Analytica Chimica Acta 980 (2017) 1–24

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Tutorial

Quantitative NMR analysis of intra- and extracellular metabolism of mammalian cells: A tutorial

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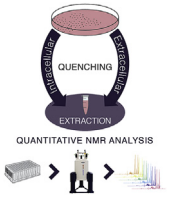
HIGHLIGHTS

- Quenching, extraction and analysis of mammalian cells.
- Quantitative NMR analysis of intra- and extra-cellular metabolites.
- Tutorial describing a step by step procedure.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 17 January 2017
Received in revised form
11 April 2017
Accepted 13 May 2017
Available online 24 May 2017

Keywords:
NMR
Mammalian cells
Metabolomics
Quantitation

ABSTRACT

Metabolomics analysis of body fluids as well as cells is depended on many factors. While several well-accepted standard operating procedures for the analysis of body fluids are available, the NMR based quantitative analysis of cellular metabolites is less well standardized. Experimental designs depend on the cell type, the quenching protocol and the applied post-acquisition workflow. Here, we provide a tutorial for the quantitative description of the metabolic phenotype of mammalian cells using NMR spectroscopy. We discuss all key steps of the process, starting from the selection of the appropriate culture medium, quenching techniques to arrest metabolism in a reproducible manner, the extraction of the intracellular components and the profiling of the culture medium. NMR data acquisition and methods for both qualitative and quantitative analysis are also provided. The suggested methods cover experiments for adherent cells and cells in suspension. We ultimately describe the application of the discussed workflow to a thyroid cancer cell line. Although this tutorial focuses on mammalian cells, the given guidelines and procedures may be adjusted for the analysis of other cell types.

Contents

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http://dx.doi.org/10.1016/j.aca.2017.05.011

1. Introduction

In vitro cell based metabolomics studies, often combined with other −omics, have found widespread use in many areas of research. These include: studies on the effect, action and toxicology of drugs [1,2], characterization and understanding of cancer cell metabolism [3], regenerative medicine [4,5], immune metabolism [6,7] and many more. The common goal of all of them is to understand and decipher the influence and involvement of metabolism on/in biological effects and mechanisms, and integrate this information onto metabolic maps [8]. When working with cellular systems, detailed quantitative metabolic data is required for both the intra- and extra-cellular compartment. In recent years, several targeted metabolomics approaches have been developed in order to obtain such quantitative metabolic data, including mass spectrometry (MS) and NMR based methods [9–22]. Particularly MS based approaches are jeopardized by ionization suppression, matrix effects and linearity issues, of which some have been overcome by global internal standard methods such as the MIRACLE approach [21,23]. On the other hand, NMR spectroscopy is much less sensitive when compared to MS based techniques. Nevertheless, firstly, many core metabolites needed for constructing metabolic maps (i.e. amino acids, sugars, TCA cycle intermediates) are present at levels which can be analysed using NMR spectroscopy. Secondly, NMR is a non-destructive and much more robust technique when compared to MS and thirdly, quantitation using NMR can be carried out in a straightforward manner without the need for specific internal standards spanning as much as six orders of magnitude. In summary, NMR is a robust, quantitative technique which can be used to analyse several core features of cellular metabolism. There are two options to measure cellular metabolism using NMR, either using intact cells and high resolution magic angle spinning (HR-MAS) NMR [24], or using extracts reconstituted in a solution sample. The first option, although useful, requires special equipment and is beyond the scope of this tutorial. Instead, here we present a detailed step by step tutorial on how to use NMR for obtaining quantitative cellular metabolic data for more than 65 key metabolites from several chemical classes.

2. Sampling of cells and culture media

The overall enzymatic activity within cells is sensitive to the extracellular environment, and specifically to the availability of substrates, the pH and the local temperature. Therefore, the choice of culture medium and the growth environment is a critical step in the experimental design. First, cells need to be cultured under standardized conditions, optimized for the needs of the study and able to provide reproducible cellular phenotypes between batches of cell lines [25]. Second, as cell culture conditions can vary greatly between different cell types, care has to be taken that none of the medium components compromises the quality of the metabolic profiling with the selected analytical platform. The next step of the sampling process, and often the most difficult one, is the selection and optimization of a quenching method that produces reproducible and valid screenshots of intracellular metabolism. The components of the latter have to be extracted quantitatively with a method that provides high metabolic recovery. Most often, it is desirable to collect data from both, the extra- and intra-cellular compartment- and the separation of both is required prior to or during the quenching step. This process may vastly depend on whether the cells are in suspension or adherent. There is a plethora of methods proposed for the sampling of mammalian cells [26] and in the following sections we discuss the most suited ones and provide some practical guidelines for their facile implementation. The general outline of the proposed workflow is depicted in Fig. 1.

2.1. Culture medium selection

There is a broad range of synthetic culture media, with optimized formulations to fulfil the growth needs of a wide range of cells. However, the culture environment is influenced by the presence and levels of a number of factors [25]. These are: essential

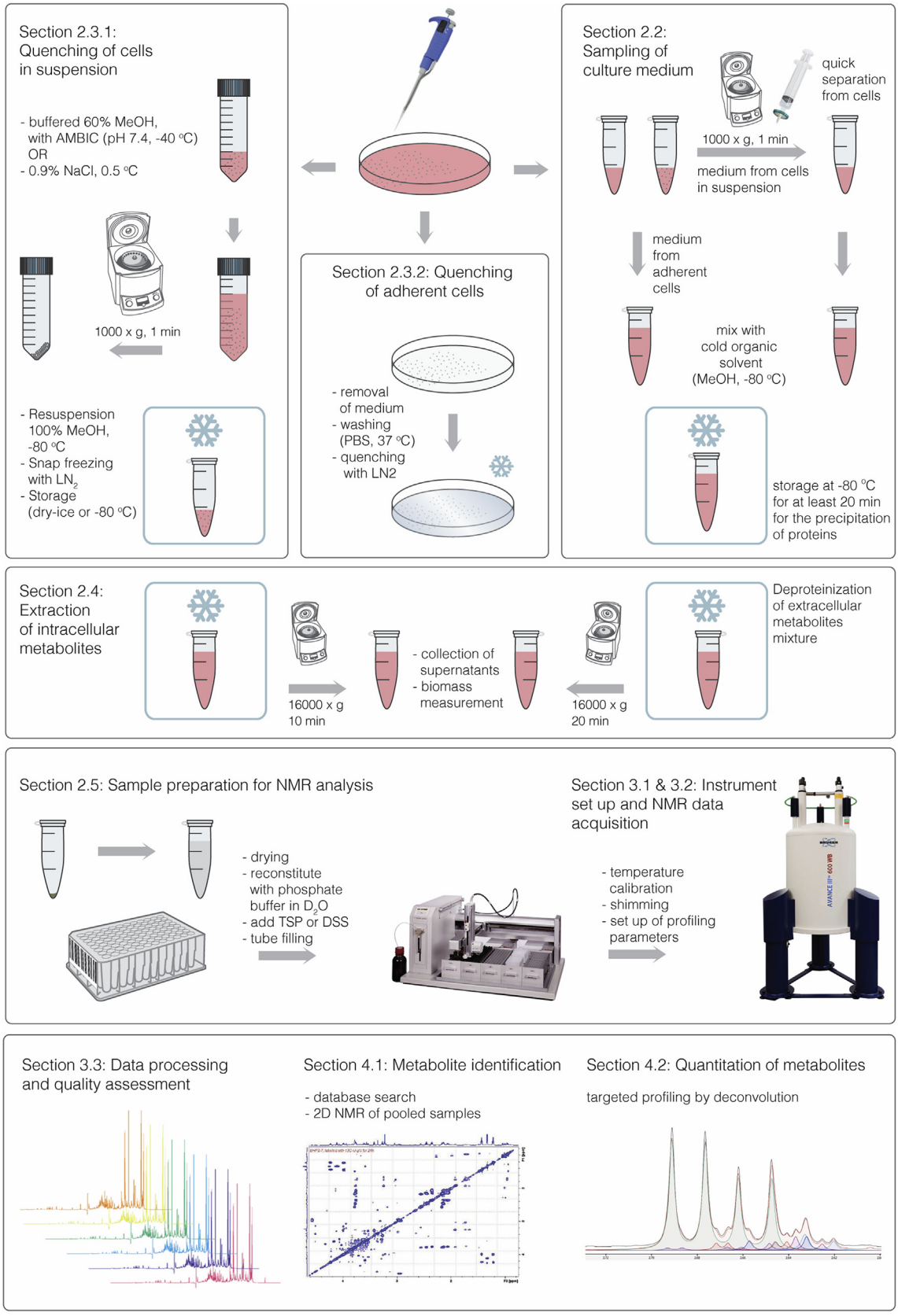


Fig. 1. Schematic workflow diagram for the presented NMR-based targeted profiling of cell culture medium and intracellular metabolites from adherent and in suspension mammalian cells.

nutrients (supplied by the culture medium), growth factors, hormones, gases (O_2 and CO_2) and a regulated environment (pH and temperature). The proper choice of a culture medium is based on the type of cells, with Dulbecco's Modified Eagle's Medium (DMEM) and Roswell Park Memorial Institute (RPMI) being the most frequently used media for a wide range of mammalian cells. However, also other types of medium as for example keratinocyte growth medium (known as KGM) were successfully analysed with the described protocol. In general, any type of standard mammalian cell medium should be analysable with the here described method. We however recommend to double check this, by analysing medium samples and comparing the obtained quantitative results with the supplier's specifications. Growth factors and hormones are usually supplied either as isolated serum components or by the addition of Fetal Bovine Serum (FBS) to the medium, in the range of 2%–10%. Stable pH, typically at neutral values (~7.4), is maintained by the addition of sodium bicarbonate ($NaHCO_3$) and the supply of 5–10% gaseous CO_2 in the incubator. In order to keep pH stable when CO_2 supply is not possible (e.g. outside the incubator) it is typical for media to include 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES). While HEPES has a superior buffering capacity compared to $NaHCO_3$, it is very problematic for NMR-based metabolic profiling as it is usually present in high concentrations (25 mM in DMEM) and its broad and intense resonances are overlapping with those from several metabolites of interest (Fig. 2). Therefore, if possible, HEPES should be avoided and extra care should be taken for fast handling of samples when placed outside the incubator, to avoid pH variation before quenching.

Another common reagent used in culture media is the pH indicator phenolsulfophthalein (phenol red). It is typically found in the range of ~25–40 μM and contributes two peaks in the aromatic region of the proton NMR spectrum at δ 6.62 and 7.37 ppm (at pH = 7.4). These peaks have a rather low intensity and appear at a less crowded spectral region and thus their presence is not hampering the quantitation of other compounds. None of the other components commonly used in culture media cause any problems associated to data quality, at least with regard to NMR analysis, except for intact FBS or isolated serum components for which a protein precipitation step is needed (*cf* section 2.2).

