. Svendsen and J. G. Bundy, *Biol. Lett.*, 2014, **10**, 20140615–201406113.
74 J. G. Bundy, D. J. Spurgeon, C. Svendsen, P. K. Hankard, J. M. Weeks, D. Osborn, J. C. Lindon and J. K. Nicholson, *Ecotoxicology*, 2004, **13**, 797–806.
75 J. G. Bundy, H. C. Keun, J. K. Sidhu, D. J. Spurgeon, C. Svendsen, P. Kille and A. J. Morgan,

Environ. Sci. Technol., 2007, **41**, 4458–4464.
76 J. Overgaard, A. Malmendal, J. G. Sørensen, J. G. Bundy, V. Loeschcke, N. C. Nielsen and M. Holmstrup, *Journal of Insect Physiology*, 2007, **53**, 1218–1232.
77 A. Malmendal, *AJP: Regulatory, Integrative and Comparative Physiology*, 2006, **291**, R205–R212.
78 B. J. Blaise, J. Giacomotto, B. Elena, M.-E. Dumas, P. Toulhoat, L. Ségalat and L. Emsley, *Proc. Natl. Acad. Sci. U.S.A.*, 2007, **104**, 19808–19812.
79 B. J. Blaise, J. Giacomotto, M. N. Triba, P. Toulhoat, M. Piotto, L. Emsley, L. Ségalat, M.-E. Dumas and B. Elena, *J. Proteome Res.*, 2009, **8**, 2542–2550.
80 S. K. Davies, J. G. Bundy and A. M. Leroi, *J. Proteome Res.*, 2015, **14**, 4603–4609.
81 S. Fuchs, J. G. Bundy, S. K. Davies, J. M. Viney, J. S. Swire and A. M. Leroi, *BMC Biology*, 2010, **8**, 14.
82 J. Swire, S. Fuchs, J. G. Bundy and A. M. Leroi, *Proceedings of the Royal Society B: Biological Sciences*, 2009, **276**, 2747–2754.
83 Y. S. Ling, H.-J. Liang, M.-H. Chung, M.-H. Lin and C.-Y. Lin, *Mol Biosyst*, 2014, **10**, 1918–1931.
84 M.-F. Penet, Z. M. Bhujwalla and K. Glunde, in *Methodologies for Metabolomics*, eds. N. Lutz, J. Sweedler and R. Wevers, Cambridge University Press, Cambridge, 2012, pp. 335–376.
85 M. Cuperlovic-Culf, I. C. Chute, A. S. Culf, M. Touaibia, A. Ghosh, S. Griffiths, D. Tulpan, S. Léger, A. Belkaid, M. E. Surette and R. J. Ouellette, *Chemical Science*, 2011, **2**, 2263–2270.
86 S. A. Bradley, A. Ouyang, J. Purdie, T. A. Smitka, T. Wang and A. Kaerner, *Journal of the American Chemical Society*, 2010, **132**, 9531–9533.
87 J. K. Nicholson, E. Holmes, J. M. Kinross, A. W. Darzi, Z. Takats and J. C. Lindon, *Nature*, 2012, **491**, 384–392.
88 C. Rose, A. Parker, B. Jefferson and E. Cartmell, *Crit Rev Environ Sci Technol*, 2015, **45**, 1827–1879.
89 F. Savorani, M. A. Rasmussen, M. S. Mikkelsen and S. B. Engelsen, *Food Research International*, 2013, **54**, 1131–11413.
90 T. Bezabeh, R. Somorjai, B. Dolenko, N. Bryskina, B. Levin, C. N. Bernstein, E. Jeyarajah, A. H. Steinhart, D. T. Rubin and I. C. P. Smith, *NMR Biomed*, 2009, **22**, 593–600.
91 O. Deda, H. G. Gika, I. D. Wilson and G. A. Theodoridis, *Journal of Pharmaceutical and Biomedical Analysis*, 2015, **113**, 137–150.