2.2. Sampling of culture medium

In the case of adherent cell cultures, collection of medium is straightforward and can be done at several time points during the growth period without affecting the cells. For NMR-based studies, about 50–300 μL are directly collected from the culture plate using a pipette. However, it should subsequently be secured that the remaining medium volume is not becoming a limiting factor, in that cell growth or metabolism might be affected. In our lab, cells are usually cultured in approximately 6–10 mL of medium from which a maximum of 0.3 mL is collected for analysis. However, in case of cells growing in suspension, there is no reliable manner to collect culture medium without affecting the state of the cells. Still an option can be when, either the whole culture broth or a portion of it is collected and separation of cells from the culture medium is rapidly performed by filtration or centrifugation. Filtration has the advantage that it can be carried out in a few seconds by immediately passing a small quantity of the culture broth through sterile syringe filters with 3 kDa cut-off membranes. The pore size of the filters depends on the size and the number of cells, since too many cells can block the filters and reduce the recovery of the medium [27,28]. Pore sizes ranging from 0.2 to 5 μm have been used [27,29,30] and a study specific evaluation is necessary for the proper selection of filters. Another critical issue when using filters is to remove membrane additives, e.g. glycerol on cellulose filters, which can contaminate the filtrate. We recommend thorough washing of the filters with phosphate buffered saline (PBS) or 0.9% (w/v) NaCl solutions at room temperature prior to usage and

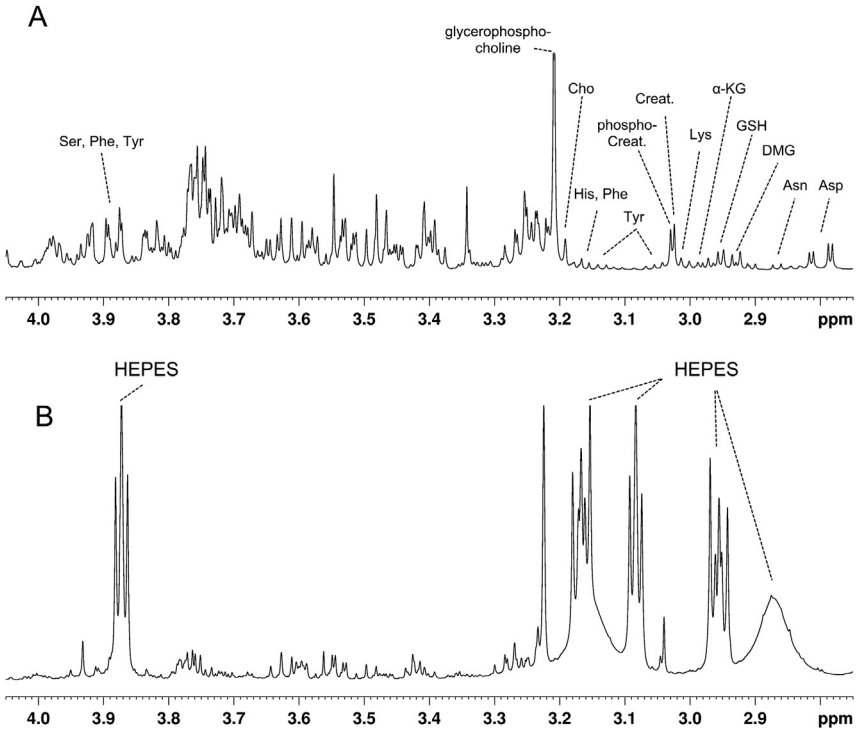


Fig. 2. 1D ^{1}H NMR spectra of intracellular metabolites from cells cultured in a medium with (A) or without HEPES (B). Several metabolites shown in (B) are masked by the broad and intense resonances of HEPES (A).

collection of a blank washing filtrate as a control for impurities.

Applying centrifugation as a method to separate extracellular from intracellular metabolites, has the disadvantage of being rather slow, possibly leading to a skewing of intracellular metabolism. On the other hand, it has the advantage that a high number of cells can be sampled in contrast to filtration. If only the exometabolome is analysed, and fast filtration is not an option, a mild centrifugation (<1000×*g*) at 4 °C or lower (down to −20 °C) for 1 min should be sufficient to separate cells from medium. Unfortunately, in this case, leakage of intracellular components cannot be excluded. Centrifugation is generally preferred when both intracellular and the extracellular metabolites are to be analysed from the same sample and a high number of cells per sample is employed. For this purpose, separation of the cells from the culture medium and quenching are performed simultaneously, a topic that will be covered in the following section 2.3.

Regardless of the method used for collection, the culture medium samples should be processed immediately with an organic solvent to remove proteins (intact FBS or serum isolated macromolecules) and immediately quench any possible residual metabolism. Macromolecules can induce errors [31] in the subsequent NMR analysis for two reasons. First, metabolites might become non-specifically bound to proteins thus altering the sample's observed metabolic composition. Second, protons from macromolecules as well as those from their weakly bound ligands (metabolites) appear as broad resonances in the NMR spectrum due to their shorter transverse relaxation (T2 relaxation time) compared to small molecules. This ultimately compromises data quality and induces laborious processing steps (e.g. correction of baseline) and analytical errors due to broadened peak shapes (e.g. complex deconvolution of overlapped peaks, inaccurate fitting of peaks, etc.). Although, broad peaks from macromolecules can be technically attenuated by performing T2-filtered NMR experiments, like the Carr-Purcell-Meiboom-Gill (CPMG), the NMR intensities from other metabolites will also be partially affected due to small differences in T2 times, and thus their absolute quantitation will not be possible [32]. Protein precipitation of samples is therefore recommended. In most cases either cold methanol or acetonitrile [28,32] for macromolecule precipitation are used. In our laboratory, we routinely mix one volume of culture medium with two volumes of 100% LC-grade cold methanol (left overnight at −80 °C and then placed on dry-ice for a short time during sample collection) in a microcentrifuge tube and allow the proteins to precipitate at −20 °C for 30 min followed by centrifugation at 16000×*g* at 4 °C for 20 min. The supernatant is then collected and stored at −80 °C, or immediately dried to remove the methanol for subsequent NMR measurements.

2.3. Quenching of intracellular metabolism

Some intracellular metabolites present very fast turnover rates, such as ATP (1.5 mM/s), ADP (2.0 mM/s) and D-glucose (1.0 mM/s) [33,34]. Hence, prolonged sampling times may induce variations in the intracellular metabolome. Therefore, an immediate and effective quenching step is required, in order to obtain an accurate and reproducible description of the cellular phenotype. Today's most common methods for quenching include the use of cold organic solvents [20,35–37], cold isotonic solutions [17,27], or liquid nitrogen [38]. Often, a washing step is additionally performed to remove residual culture medium from the cells [17,20,37,38]. The selection of the right method is dependent on whether the cells are growing in suspension or attached to the plate wall. A careful evaluation and optimization should always be performed to match the study specific experimental design. A critical overview of the best suited protocols is provided in the following sections 2.3.1 to 2.3.3 together with practical recommendations.

2.3.1. Quenching of cells in suspension

A critical aspect of quenching with cold organic solvents and in particular methanol, is the reported membrane leakage and loss of intracellular components [27,39]. Two methods have been suggested to solve this problem, both tested with Chinese hamster ovary (CHO) cells. The first includes the addition of 0.85% (w/v) ammonium bicarbonate (AMBIC) as a buffering system (pH 7.4) in a cold methanol solution methanol/AMBIC 6:4 (v/v) at −40 °C) [35,39]. Quenching was performed by mixing one volume of suspension cultured cells (~1 × 10^7 cells) and 5 vol of quenching solution followed by centrifugation for 1 min at 1000×*g* at −20 °C. The supernatant (quenching solution) was then removed and 0.5 mL of cold 100% methanol (−80 °C) was added to the cell pellet and the mix was snap frozen in liquid nitrogen and stored at −80 °C until extraction. The critical steps of this method are, i) to maintain the quenching solution at −40 °C but not colder than −45 °C, in order to avoid membrane leakage and ii), to perform a mild centrifugation at −20 °C for no longer than 1 min. An adjustment could be made to the duration of centrifugation, if for example the available instrument cannot operate at −20 °C, shorter times should be evaluated in order to ensure the temperature remains low [40] while still managing to separate the cell pellet.

In the second method, the use of an isotonic aqueous solution (0.9% (w/v) NaCl at 0.5 °C) instead of methanol is proposed for quenching [27]. In this study, one volume of suspension cultured cells was mixed with four volumes of 0.9% (w/v) NaCl and centrifuged at 1000×*g* for 1 min at 0 °C. The cell pellet was then placed on ice for a maximum of 10 min until extraction. However, in our experience, it can also be frozen and stored at −80 °C or on dry-ice until extraction if more time is required. This method appears to be less laborious, e.g. there is no need for narrow temperature control at −40 °C. However, both studies [27,39] have used the same cell line (CHO cells) but failed to reproduce exactly the same results after comparison of the two methods [27,35]. At the moment, there isn't any definite answer found in literature concerning the most suited method for quenching suspension cells and in most cases, adaptations of the above mentioned methods are needed [26]. Ideally the ultimate choice should rely on some study specific quality assurance criteria which are discussed below in section 2.3.3.

2.3.2. Quenching of adherent cells

Adherent mammalian cell metabolism can be quenched with either cold organic solvents [20,37], cold buffer solutions [17] or liquid nitrogen (lN_2) [38]. While the majority of the culture medium is easily removed by aspiration, unfortunately some traces will remain in the dish. Since many intracellular metabolites are also present in the culture medium, incomplete removal will quantitatively affect the obtained data for intracellular metabolites. Therefore, one or two rinsing steps are performed with either ice-cold PBS [20], ice-cold saline solution (0.9% NaCl (v/v)) [17], warm PBS (37 °C) [37], or warm deionised water (37 °C) [38]. Washing with cold PBS or saline solution has the advantage that metabolism is slowed down due to the low temperature obtained and therefore provides a time window needed to add the quenching solution. On the other hand, applying a cold shock to the cells might increase the risk of membrane leakage and loss of intracellular metabolites. From this point of view, washing with a solution at the same temperature as the culture medium, reduces the cold shock, but has to be performed quickly enough (in less than 5 s) to minimize turnover of labile metabolites. While direct comparison of warm PBS with warm deionised H_2O resulted in higher metabolic recovery for cells washed with water [38], the very low ionic strength

of the latter increases osmotic pressure possibly affecting membrane integrity [41]. PBS on the other hand is a balanced osmotic salt solution and is well suited for short term cell storage. From our experience, rinsing cells once with warm PBS (in 3–5 s) is sufficient for complete removal of culture medium prior to quenching.

While quenching with cold organic solvents or buffers are feasible techniques, the choice of lN$_2$ is the quickest, most simple and effective method [38]. As soon as the culture medium is completely removed, there is no need to maintain cellular membrane integrity. On the contrary, snap freezing with lN$_2$ aids the subsequent extraction step by inducing some damage to the cell membranes and thus increasing metabolite extraction efficiency. However, since lN$_2$ is quickly evaporated, it is critical that cells are not allowed to thaw until the extraction step. This is easily achieved by keeping the dishes on dry-ice until all samples are processed. For a culture dish with a diameter of 10 cm, about 5 mL of warm PBS are needed for washing and 10 mL lN$_2$ for quenching.

2.3.3. Design and evaluation of optimal quenching conditions

As it was discussed in section 2.3.1, the selection of the optimum quenching protocol for cultured cells in suspension is often a study specific question and there is some confusion about what method to follow. In our opinion, an optimal outcome can be obtained when, evaluation experiments using either one of the suggested methods as the basis are performed. Subsequently small adaptations to the method of choice should be applied until the demanded quality parameters are fulfilled, i.e. effective quenching of metabolism and no (or at least minimal) membrane leakage. Firstly, cellular membrane integrity during quenching should be evaluated by analysing both, intracellular and extracellular metabolites and comparing their levels with control culture medium (cell-free) as well as culture medium from unquenched cells. Two sets of metabolites can be used as measures for membrane integrity. The first set consists of labile molecules, like ATP, ADP, AMP, NADH and NAD^+. These metabolites are typically found inside the cell, and their identification in the medium is an indication of a disrupted membrane. This set of metabolites can also be measured by enzymatic assays. The second set of metabolites are the tricarboxylic acid intermediates, citrate, succinate, malate and α-ketoglutarate (oxaloacetate can be confused with pyruvate in the proton NMR and should therefore be avoided here), which are indicators of membrane leakage [35]. We recommend evaluation of the levels of these two sets of compounds in the intracellular extract, the culture medium after quenching and the medium from unquenched samples. Direct comparison of recoveries in each of the quenching methods used, as well as the presence of the labile compounds in the medium will provide the basis for choosing the best quenching method with regard to membrane integrity. In those cases that membrane leakage is found for all suggested methods described here, additional adaptations are needed, specifically investigating centrifugation time and g-force, as well as temperature and increase of ionic strength of the applied quenching solution.

Secondly, quenching efficiency should be investigated (for both adherent and suspension cells) using the adenylate energy charge (AEC) as an estimation [42]. This is done employing the following equation: AEC = (([ATP] + [ADP]/2)/([ATP + [ADP] +[AMP])) and provides a good readout of the cells state after quenching. Actively metabolizing cells exhibit a high excess of energy-rich adenylate phosphate resulting in high AEC values (0.80–0.95) after quenching, indicating an effective quench of metabolism. ATP, ADP and AMP can be evaluated by commercial assay kits.

A final critical issue of the quenching procedure that should be noted is the often-underestimated issue, of maintaining the quenched state until extraction has occurred. Typically, the entire experiment includes the quenching of several samples, hence a considerable time interval until extraction has to be taken into account, during which the temperature of the quenched cells has to remain low (<-50 °C). Moreover, it has to be considered that the temperature of the quenching solution increases when added to a plate or tube with cells being at 37 °C. Therefore, if for example, ice-cold saline solution is used, a slight increase of temperature is initially expected, which cannot be circumvented by placing the samples on ice. A study using the model microorganism *Corynebacterium glutamicum*, quenching with 60% (*v/v*) methanol at −50 °C, has shown extensive labelling of intracellular metabolites upon addition of uniformly ^{13}C-labelled D-glucose to the quenched mixture. After evaluation of the mixture's temperature, it was found that this activity occurred at −20 °C but not at −50 °C (quenching temperature) or at −80 °C (upon sample storage) [40]. It is therefore, strongly suggested that quenched samples are snap-frozen in lN$_2$ and temporarily stored −80 °C or kept in a box with dry-ice until extraction to avoid any post-quenching skewing of metabolism.

2.4. Extraction of intracellular metabolites

Several methods have been evaluated for their performance in extracting intracellular metabolites. The majority concluding that the use of organic solvents and their aqueous mixtures are the best option. Unfortunately, there remains much confusion about which exact solvent and mixture to use, furthermore considering polarity, temperatures and extraction cycles. For instance, ice-cold 50% (v/v) aqueous acetonitrile [27], 82% (v/v) ice-cold aqueous methanol [20], pure methanol [17,43] and 90% (v/v) 9:1 aqueous methanol/chloroform [12,38] have all been evaluated and described as best extraction solutions after quenching. On the other hand, 70% (v/v) ice-cold aqueous acetonitrile [37], and ice-cold 80% (v/v) aqueous methanol [44] have been proposed to perform quenching and extraction in a single step. However, the inhomogeneity of the intracellular metabolites, the various cell lines and the different analytical methods used in the described studies might explain the different outcomes. In turn, extraction should be evaluated individually per researcher per study.

Also, it should be kept in mind that, except for good metabolite recoveries, protein and macromolecule precipitation are important characteristics of extraction solutions. In our experience the aqueous solutions of acetonitrile (ACN), methanol (MeOH) and methanol/chloroform (MC) mentioned above, all fulfill these criteria, however, only for MC, a systematic evaluation of the degree of polarity is described in literature [38]. It was found that a ratio of 90% (v/v) aqueous MeOH/$CHCl_3$ 9:1 (MeOH/$CHCl_3$/H_2O (v/v/v), 8.1:0.9:1) results in approximately 75% (v/v) aqueous MeOH/$CHCl_3$ 9:1 when the residual water in the 10 cm culture dish is taken into account (~0.3 mL). For this condition, the extraction resulted in highest recovery for a broad range of metabolites [38], while effectively removing intracellular macromolecules. However, and as the authors suggest, they achieved similar results with a 70% (v/v) MeOH solution but with slightly less stable extracts. In our lab, we have successfully implemented the 90% MC extraction with an adherent cell line, as we outline in the last part of this tutorial (Section 5).

The temperature of the extraction process is another critical aspect. We have already discussed in 2.3.3 that some enzymatic activity occurs after quenching if the temperature is not low enough. The same is known to occur even after extraction of the intracellular metabolites, probably due to incomplete removal of proteins or chemical interconversion of metabolites in the extract [38,45]. Based on our experience, keeping the sample temperature as low as possible throughout the whole process from post-quenching to extraction and cell pellet removal, is an important

factor to achieve minimal variability and optimal stability. We suggest keeping dishes or tubes always on dry-ice during sampling and using a −80 °C extraction solution (MeOH/$CHCl_3$ or aqueous MeOH). The centrifugation step for the separation of the extract from the cell debris should also be carried out at the lowest possible temperature. We have obtained highly reproducible results with centrifugation at 16000×*g* and 4 °C for 15 min.

Finally, it has been shown that repeated extraction cycles using the same solvent system as initially applied helps in increasing metabolite recovery [35,43]. In addition, methods that increase cell membrane disruption like ultra-sonication [46], mechanical homogenization [20] and freeze-thaw cycles [35] can also increase metabolite recovery. From our own experience in several cell lines, repeating the 90% (v/v) MC 9:1 extraction step did not alter total metabolite recovery, being in agreement with literature [38]. However, when testing ultra-sonication as an additional extraction step in combination with the MC protocol in murine macrophages, we observed increased metabolites levels. Once more, it is apparent that several tests should be performed to optimize the conditions of sampling to ensure valid results.

We recommend a multilevel evaluation with a limited number of biological replicates of the same type of cell lines that will be used in the study. First, the optimum polarity of the extraction solvent system should be tested, with regard to protein precipitation and the recovery of the most polar metabolites. The nucleotides and especially the triphosphates (ATP, CTP, GTP) and some polar amino acids as for example arginine and asparagine together with some less polar ones like phenylalanine, proline and leucine, can be used in these tests. We suggest to use 3 different polarities, starting from 50% (v/v) MeOH (or ACN), to 75% (v/v) aqueous MeOH/$CHCl_3$ 9:1 (or the 70% (v/v) aqueous MeOH) and finally 100% MeOH. All extraction solvents should be cooled to at least −40 °C or lower. The ideal extraction solvent is the one presenting highest overall recovery with lowest residual protein content. The decision upon this can be directly taken with a simple and quick one dimensional (1D) proton NMR measurement of these replicates (for details about the NMR experiment see section 3.2 below). As NMR is a quantitative method, there is no need for calibration functions at this point, but rather a straightforward judgement from spectral overlay should suffice. Protein presence can be easily evaluated by the intensity of the broad resonance at 0.95–0.85 ppm (methyl protons). Observation of the internal standard used for chemical shift and concentration reference is an additional way to decide if proteins are sufficiently removed. In the absence of non-specific binding, caused by proteins, the intensity and the linewidth of the internal standard peak should be identical in all spectra (provided they are all acquired with exactly the same parameters; see below sections 2.5 for NMR sample preparation and 3.1–3.3 for NMR experiments).

Once the optimum polarity is determined, the number of extraction cycles should be tested with the selected solvent system. As described above, a second extraction step with 75% (v/v) aqueous MeOH/$CHCl_3$ 9:1 did not add to the recovery of metabolites in our and others experience [38]. However, we do recommend an evaluation of this step prior to studies where a specific cell type is being used for the first time. Again, direct comparison of 1D proton NMR spectra by overlay is the most straightforward method to quickly evaluate the possible gain of a second extraction step.

Finally, an overall judgement should be made considering extraction efficiency with regard to the number of metabolites present in the spectra and the cell numbers or the total biomass of each sample. Poor spectra, in terms of “compounds detected” and low signal intensity of some compounds, indicate poor extraction efficiency. The judgement of the number of compounds detected is somewhat empirical but in general, one should expect to identify the vast majority of amino acids, even those that are usually present in the low micromolar range like tryptophan. On the other hand, the intensity of the peaks originating from the lactate methyl protons and/or some cholines likes phosphocholine or glycerophosphocholine should be comparable to the intensity of the internal standard (see section 2.5 below). If this is not the case, even after a sustained 1D experiment (e.g. 512 or 1024 scans), this is a clear indication for poor extraction efficiency. In these instances, additional extraction measures should be tested. This can be either ultra-sonication for 30 s or 1 min or 3 × freeze-thaw cycles [35,46].

2.5. Sample preparation for NMR analysis

Sample preparation for NMR analysis is typically much less laborious than the methods described in the previous sections. Both the culture media and the cellular extracts need to be dried for the removal of the organic solvent(s) used for the quenching and/or the extraction. Drying can be done either using a SpeedVac or by applying a stream of nitrogen gas. The procedure typically takes a few hours (2–3 h), depending on the solvent system. Importantly, samples should be handled under reduced lighting and contact with oxygen should be avoided, in order to keep any possible metabolite degradation as low as possible. Evaporation should be carefully monitored and samples processed further as soon as possible.

The dried material is then reconstituted with phosphate buffer (0.15 M K_2HPO_4, pH 7.0 or 7.4) in deuterated water (D_2O) (~250 μL). Several key points need to be further addressed here. First, the regulation of the sample pH with the buffer to a small range either close to 7.0 or 7.4, ensures minimal or no shifting of NMR resonances across the whole set of monitored analytes, facilitating quantitative analysis of complex samples. In our studies, we have obtained excellent reproducibility using 0.15 M K_2HPO_4 at pH 7.4, with only minor shifting of, for example, histidine's protons. However, regulation at pH 7.0 is also acceptable and followed by others [47]. The second point concerns the selected volume to reconstitute the dried material. In our laboratory, we routinely use 3 mm NMR tubes and we reconstitute the dried extracts in 250 μL, however, this volume should be adjusted if other tube sizes (e.g. 5 mm NMR tubes) are to be used. Three mm tubes require 165 μL of NMR sample volume. The additional volume (up to 250 μL) ensures that there is enough sample left after tube filling to make a pool sample for each group of cell extracts (e.g. triplicates from a time point or from a specific cell line). We highly recommend the preparation of pool samples for two purposes. First, a pool sample can be used for the optimization of the spectrometer prior to automatic measurements (see section 3.1) and second, they can be used for two-dimensional (2D) NMR experiments and (if needed) for spiking experiments with reference compounds for metabolites identification. In the case that more than 3 biological replicates are used per group, the dried extract can be reconstituted in less than 250 μL of buffer, which will result in even more concentrated samples and thus increased sensitivity.