92 J. Wu, Y. An, J. Yao, Y. Wang and H. Tang, *Analyst*, 2010, **135**, 1023–1030.
93 S. Lamichhane, C. C. Yde, M. S. Schmedes, H. M. Jensen, S. Meier and H. C. Bertram, *Anal. Chem.*, 2015, **87**, 5930–5937.
94 J. Saric, Y. Wang, J. Li, M. Coen, J. Utzinger, J. R. Marchesi, J. Keiser, K. Veselkov, J. C. Lindon, J. K. Nicholson and E. Holmes, *J. Proteome Res.*, 2008, **7**, 352–360.
95 H. C. Keun and T. J. Athersuch, *Methods Mol. Biol.*, 2011, **708**, 321–334.
96 D. M. Jacobs, N. Deltimple, E. van Velzen, F. A. van Dorsten, M. Bingham, E. E. Vaughan and J. van Duynhoven, *NMR Biomed*, 2008, **21**, 615–626.
97 J. T. Bjerrum, Y. Wang, F. Hao, M. Coskun, C. Ludwig, U. Günther and O. H. Nielsen, *Metabolomics*, 2014, **11**, 122–133.
98 F.-P. J. Martin, S. Collino, S. Rezzi and S. Kochhar, *Frontiers in Physiology*, 2012, **3**.
99 L. Sakka, G. Coll and J. Chazal, *European Annals of Otorhinolaryngology, Head and Neck Diseases*, 2011, **128**, 309–316.
100 B. MORRISON, in *Cerebrospinal Fluid in Clinical Practice*, Elsevier, 2009, pp. 11–17.
101 B. C. Sweatman, R. D. Farrant, E. Holmes, F. Y. Ghauri, J. K. Nicholson and J. C. Lindon, *Journal of Pharmaceutical and Biomedical Analysis*, 1993, **11**, 651–664.
102 R. A. Wevers, U. Engelke, U. Wendel, J. G. de Jong, F. J. Gabreëls and A. Heerschap, *Clin. Chem.*, 1995, **41**, 744–751.
103 T. Cruz, S. Balayssac, V. Gilard, R. Martino, C. Vincent, J. Pariente and M. Malet-Martino, *Metabolites*, 2014, **4**, 115–128.
104 F. Kork, J. Holthues, R. Hellweg, V. Jankowski, M. Tepel, R. Ohring, I. Heuser, J. Bierbrauer, O. Peters, P. Schlattmann, W. Zidek and J. Jankowski, *Curr Alzheimer Res*, 2009, **6**, 519–524.
105 F. Kork, A. Gentsch, J. Holthues, R. Hellweg, V. Jankowski, M. Tepel, W. Zidek and J. Jankowski, *Biomarkers*, 2012, **17**, 36–42.
106 N. W. Lutz, S. Maillet, F. Nicoli, P. Viout and P. J. Cozzone, *FEBS Lett.*, 1998, **425**, 345–351.
107 D. S. Wishart, M. J. Lewis, J. A. Morrissey, M. D. Flegel, K. Jeroncic, Y. Xiong, D. Cheng, R. Eisner, B. Gautam, D. Tzur, S. Sawhney, F. Bamforth, R. Greiner and L. Li, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, 2008, **871**, 164–173.
108 R. Mandal, A. C. Guo, K. K. Chaudhary, P. Liu, F. S. Yallou, E. Dong, F. Aziat and D. S. Wishart, *Genome Med*, 2012, **4**, 38.
109 J. Levine, K. Panchalingam, McClure RJ2, S. Gershon and J. W. Pettegrew, *J Neural Transm (Vienna)*, 2000, **107**, 843–848.

110 S. I. Paskevich, M. V. Molchanov, M. A. Timchenko and V. P. Kutyshenko, *Journal of Analytical Chemistry*, 2013, **68**, 862–870.