Finally, although we use and recommend the use of 3 mm tubes for studies with cell extracts, clearly the selection of the tube size is dependent on the available equipment of each laboratory. The volume needed for the 3 mm tubes is 3.5 times less than the one needed for the more common 5 mm tubes (560 μL) and thus, a significant gain in concentration of metabolites in the sample is achieved. However, as most NMR laboratories are equipped with 5 mm probes, measuring in 3 mm tubes induces a compromise of the increased thermal noise, which eventually reduces the signal to noise (S/N) in the recorded spectra. This noise occurs due to a longer distance between the sample in the probe and the coils used to apply and record the radiofrequencies (RF) during the NMR

experiment. The loss of S/N is significantly reduced with cryoprobes allowing for the use of 3 mm tubes with less concentrated samples. However, this gain might not apply in room-temperature probes and in this case the use of 5 mm tubes should be preferred. Another option to improve sensitivity, would be to use 1 or 1.7 mm microprobes. These probes operate with capillary tubes with diameter of 1 or 1.7 mm, and require just 5 or 30 μL of sample, respectively. Such significant decrease of sample volume would allow for much more concentrated samples and thus boost sensitivity. One potential drawback however, could be the sample preparation accuracy (i.e. accurate addition of buffer and internal standard). With such small volumes, the analytical error can be large and detrimental for the accuracy of the quantitative analysis.

In contrast to other analytical methods, only one internal standard is necessary for NMR analysis, being either 3-(trimethylsilyl)-propionic-d_4 acid sodium salt (TSP-d_4) or 3-(trimethylsilyl) propanesulfonic-d_6 acid sodium salt (DSS-d_6). Both compounds can be used as chemical shift references for the calibration of the NMR data (at δ 0.0 ppm) as well as internal standard for quantitation. It should be noted however, that in contrast to DSS, TSP is sensitive to pH and in some cases, it is preferably referenced at δ −0.0159 ppm, with respect to DSS (see section 4.2 for more details). We recommend a concentration of about 0.02 mM of TSP or DSS in the NMR samples of intracellular metabolites and about 0.2–0.4 mM for culture medium samples. These concentrations ensure that the internal standard will have a resonance with sufficient S/N for accurate quantitation and at the same time an intensity within the average dynamic range of the typically observed components of the samples. Addition of the buffer and the included internal standard should be done with the highest possible accuracy. Analytical variation at this step will induce significant errors if absolute concentrations of metabolites are to be determined. Upon addition of the buffer the samples should be vortexed for 30 s and centrifuged at ~16000×g at 4 °C for 15 min to remove any insoluble material. Supernatants are then transferred to NMR tubes. If possible, a robotic liquid handler can be used at this point for optimal reproducibility of NMR tube filling [48].

As additional step to ensure proper quality of the generated data, we suggest the preparation of a few quality control samples (QC) of the same matrix with at least one component of known concentration added. An easy way to do this is by making aliquots from a pooled mix of all samples of the intracellular extracts samples used in the particular experiment or from the culture medium. While for the latter the preparation is straightforward due to the excess of the available volume, for the intracellular extracts, care has to be taken to dissolve the dried material in more volume than the one require for the NMR tube filling, as described above, and then prepare some QC samples (at least 2 per 96-well-plate) from the leftovers. Except for the TSP or DSS, an additional reference compound (e.g. formate or sodium acetate) can be spiked to these QC samples, which will be used as a second correction measure of the quantitative data. All NMR samples should remain stored at 4–6 °C until analysis but no longer than 48 h, as stability might become an issue.

3. NMR spectroscopy

NMR is used for both structure elucidation and quantitation. It is considered a robust and quantitative analytical platform, well known for high reproducibility [49], exhibiting a quantitative inaccuracy of less than 2% [50]. In order to take full advantage of these features, both physico-chemical and instrumental parameters need to be optimized prior to data acquisition. We have already referred to the pH regulation in the previous section (2.5). Both the pH and the ionic strength affect the chemical shifts and the relaxation properties of the protons. Keeping the sample pH within a narrow range - for cell extracts a pH at 7–7.4 is chosen – results in better data consistency for both the positions and the intensities of the peaks across all spectra. Besides sample properties, a number of other, instrument – related factors can have a significant influence on the accuracy and precision of the generated NMR data and thus, they all have to be adjusted and optimized prior to data acquisition [51]. These parameters are: temperature, homogeneity of the magnetic field, water signal suppression, tuning and matching of the probe and the pulse for excitation as well as experimental parameters like resolution, measurement duration and the sensitivity. With modern NMR instruments, experiments can be performed in full automation by loading a pre-optimized set of the above-mentioned parameters for each sample and applying small adjustments for further sample-specific optimization. An overview of the instrument set up is covered in section 3.1. With quantitative NMR in mind, some processing of the raw data is also performed to enhance the appropriateness of the spectra and the latter is evaluated by applying several quality criteria. In section 3.2 we provide some general guidelines to assist the readers implementing these steps in their own studies.

3.1. NMR spectrometer set up

Prior to any measurements, a calibration of the probe's internal temperature should be carried out. This is the actual temperature that every sample will adopt and maintain throughout the whole experiment. The most common method is to use 99.98% deuterated methanol in a sealed NMR tube (it is strongly recommended to use a fresh methanol solution from an ampule) and acquire a short ^{1}H NMR experiment (~2 scans). The actual sample temperature can be calculated accurately form the distance (in Hz) between the two methanol peaks (CH_3 and OH) [52]. Appropriate adjustments are made until an exact temperature of 300 K (27 °C) is achieved. All samples should then be measured at this temperature with an acceptable deviation range of ±0.02 K. In our system, we have observed that the sample requires about 3 min in the probe to adopt to a stable temperature of 300 K. However, we are applying and strongly recommend, to wait 5 min after each sample is placed in the probe and before any automatic (or manual) routines are started, to ensure proper stability of the temperature. If the temperature is not stable beyond 5 min the temperature control system should be tested for malfunction. In some systems, like the SampleJet form Bruker, there is the possibility to pre-heat the sample before insertion to the magnet. If such a system is available, then the sample can adopt the desired temperature faster and the automation routines can start earlier than the suggested 5 min.

As long as the temperature is calibrated, a standard aqueous solution is used to optimize the magnetic field homogeneity (shimming). Improper shimming will result in distorted peak shapes and reduce the resolution, therefore it will directly affect the quality of the quantitation [51]. A pooled sample from the study in progress is an ideal candidate for this step. The optimum shimming is stored and can be used as the basis for the automatic shimming routine which is applied to each sample. Since, the stored shim file is created using the same matrix as the one of all samples in the study, an on-axis (z-axis) automatic shimming for each sample is sufficient to provide good homogeneity for the automatic measurements. The shimming quality can be monitored by using the peak shape of the singlet from the internal standard (TSP or DSS). The linewidth at half height should not exceed 0.7 Hz.

Although the samples are prepared in D_2O (99.98% ^{2}H), there will be some residual protonated water and its resonance should be suppressed. This is achieved by a continuous soft pulse (presaturation) with a bandwidth of 25–50 Hz [49] implemented in the 1D

version of the NOESY experiment [53]. Ideally, a good water suppression experiment should result in a flat baseline at both sides of the water resonance and a proper phase of all peaks in the spectrum after correction. Although the water suppression efficiency will probably vary slightly across spectra, this variation will be negligible for most spectra if an optimization step is performed before the study measurements. The proper way to do this, is to use the same pool sample as the one used for shimming optimization above, and optimize the bandwidth and the irradiation frequency for water suppression. This can be done by small adjustments of the water resonance frequency and acquisition of 1D NOESY experiments with 8 scans and all other parameters fixed (delays and mixing time), until the optimum result is achieved. As a general rule, we suggest that the width at the baseline of the suppressed water peak should not exceed 0.15 ppm (~90 Hz in a 600 MHz NMR) when D_2O (99.98%) is used as solvent as we propose in this tutorial. In the case that 10% of D_2O is used, then the H_2O content is much higher and an increased bandwidth (50–100 Hz) should be used for sufficient suppression. One drawback of the presaturation method however is the partial saturation of the resonances from other protons in the same region (close to 4.7 ppm). A common example are the anomeric protons of α- and β-glucose, resonating at δ 5.23 and 4.67 ppm, respectively. These resonances will be slightly reduced and this has to be taken into account for quantification as we will discuss in section 4.2.1 below. Despite this disadvantage of presaturation, the robustness of the method, the limited optimization needed and the ease of automation make it the best method for studies with biological mixtures [54,55].

Modern NMR spectrometers and probes technology, allow for automatic tuning and matching of the probe and pulse calibration for each sample that is inserted into the magnet. Both are depended on the sample and it's physico-chemical properties as well as the temperature and their proper calibration is crucial for data consistency. The full set of optimized parameters can be initially obtained using the pooled sample and then used throughout the whole study with only small per-sample adjustments of tuning, matching of the probe, shimming and the 90° pulse calibration [48].

3.2. NMR data acquisition

For cellular extracts and culture media samples, two experiments are performed per sample, a 1D ^{1}H NOESY and a fast 2D ^{1}H *J*-resolved (*J*res) (Fig. 3). The 1D NOESY provides excellent quantitative spectra with good solvent suppression [53]. The *J*res experiment displays the coupling patterns of protons (indirect dimension) in addition to their chemical shift (direct dimension) in two dimensions and is very useful for identification of metabolites, especially in crowded spectral areas [56]. For both experiments, the optimized presaturation bandwith and frequency is followed, as determined earlier in the pool sample. In addition to the optimized, instrumental and experimental parameters, there are also two additional requirements in order to get accurate quantitative spectral data with the 1D NOESY. These are, a long enough repetition time (i.e. the sum of the relaxation delay and the acquisition time, per scan) to allow the proton nuclei to fully relax after excitation, and sufficient number of data points for high resolution spectra.

The repetition time is a critical parameter, which in relation to the longitudinal relaxation time (T_1) of the protons, has an important role for the resonances of the peaks in the spectrum. A repetition time of about $5 \times T_1$ is needed to get 99% relaxed protons after excitation [49,57]. As a consequence, resonances from protons with longer $5 \times T_1$ than the repetition time used, will exhibit lower intensities and thus, their concentration cannot be correctly quantified. In complex mixtures such as those from cell extracts and culture media, the T_1 times vary significantly from a few hundred milliseconds to some seconds [57]. This practically means that in order to fulfil the quantitative NMR (qNMR) requirement, ideally, very long repetition times (30–50 s) should be used. Unfortunately, this would not be practical for the analysis of large samples numbers (typically tens or hundreds, for studies with mammalian cell lines) hence the use of shorter repetition times is common in NMR-based metabolomics studies.