111 G. F. Hoffmann, W. Meier-Augenstein, S. Stöckler, R. Surtees, D. Rating and W. L. Nyhan, *J. Inherit. Metab. Dis.*, 1993, **16**, 648–669.
112 J. Lynch, J. Peeling, A. Auty and G. R. Sutherland, *Can J Neurol Sci*, 1993, **20**, 194–198.
113 N. W. Lutz and P. J. Cozzone, *Curr Pharm Biotechnol*, 2011, **12**, 1016–10213.
114 H. Nagashima, Y. Morio, S. Meshitsuka, K. Yamane, Y. Nanjo and R. Teshima, *Eur Spine J*, 2010, **19**, 1363–1368.
115 A. Hiraoka, I. Miura, M. Hattori, I. Tominaga, K. Kushida and M. Maeda, *Biol. Pharm. Bull.*, 1994, **17**, 1–4.
116 E. Holmes, T. M. Tsang, J. T.-J. Huang, F. M. Leweke, D. Koethe, C. W. Gerth, B. M. Nolden, S. Gross, D. Schreiber, J. K. Nicholson and S. Bahn, *PLoS Med.*, 2006, **3**, e327.
117 R. A. Wevers, U. F. Engelke, S. H. Moolenaar, C. Bräutigam, J. G. de Jong, R. Duran, R. A. de Abreu and A. H. van Gennip, *Clin. Chem.*, 1999, **45**, 539–548.
118 F. Commodari, D. L. Arnold, B. C. Sanctuary and E. A. Shoubridge, *NMR Biomed*, 1991, **4**, 192–200.
119 U. K. Sundekilde, L. B. Larsen and H. C. Bertram, *Metabolites*, 2013, **3**, 204–222.
120 F. C. Marincola, A. Noto, P. Caboni, A. Reali, L. Barberini, M. Lussu, F. Murgia, M. L. Santoru, L. Atzori and V. Fanos, *J. Matern. Fetal. Neonatal. Med.*, 2012, **25**, 62–67.
121 U. K. Sundekilde, N. A. Poulsen, L. B. Larsen and H. C. Bertram, *J. Dairy Sci.*, 2013, **96**, 290–299.
122 U. K. Sundekilde, P. D. Frederiksen, M. R. Clausen, L. B. Larsen and H. C. Bertram, *J. Agric. Food Chem.*, 2011, **59**, 7360–7367.
123 F. Cesare Marincola, A. Dessì, S. Corbu, A. Reali and V. Fanos, *Clin. Chim. Acta*, 2015, **451**, 103–106.
124 N. J. Andreas, M. J. Hyde, M. Gomez-Romero, M. A. Lopez-Gonzalvez, A. Villaseñor, A. Wijeyesekera, C. Barbas, N. Modi, E. Holmes and I. Garcia-Perez, *Electrophoresis*, 2015, **36**, 2269–22813.
125 J. T. Smilowitz, C. B. Lebrilla, D. A. Mills, J. B. German and S. L. Freeman, *Annual Review of Nutrition*, 2014, **34**, 143–169.
126 J. Wu, M. Domellöf, A. M. Zivkovic, G. Larsson, A. Öhman and M. L. Nording, *Biochem. Biophys. Res. Commun.*, 2016, **469**, 626–632.
127 G. Praticò, G. Capuani, A. Tomassini, M. E. Baldassarre, M. Delfini and A. Miccheli, *Nat. Prod. Res.*, 2014, **28**, 95–101.
128 M. Longini, M. L. Tataranno, F. Proietti, M. Tortoriello, E. Belvisi, A. Vivi, M. Tassini, S. Perrone and G. Buonocore, *J. Matern. Fetal. Neonatal. Med.*, 2014, **27**, 27–33.
129 M. J. Lynch, J. Masters, J. P. Pryor, J. C. Lindon, M. Spraul, P. J. D. Foxall and J. K. Nicholson, *Journal of Pharmaceutical and Biomedical Analysis*, 1994, **12**, 5–19.