Frequently, the comparison of the relative changes of metabolites within a particular study is required. In these cases, the short repetition times used in the 1D NOESY are not problematic since the T_1 effect on each resonance, will be the same for the same resonance across all spectra if identical parameters and conditions are applied. Even when absolute concentrations are to be obtained (e.g. to compare with the results from other laboratories or different analytical methods) there are ways to correct the quantification error due to insufficient relaxation time. One way is to apply a T_1 correction factor to each quantified resonance [51,57]. In order to do so, knowledge of all T_1 times is necessary, including the one of the internal standard used (TSP or DSS). Another option is provided by the Chenomx NMR suite software (Chenomx Inc. Edmonton, Canada). If this tool is used for quantification, then it is possible to extract accurate concentrations without any T_1 correction, while still using a short repetition time of 5 s. The only requirement is to prepare the sample and acquire the 1D NOESY experiment in the exact way suggested by the developer. In our laboratory, we typically use a slightly different set up, optimized for all our metabolomics analyses [48] with a repetition time of 6.72 s. In section 4.2 we discuss in more detail about the quantification methods and corrections with regard to experimental repetition times.

The spectral resolution requirement is much more straightforward and easily achievable in all modern NMR spectrometers. We suggest to collect 64 k data points in the 1D NOESY to assist a higher accuracy for quantitation. This amount or raw data together with a special processing method (zero filling, see below 3.3) provides high resolution spectra, which facilitate the quantification and deconvolution of overlapped resonances. For the interested reader, a detailed description of the experimental parameters and optimization routines, can be found in the paper of Dona et al. [48]. Although it is mainly written for application in body fluids and describes the procedures implemented on Bruker NMR instruments, with some adaptations the protocols can be implemented for studies with mammalian cells on any NMR spectrometer. In section 5 and in the supplementary information we also provide the experimental parameters we have used for the analysis of the BHP2-7 cell line on a 14.1 T NMR instrument. With the exception of the number of scans, which are depended on sample concentration, all other parameters can be used by the readers on their own NMR instrumental set up. Furthermore, we also provide the experimental parameters suggested by Chenomx and other labs [47] in the supplementary material.

With regard to experimental times and the number of scans used per experiment for cellular extracts and culture media, an evaluation has to be made using the pool sample(s) as representative measure and also some culture media replicates. The pool sample (or a sample from a trial experiment prior to the actual study) provides the right matrix to test for the measurement time needed. An easy method is to measure the S/N of some resonances, which exhibit low intensities. This will directly provide the basis of how many scans are needed. In general, a S/N of 3 represents the limit of detection (LOD) [58] but in most cases, the quantification of such small peaks might result in lower precision. For these cases

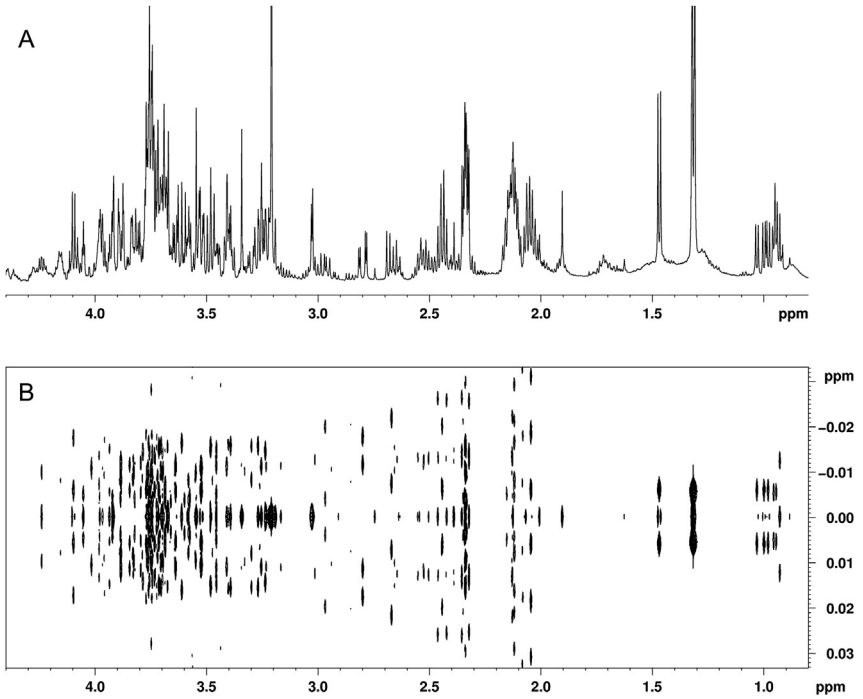


Fig. 3. Typical set of NMR experiments for the automatic profiling of cell extracts and culture media samples. A: spectral region from the 1D ^{1}H NOESY experiment of cell extract. The intensities and areas of peaks are dependent on the concentrations of the metabolites, and thus provide a quantitative fingerprint of the complex mixture. B: the same spectral region from the 2D *J*res spectrum, retaining the protons chemical shifts (horizontal axis) and displaying their coupling constants (in Hz) on the vertical axis. Singlets (no spin − spin coupling) are displayed as a single peak at the center frequency (0 Hz), while multiplets (doublets, triplets, quartets, etc.) are shown as two peaks symmetrically positioned around 0 Hz, with the distances between them being equal to their coupling constants in Hz. The dispersion of couplings fine structure in a second dimension, allows for the resolution of complex multiplets, which are otherwise overlapped in the 1D spectrum. This information (chemical shifts and couplings) is extremely valuable for the accurate fitting of pure compounds with the peaks in the complex mixture.

the limit of quantitation (LOQ) should be measured, i.e. the minimal S/N, for which an acceptable precision is achieved [51]. In a pilot study with cell culture media, we have used tryptophan quantification as a representative measure to decide upon the precision and the experimental time needed to achieve it. Tryptophan is commonly present in both culture media and intracellularly, however it is usually one of the less abundant metabolites and thus, it is a good example to estimate the experimental time. We used the BATMAN peak fitting software (see section 4.2.1 for BATMAN software details) to fit the 2 doublets at δ 7.55 and 7.74 ppm. We obtained a coefficient of variation (CV) of 10.61% at a S/N = 12.55, while for a S/N = 43.62, the CV was slightly reduced to 9.72%. A CV in this range (~10%) is frequently acceptable and therefore, we concluded that for this study a S/N of ~15 was sufficient to achieve our limit of quantitation. We should note at this point however, that often, the precision is also dependent on the complexity of the resonance and the method used for the quantification. We discuss further about this topic and the accuracy of quantification in section 4.2. In section 5, we also comment on the results of our quantification of cell extracts as well as, cells-free DMEM/F12 medium, with and without FBS.

As a general indication about NMR measurement times, using 3 mm tubes and 165 μL of sample, on a 600 MHz NMR spectrometer equipped with a cryoprobe, 25–40 min are needed to acquire both profiling experiments for samples in the range of 1×10^7 cells. However, with less cells (e.g. $1–5 \times 10^6$), measurement times can reach up to an hour (e.g. 512 scans for 1D NOESY) in order to increase metabolic coverage within the LOQ and improve the precision of the quantification in the low μM range.

3.3. Data processing and quality assessment

Typically, NMR data are processed automatically after data acquisition. This includes phase and baseline correction, referencing to the chemical shift of the TSP (or DSS) methyl groups at δ 0.0 ppm and line broadening (LB 0.1–1.0 Hz) to increase S/N. However, it is common practice to perform manual corrections in addition to the automated ones, especially for the correct phasing of the spectra [51]. On the other hand, the choice of the appropriate line broadening is also dependent on the method to be used for quantification. In general, a small line broadening of 0.1–0.3 Hz, will induce a small increase in S/N without compromising resolution and it is recommended for the analysis of complex mixtures with many overlapping resonances. If Chenomx is used for quantification, often a line broadening of 0.5–1 Hz, provides better fitting of the reference compounds to the metabolites in the spectra, even though the resolution is reduced. For example, the company mentions that the best overall fitting is achieved for DSS (or TSP) half-height linewidths from 1 to 1.5 Hz. Overall the choice of the LB factor is case dependent, but since this is a processing parameter, it can be changed easily on the same raw dataset as many times as needed, without destroying the recorded data (stored in the form of free induction decay; FID).

The quality of the processed spectra can be quickly evaluated by calculating the linewidths of the TSP (or DSS) singlet. We also routinely use Alanine's methyl protons doublet (at δ 1.48 ppm), using a half height linewidth value of 1.2 Hz as a cut-off above which the sample is examined further whether it should be re-measured or discarded from subsequent analysis. Moreover, multiple spectra overlay helps to identify inconsistencies in the data. NMR spectra from samples that were properly prepared and

measured in a well optimized instrument should have no phase and baseline offsets after the corrections and the residual water peak should not affect the spectral baseline outside of 4.65–5.1 ppm for cryoprobes or 4.7 to 4.9 for the room-temperature operating inverse probes. Furthermore, there should not be any peak drifting or only minimal for the protons with pK_a values close to the pH of the sample (e.g. histidine's imidazole proton and in some cases those from histamine and citrate). Finally, the integrals and the peak shapes of the internal standard's singlet should be identical across all spectra so that it can be used for quantification of the other resonances. Large deviations of the TSP peak area and shape might be due to insufficient protein removal during sample preparation, as TSP (and DSS) is known to bind non-specifically to several macromolecules present in plasma and serum. In that case, only relative quantitation is possible, unless a second measurement is carried out or a correction based on other standards is available (Fig. 4).

4. NMR data analysis

In metabolomics studies, it is common to initially analyse the NMR data in a non-targeted manner [59–62]. Hence, the full spectral area is taken into account with no prior knowledge of the compounds ID and multivariate models are constructed that seek how the NMR fingerprints of each sample and between groups of samples, are related to each other [61]. While this approach provides a first global view of the underlying trends and differentiation patterns in the data, it is often not valuable for the analysis of mammalian cell lines [63]. It is rather the metabolites ID and their relative fluctuation in the data which are ultimately needed in order to understand metabolic processes and thereby address the study's specific questions and understand the underlying biology. In turn we will here focus on the targeted identification and quantification of metabolites in (mammalian) cell lines.

4.1. Metabolite identification

Spectra annotation in NMR spectral data of cell extracts and culture media can be, to some extent, a straightforward and relatively easy task for an experienced user. This is because all commonly detectable metabolites in this kind of samples (e.g. amino acids, carbohydrates, amines, carboxylic acids, etc.) have a unique peak pattern (fingerprint), which is well-defined and repeated across all spectra for a specific pH, solvent and temperature [59]. Moreover, it is common to compare the data with spectral databases using search tools. The most commonly used and freely available metabolite databases are the Human Metabolome Database (HMDB; http://hmdb.ca) [47], the Biological Magnetic Resonance Data Bank (BMRB; http://www.bmrb.wisc.edu) [64] and the Birmingham Metabolite Library (BML; http://www.bml-nmr.org/) [65]. Other well-known commercial databases are the Bbiorefcode (Bruker Biospin Ltd.) and Chenomx library (Chenomx Inc.).

While searching in databases is very helpful, there will usually be a considerable number of resonances with ambiguous annotations that cannot be identified solely on database related information. Such assignments need further verification using 2D NMR data as well as the information from nuclei other than ^{1}H, e.g. ^{13}C or ^{15}N [63,66]. For example, overlapping of methyl protons peaks of several metabolites at δ 0.85–1.15 ppm, is a frequent case where assignment from only the 1D spectra is not sufficient. In such cases, analysis of the spin – spin correlations using $^{1}H-^{1}H$ COSY and TOCSY experiments, usually provides the necessary information for deducing the correct ID of the compound(s). In Fig. 5A we show two characteristic examples where the TOCSY spectrum is used for the assignment of 3-methyl-2-oxoavalerate and 2-oxoisocaproate. On the other hand, the proton - proton correlation experiments are not

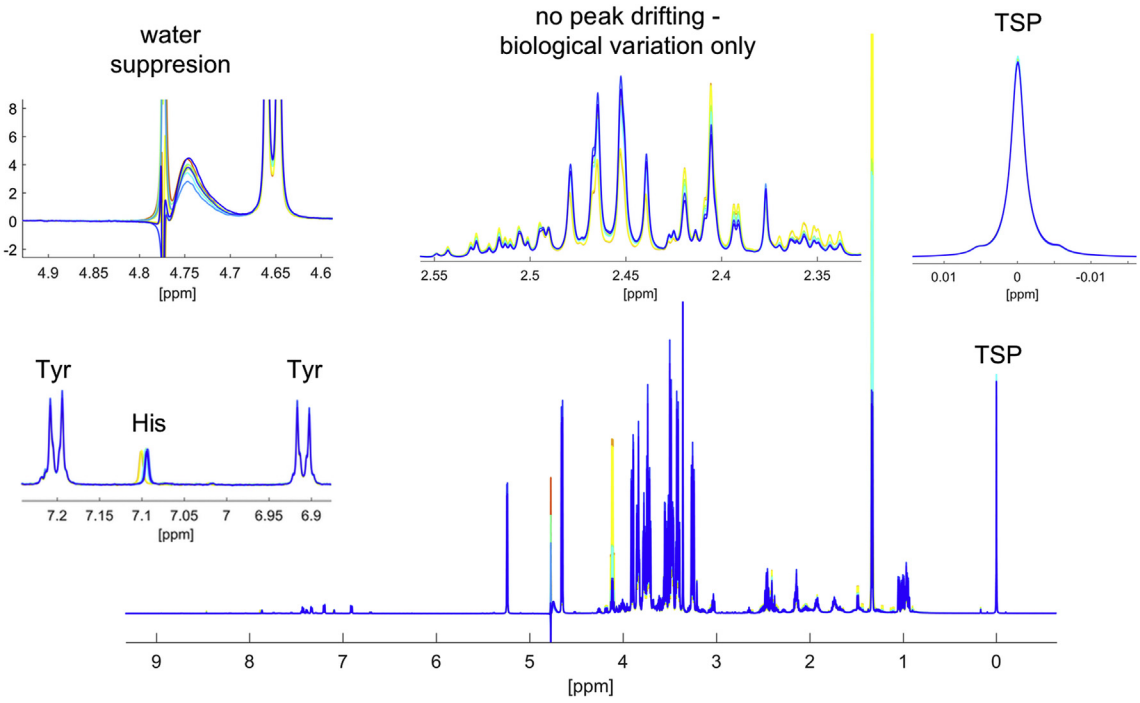


Fig. 4. Overlay of spectral regions from nine ^{1}H NMR automatically processed spectra of culture medium from BHP2-7 cells. The samples shown here, represent 3 time points of medium collection. While further manual processing is possible, even after the automatic processing only, the biological variation is evident (i.e. 3 groups of triplicates for some of the metabolites). This is a result of the standard sampling procedure and the optimized instrumental parameters. Specifically, peak drifting is minimal and occurred mainly for Histidine, while there is no baseline offset around the residual water resonance (baseline at 0). Furthermore, the reproducible linewidth and integral of the internal standard peak (TSP in this case) in all spectra, allows the direct use of it for quantitation of the extracellular metabolites.

informative in the case of singlets. For example, betaine and trimethylamine-*N*-oxide (TMAO) both resonate closely at δ 3.26 ppm. However, the carbon chemical shifts of the methyl groups are at δ 55.8 ppm for betaine and at δ 62.2 ppm for TMAO and this information is easily obtained by the $^{1}H-^{13}C$ 2D Heteronuclear Single Quantum Correlation (HSQC) experiment (Fig. 5B).

These examples demonstrate the need to acquire 2D NMR experiments in addition to the 1D NOESY and 2D *J*res. However, in practice this cannot be done for all samples as 2D NMR experiments (with the exception of the ultra-fast *J*res) require long experimental times. Instead, a small selection of samples can be made, e.g. one sample from each group of cells, for which a 2D NMR spectrum will be recorded. Alternatively, it is even better practice to create pool samples (e.g. from the leftovers of each sample during sample preparation) which will provide a closer representation of the metabolites in all samples rather than those present in a subset. A typical set of 2D NMR experiments include the $^{1}H-^{1}H$ COSY and TOCSY, a *J*res spectrum as well as a $^{1}H-^{13}C$ HSQC while often, an HMBC (Heteronuclear Multiple Bond Correlation) can also be useful [54]. The HSQC experiment provides information on the single bond connectivity between protons and heteroatoms, most commonly ^{13}C, but ^{15}N or ^{31}P are also used. It is a very versatile experiment for metabolites identification in complex mixtures for 3 reasons. First, it simultaneously provides the chemical shifts of the protons and their attached heteroatoms (e.g. carbons). Second, the much broader dispersion of carbon chemical shifts (up to ~ 220 ppm), assists greatly the analysis of the otherwise heavily overlapped resonances in the proton spectrum. And third, a very common variant of HSQC can provide carbon multiplicity information as well. In this multiplicity edited-HSQC, the $^{1}H-^{13}C$ cross peaks are phase-sensitive, so that the methyl (CH_3) and methine (CH) moieties are of opposite phase than the methylene (CH_2) ones [67]. The HMBC experiment, shows correlations through coupling of protons with heteroatoms that are further away, up to 3 or 4 bonds. These long range couplings are very different in magnitude from the one-bond ones (for $^{1}H-^{13}C$, $^{1}J_{CH}$ = 100–300 Hz, $^{2-4}J_{CH} \leq 10$ Hz) and since the one-bond correlations are depicted in the HSQC, it is common to simplify the HMBC spectra by excluding the one-bond cross peaks using a low-pass filter [54]. Although less used, the HMBC can provide valuable, complementary information for metabolites structure and identity. An example of the parameters used in some of these experiments is provided in the supplementary material S2.

In most cases, the combined use of database search and 2D NMR helps to annotate most, if not all detectable peaks in the spectra. However, in some cases even more work needs to be done. Characteristic examples are the uridine diphosphate (UDP) conjugates often found in NMR spectra of cellular extracts, e.g. UDP-*N*-acetylglucosamine (UDP-Gluc-NAc), UDP-glucose (UDP-Glc) and UDP-glucuronic acid, among others. These molecules are typically present at very low concentrations and most of their peaks are heavily overlapped with other more intense signals (e.g. UDP-glucose with the more abundant D-Glucose) and thus are not easily resolved for their identification, even in 2D NMR. However, their peaks at δ 5.50–5.65, δ 5.90–6.00 and δ 7.85–7.95 ppm, have sufficient S/N and can be used for quantitation. The only prerequisite for this is the knowledge of the exact chemical shifts for each compound, as they resonate closely to each other (Fig. 6). Unfortunately, due to their usually low concentration in the sample, 2D NMR experiments are often not sensitive enough to display these protons and their corresponding carbons. In these or similar cases of ambiguity, spiking of reference compounds is recommended as the best option for unambiguous identification and quantitation. The reference compounds are added in concentrations of about 10–100 μM, depending on the compound, subsequently the 1D NOESY and 2D *J*res experiments are repeated. In Fig. 6 we show one example where spiking of pure UDP-*N*-acetyl-glucosamine was performed. In section 5 we also provide a table with all metabolites found in the BHP2-7 cell line using all the above-mentioned methods. Obviously, this list is cell specific and can only be seen as a framework representing what should be anticipated from NMR studies of cellular extracts.

4.2. Quantification of metabolites in NMR spectra

The quantification of NMR spectral resonances relies on the fundamental property that the areas of these resonances are directly proportional to the number of protons they consist of. Once the latter is known after spectra annotation, the peak areas have to be extracted in order to generate quantitative data of the metabolites. Metabolite relative concentrations can simply be obtained by comparing peak areas of analyte and internal or external standard [51,59]. Since no form of chromatographic separation is usually applied upfront NMR-based profiling approaches, a huge dynamic range of metabolite concentrations can be observed in a single proton spectrum, ranging from low μM to high mM. However, NMR benefits from its large linear dynamic range and signal response (up to 6 orders of magnitude) and this, together with the use of modern digitizers, allows for quantification without the need of calibration lines (in case of calculation of ratios) such as in the case of MS-based methods.

Several approaches have been developed, to generate a "reference" for the absolute quantitation in the field of biomolecular NMR [51]. Among them, the addition of an internal standard (TSP or DSS) is commonly applied for the targeted profiling of mammalian cells. This method also benefits from the fact that the samples are deproteinized before the addition of the buffer and the internal standard and therefore, no variation is expected due to binding effects. Using this approach, the relative concentration of a metabolite is calculated in relation to the known concentration of the internal standard using the areas of the corresponding resonances in the spectrum:

$$\frac{M}{S} = \frac{I_M}{I_S} \times \frac{N_S}{N_M} \quad (1)$$

where M and S, are the concentrations of the metabolite and the internal standard (S is known), I_M and I_S are the integrals of their resonances and N_M and N_S are the number of protons contributing to these resonances, respectively. However, since both DSS and TSP methyl protons have long T_1 relaxation times and short repetition times (t) are used (see section 3.2) t becomes $< 5 \times T_1$ and therefore, the TSP (or DSS) resonances are attenuated. Consequently, their integrals do not reflect their real concentration and the quantities of metabolites will also be affected by this error. As mentioned earlier, this is not a problem if relative concentrations are sufficient for the purposes of the study. However, if absolute concentrations are required then a correction for the T_1 times should be applied with regard to the repetition time used [51]. This is done by the following formula:

$$[M_{real}] = [M_{calc}] \left[\frac{\left(1 - e^{-t/T_1^S}\right)}{\left(1 - e^{-t/T_1^M}\right)} \right] \quad (2)$$

where, $[M_{real}]$ and $[M_{calc}]$ are the corrected and quantified concentrations for repetition time t, and T_1^S and T_1^M are the T_1 relaxation times of the resonances of the internal standard S and from metabolite M, respectively. The T_1 times can be easily calculated

using an inverse-recovery NMR experiment (t1ir for Bruker instruments). The NMR dedicated software used for each system (e.g. Topspin for Bruker) usually provides automatic routines to extract the relaxation times from the inverse-recovery experiments together with detailed instructions how to perform them. Using the same buffer as we propose in this tutorial and a temperature of 300 K in our 600 MHz instrument, we have calculated a T_1 of 3.22 s for TSP and 3.19 s for DSS.