130 S. Hamamah, F. Seguin, L. Bujan, C. Barthelemy, R. Mieusset and J. Lansac, *Human Reproduction*, 1998, **13**, 132–1313.
131 C. Bonechi, G. Collodel, A. Donati, S. Martini, E. Moretti and C. Rossi, *Syst Biol Reprod Med*, 2015, **61**, 353–359.
132 T. AVERNA, E. KLINE, A. SMITH and L. SILLERUD, *The Journal of Urology*, 2005, **173**, 433–438.
133 I. F. Duarte, C. Legido-Quigley, D. A. Parker, J. R. Swann, M. Spraul, U. Braumann, A. M. Gil, E. Holmes, J. K. Nicholson, G. M. Murphy, H. Vilca-Melendez, N. Heaton and J. C. Lindon, *Mol Biosyst*, 2009, **5**, 180–190.
134 S. Barnes and D. N. Kirk, in *The Bile Acids: Chemistry, Physiology, and Metabolism*, Springer US, Boston, MA, 1988, pp. 65–136.
135 D. V. Waterhous, S. Barnes and D. D. Muccio, *J. Lipid Res.*, 1985, **26**, 1068–1078.
136 O. B. Ijare, B. S. Somashekar, Y. Jadegoud and G. A. Nagana Gowda, *Lipids*, 2005, **40**, 1031–1041.
137 G. A. N. Gowda, B. S. Somashekar, O. B. Ijare, A. Sharma, V. K. Kapoor and C. L. Khetrapal, *Lipids*, 2006, **41**, 577–589.
138 T. Bezabeh, O. B. Ijare, N. Albiin, U. Arnelo, B. Lindberg and I. C. P. Smith, *MAGMA*, 2009, **22**, 267–2713.
139 G. A. N. Gowda, *Bioanalysis*, 2011, **3**, 1877–1890.
140 G. A. Nagana Gowda, N. Shanaiah, A. Cooper, M. Maluccio and D. Raftery, *Lipids*, 2009, **44**, 27–313.
141 C. J. Sims, D. T. Fujito, D. R. Burholt, J. Dadok, H. R. Giles and D. A. Wilkinson, *Prenatal Diagnosis*, 1993, **13**, 473–480.
142 L. C. Robosky, K. Wade, D. Woolson, J. D. Baker, M. L. Manning, D. A. Gage and M. D. Reily, *J. Lipid Res.*, 2008, **49**, 686–692.
143 A. Z. Damyanovich, J. R. Staples, A. D. Chan and K. W. Marshall, *J. Orthop. Res.*, 1999, **17**, 223–231.
144 S. Meshitsuka, E. Yamazaki, M. Inoue, H. Hagino, R. Teshima and K. Yamamoto, *Clin. Chim. Acta*,

1999, **281**, 163–167.
145 L. Bala, A. Sharma, R. K. Yellapa, R. Roy, G. Choudhuri and C. L. Khetrapal, *NMR Biomed*, 2008, **21**, 606–614.
146 V. Behrends, K. J. Williams, V. A. Jenkins, B. D. Robertson and J. G. Bundy, *J. Proteome Res.*, 2012, **11**, 3888–3896.
147 J.-N. Dumez, J. Milani, B. Vuichoud, A. Bornet, J. Lalande-Martin, I. Tea, M. Yon, M. Maucourt, C. Deborde, A. Moing, L. Frydman, G. Bodenhausen, S. Jannin and P. Giraudeau, *Analyst*, 2015, **140**, 5860–5863.
148 T. W.-M. Fan and A. N. Lane, *Prog Nucl Magn Reson Spectrosc*, 2016, **92-93**, 18–53.
149 A. N. Lane and T. W.-M. Fan, *Arch. Biochem. Biophys.*, 2017, **628**, 123–131.