During the above calculations, it is assumed that the concentration of the internal standard is accurately known. Unless a commercial certified solution is used, the accurate concentration has to be verified in house. After some recent developments, this is possible using an artificial reference standard. We provide some details on this in the next paragraphs. However, the conventional way followed so far is to mix an aliquot from the solution that contains the internal standard (in the present tutorial, this is the buffer in D_2O) and an aliquot from a solution with a reference compound. The latter, should ideally having a singlet resonance away from the TSP (or DSS), e.g. formate (δ 8.45 ppm), or acetate (δ 1.91 ppm). Then a single pulse qNMR experiment is performed using a long repetition time (t ~ 30–40 s) to ensure that t becomes $> 5 \times T_1$ for both the TSP (or DSS) and the reference compound resonance. For example, if sodium acetate is used to calibrate the TSP concentration in a 600 MHz NMR spectrometer operating at 300 K, then the repetition time should be at least 23.75 s (i.e. 5×4.75) to account for the longer T_1 of the acetate methyl protons (T_1 of acetate CH_3 is 4.75 s and T_1 of TSP $(CH_3)_3$ is 3.22 s). The absolute concentration of TSP (or DSS) is then calculated by integration of both resonances and use of the integrals in equation (1). Fortunately, this process has to be performed only once the standard solution is made and the calculated concentration can then be used as long as the same stock solution (e.g. the buffer) is used for any study at the same temperature and the same instrument. In supplementary information (S3) we provide the experimental parameters suggested by Chenomx. Even if a different reference compound is chosen (Chenomx uses 50 mM sodium acetate), the provided experiment can be performed by the readers on their own set up.

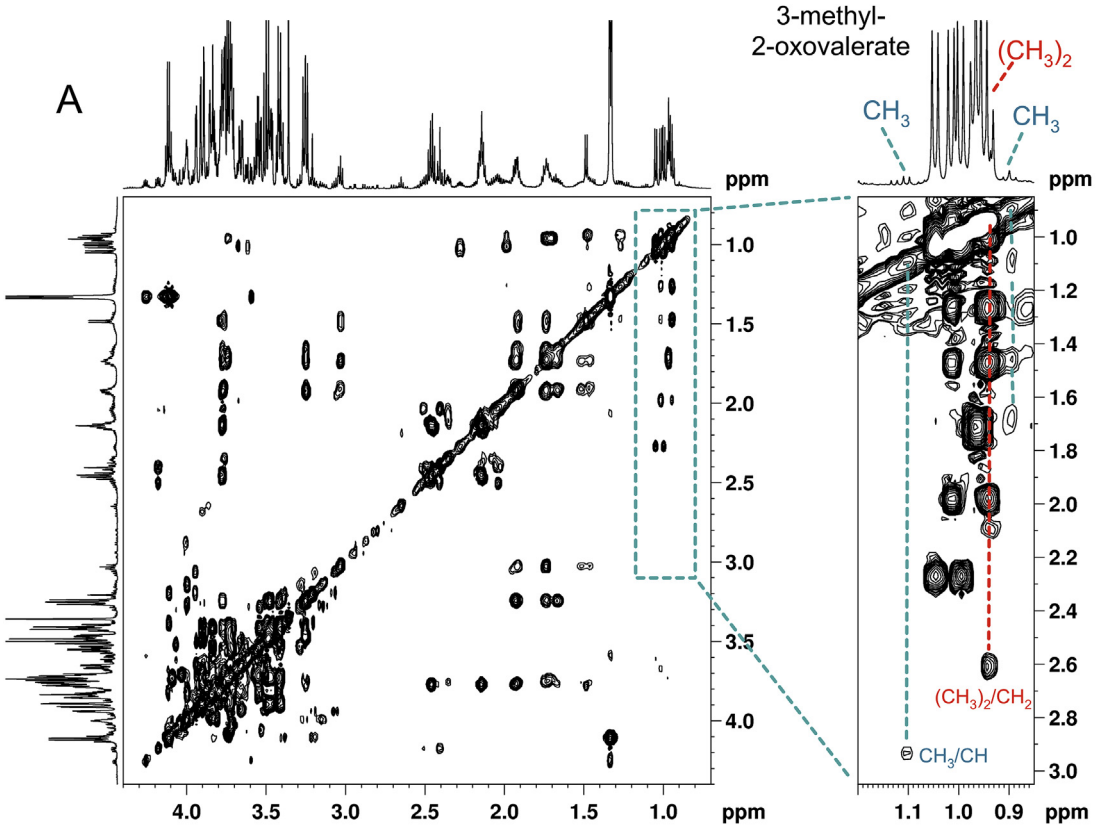
An alternative approach was recently proposed [10] for the absolute quantitation of cell cultures that does not require any reference material. The method uses the PULCON quantitative NMR (pulse length-based concentration determination), which is based on the principle that the absolute signal intensity is inversely proportional to the length of the 90° pulse used for the nuclei excitation in the active volume of the NMR tube [68]. This approach has the advantage that it requires only a single reference spectrum (calibrated once every few months) which can be used with all samples, throughout several studies. However, a serious disadvantage is that the method works for well-resolved peaks but not in the case of overlapped ones, which is typically the case in complex mixtures. Due to this, the authors have used PULCON only for the quantification of a small set of metabolites from culture media. However, the PULCON method can still provide a good alternative, for example, for the verification of the real concentrations of the internal standard in each sample of a study.

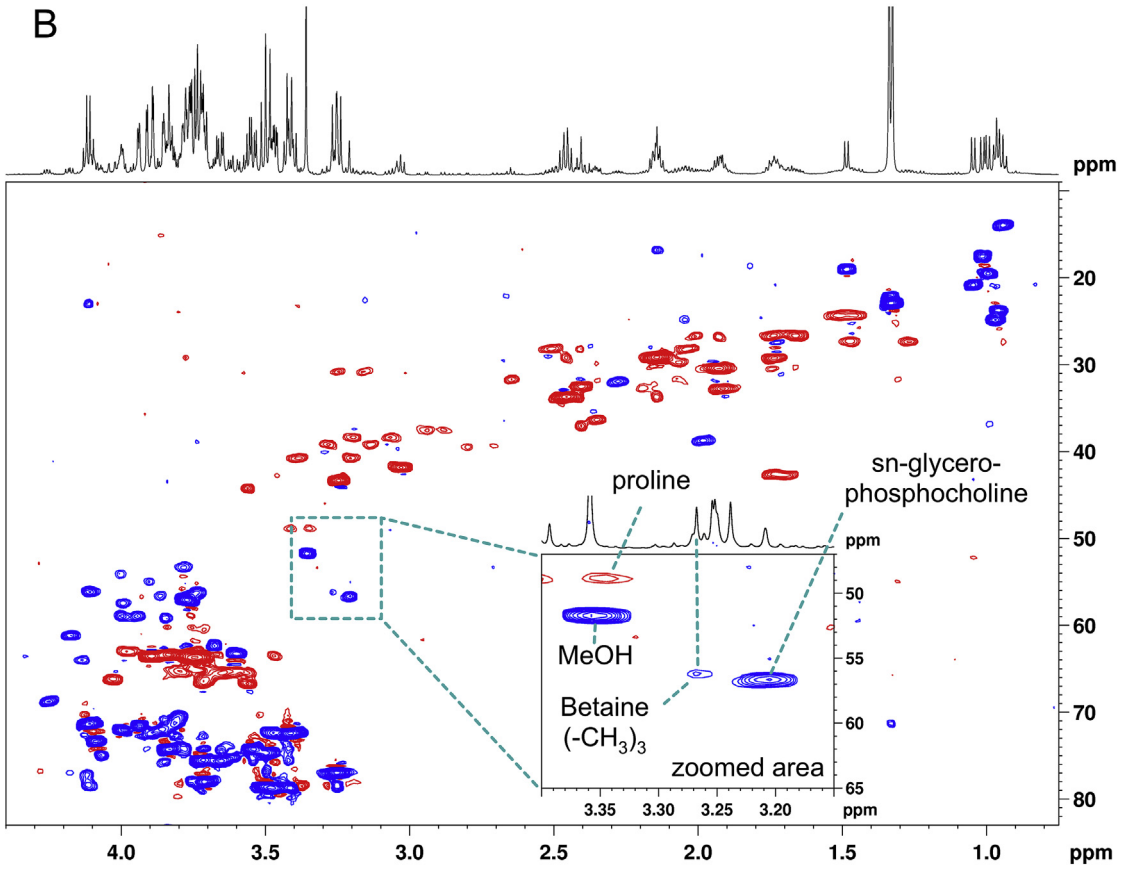
As we mentioned above, some efforts have been made to avoid using any internal standard and achieve quantification with external calibration. To this end, the Electronic Reference To access In-vivo Concentration (ERETIC) was developed [69]. ERETIC is an electronically generated signal though a second channel of the probe (e.g. ^{13}C), which is added as a pseudo-FID during the acquisition of the proton experiment. It presents all the characteristics of a regular NMR signal and only needs to be calibrated against a reference solution before it can be used for the quantification of metabolites [51]. The disadvantages of the ERETIC method are, that some hardware rearrangements are needed and also the signal is affected when the quality factor (Q-factor) of the probe is changed due to different sample properties (e.g. dielectric properties). Although, the latter has been dealt with a method that applies a correction to the ERETIC signal based on the pulse length of each sample [70], some other recent developments, namely the ERETIC2 (Bruker Biospin, Topspin 3.0) and QUANTification by Artificial Signal (QUANTAS) [71] have opened new possibilities for quantitative NMR. Both methods are generating a digital reference signal which can be positioned anywhere in the spectrum, e.g. in a clear spectral area or close to TSP and DSS and they don't require any additional hardware. Like ERETIC, one first has to calibrate the digital reference by acquiring an qNMR experiment with a verified reference solution and then this digital reference can be used for accurate quantification. We have not yet used any of these approaches in our studies with mammalian cells and therefore cannot provide more experimental details in this tutorial. However, we do anticipate that both QUANTAS and ERETIC2 can greatly facilitate the NMR-based profiling, at least, by removing any inconsistencies related with the internal standard concentration, as for example, when one needs to use very small volumes of sample, suitable for microprobes.

4.2.1. Deconvolution of NMR peaks

Simple resonance integration works well for isolated peaks and when using the appropriate corrections, absolute concentrations can be extracted. However, the requirement for well dispersed peaks is hardly met in complex mixtures, such as those from cell extracts. Quantitation of peak areas from these NMR spectra, requires more sophisticated methods than integration [51]. In such cases, line-fitting approaches (also termed as deconvolution) are preferred. Deconvolution is achieved by fitting a target peak of the compound in the complex mixture, with the one of the pure compound in reference spectra [72]. Among the available algorithms and software packages for targeted profiling, in our opinion the commercially available Chenomx NMR Suite can be considered as the gold standard. It comes with an internal database of mathematically modelled metabolites (341 in the current version, 8.2), consisting of the chemical shift and the *J*-coupling information and allows for the interactive fitting of peaks in a semi-automated manner [73]. The interactive fitting mode allows for fine adjustments of peak shapes by the user, albeit the more complex the mixture and the higher the number of the metabolites to be quantified, the more time consuming the process. In addition, the higher user interaction is a potential source of errors, hence a standardized processing practice should be followed [74].

The performance of quantification by Chenomx NMR suite, is dependent on several factors. First and most important, the library of reference compounds is built using a 1D NOESY experiment (called METNOESY; parameters are provided in supplementary material, S3) and the best fitting results can be achieved when the NMR data acquisition for each study, has been done with the exact same experimental parameters. Variations in the pulse sequence and/or the repetition time, will result in a less accurate fitting. It is important to note at this point that, if the recorded NMR data follow the protocol suggested by Chenomx then absolute concentrations of metabolites can be extracted without any T_1 corrections (we encourage the reader to visit http://www.chenomx.com; Application note 14: “Investigating the criteria for accurate quantitative results with Chenomx NMR Suite”). However, if this is not the case, the quantification results in relative concentrations. In order to assess this, further, we have compared the results obtained by our “metabolic profiling” NOESY experiment (see sections 3.1 and 5.1.3) and the METNOESY in cell-free culture medium samples and we provide the results in section 5.


A
3-methyl-
2-oxovalerate
$(CH_3)_2$
CH_3
CH_3
ppm
ppm
$(CH_3)_2/CH_2$
CH_3/CH
4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
1.1 1.0 0.9 ppm


B
ppm
proline
sn-glycero-
phosphocholine
MeOH
Betaine
$(-CH_3)_3$
zoomed area
3.35 3.30 3.25 3.20 ppm
4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm

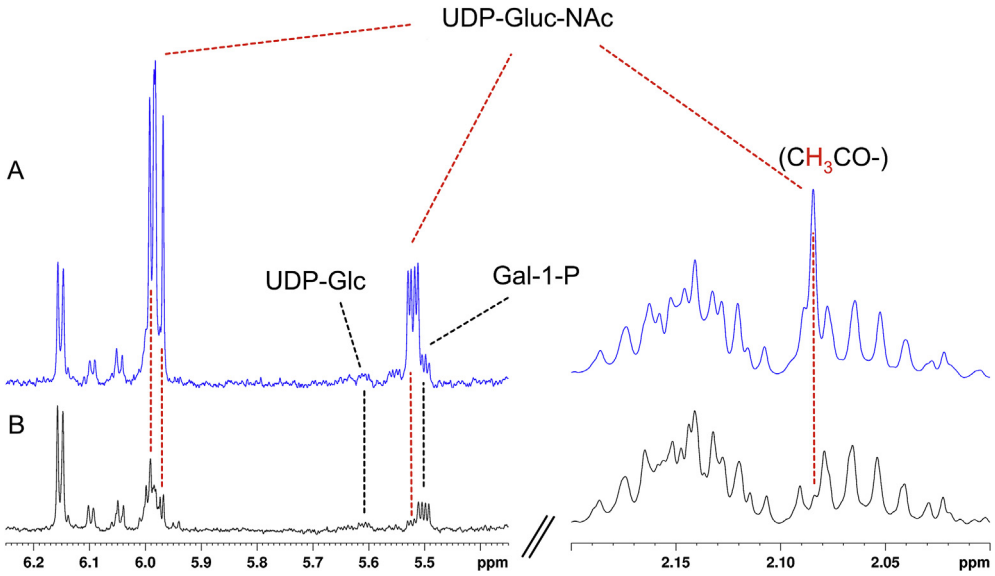


Fig. 6. Spiking of pure compounds (~20–100 μM) into a mixture helps to identify the existence of metabolites and their exact position in the spectrum. A: The spiking of UDP-*N*-acetylglucosamine (UDP-Gluc-NAc) is shown (indicated with red dotted lines), after which, the identification and quantitation of this compound was achieved by fitting the peaks shown in the two spectral regions (left and right); B: The same regions from the 1D NOESY spectrum of the mixture without the addition of UDP-Gluc-NAc. On the left side, the proton of UDP-Gluc-NAc at δ 5.50 ppm is overlapped with the one from Galactose-1-phosphate (Gal-1-P), while the two protons from the uridine moiety at δ 5.95 ppm are overlapped with those from UDP-glucose. Moreover, it is very difficult to assign the exact chemical shift of the three protons from the acetyl group for UDP-Gluc-NAc, shown on the right side, as these are overlapped with other multiplets from glutamine and glutamate as well as the singlet from methionine's methyl protons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A second source of variation which influences the fitting results is related to the properties of some protons, called labile, e.g. those attached to a nitrogen atom. These protons are often in exchange with the solvent which not only make them undetectable but also influences the resonances of the other vicinal protons causing a slight attenuation of their peaks. The consequence is that the attenuated resonances do not match exactly to the areas calculated for the other protons in the same compound. Typical examples of this are the α protons of amino acids which are vicinal to the amine. When Chenomx is used for deconvolution, one should consider other protons, preferably more isolated resonances, as better targets to achieve more accurate results. For example, in Valine profiling, the two doublets of the methyl protons at δ 0.99 and 1.04 ppm as well as the multiplet at 2.26 should be prioritized over the α-H at δ 3.67 ppm, which is usually not fitted well. In a recent study the performance of the software was evaluated using a synthetic mixture of 28 metabolites, commonly found in mammalian cell cultures [75]. The authors pointed out two major sources of bias: the incorrect interpretation of singlets and the quantification of resonances from protons in close proximity to labile protons. In addition, quantification of three metabolites, glutamate, tryptophan and arabinose was found less accurate at low concentrations.

As already mentioned in section 3.1, the presaturation of the water resonance also causes a partial peak saturation in the protons resonating close by. This is another type of error that is induced in the quantification of these resonances, and should be considered, especially with Chenomx profiling [76]. For example, it should be expected that the fitting of the anomeric protons from carbohydrates, commonly resonating close to the water resonance, will not match the fitting of other resonances of the molecules. Another typical example comes from lactate profiling for which the CH quartet at δ 4.11 ppm is usually affected by the presaturation. In this case, the doublet of the methyl protons at δ 1.33 ppm should be given preference for quantification.

We have extensively used Chenomx for both intracellular and extracellular metabolite profiling. Since an increasing number of researchers are using or intent to use this software, we consider it useful to provide the readers with some practical guidelines and recommendations, as derived from our experience with real life analysis of cells samples. As with most NMR quantification routines, one important asset of a successful quantification procedure is the pre-processing of the NMR spectra. Chenomx offers the possibility to process the raw data from scratch, i.e. phase and baseline correction, referencing to the internal standard and correction for the pH of the sample. However, some of these functions are easier done on the NMR dedicated software (e.g. Topspin). We should also note that for TSP and for samples with pH

Fig. 5. High resolution 600 MHz 2D NMR experiments of a cellular extract. A: The $^{1}H-^{1}H$ TOCSY spectrum displays the spin - spin correlation through bonds for each compound as cross peaks, symmetrically positioned around the diagonal. This experiment provides the spin system of each compound and hence allows to resolve and annotate overlapping resonances, especially for the cases of protons being in a similar chemical environment. Two examples are shown in the zoomed spectral window on the right: First, the assignment of 3-methyl-2-oxovalerate where the two methyl groups are annotated by their correlations with the other protons of the molecule (CH_2 and CH; green color); Second: the protons from the two methyl groups of 2-oxoisocaprotate (red color) are overlapped with the methyl group of Isoleucine at δ 0.93 ppm in the 1D spectrum but can be assigned by the cross peak with the methylene protons at δ 2.61 ppm. B: The 2D $^{1}H-^{13}C$ HSQC experiment of the same cellular extract as above. The HSQC displays C-H correlations with carbon chemical shift scale being on the vertical axis. It is also common to use an edited version of the pulse sequence so that additional information on carbon multiplicity can be shown. In this example, CH_3 and CH are having a positive phase (blue peaks) while CH_2 have a negative phase (red peaks). HSQC is extremely useful in cases where no spin – spin correlation occurs (i.e. singlets in the 1D spectrum and no TOCSY cross peak). An example is shown in the zoomed area with the annotation of betaine's methyl protons. These are often overlapped and misinterpreted with the ones from TMAO but they can be well resolved in the 2D HSQC base on the different carbon chemical shift. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

7.4, the software automatically positions the resonance at δ −0.0159 ppm, therefore, for convenience it is advisable to also perform this in the raw dataset. With regard to the functions provided by Chenomx, we have found the fine adjustment of the baseline, using the spline tool very useful. Moreover, since, the profiling depends on the internal standard, it is of utmost importance to properly calibrate the TSP (or DSS) singlet. This includes the careful baseline and phase correction of the area around 0 ppm, and the accurate calibration of the linewidth. Commonly, peak half-height linewidths of 1.0–1.5 Hz result in the best overall fitting to the modelled compounds of the Chenomx library. One good approach is to fit a small, representative set of metabolites until the optimal process settings are found. We also suggest the evaluation of other peaks linewidths (using different software), like the one from alanine's methyl-doublet at δ 1.48 or D-glucose α-anomeric proton at δ 5.23 ppm as a rough estimation of the best linewidth setting for the fitting of the reference spectra. On the other hand, if the linewidths are significantly broader than 1.5 Hz, a shimming correction (reference deconvolution) should be applied. However, for better accuracy and precision, samples with poor resolution, should preferably be discarded from further analysis. Using these quality criteria, we have quantified tryptophan in 8 extracts of a cell line (See, Section 5. Case study and Table 1) with a mean concentration of 7.6 μM (0.38 μM/10^6 cells) and a CV of 16.56%. In cells-free culture medium with a given concentration of 44 μM (from the supplier), we obtained 40 μM with a CV of 9.93% in DMEM/F12 with 10% FBS, and 47 μM with a CV of 1.29% in DMEM/F12 without FBS. Tryptophan has been reported [75] to result in inaccurate quantification, but in our case the results were acceptable.

Although Chenomx profiler is capable of fitting all known metabolites in the spectra automatically, our experience is that this will result in errors, especially for heavily overlapped peaks. A manual review of the fitting results is therefore necessary. In addition, the order of fitting seems to be important. As an example, starting the profiling with D-glucose resonances, will probably lead to errors as glucose is generally present at much higher levels and its resonances will mask other overlapping peaks originating from metabolites with very low concentrations. The same is true for singlets, which often resonate on top of other multiplets of lower intensity. A typical example of this is the singlet of succinate which overlaps with the multiplet from pyroglutamate. One should start the profiling with low abundant multiplets before fitting the singlets. An extremely useful feature of Chenomx is the subtraction line. This is the spectral line left after fitting of a resonance. Ideally it should be zero, but in some cases, other unknown peaks are overlapping with the targeted ones and thus, the spectral line is not fully fitted. Finally, we have found it very useful to initially analyse, a mixture of known composition, in order for the analyst to tune the fitting process. We typically use blank culture medium from the study, ideally without FBS addition. In this manner one can compare the results to the manufacturer's specifications. However, please note that for the best accuracy, NMR data should be collected as suggested by Chenomx as discussed previously. In our experience though, even with a different experimental set up, as the one we propose in this tutorial, the quantification results are close to the theoretical ones. We are addressing this point in more detail in section 5.2.2. Overall, despite the challenges mentioned above when it comes to quantification of metabolites in complex mixtures, the great advantage of Chenomx is that it can provide the absolute or relative levels (at least a close estimation) of many metabolites with heavily overlapped resonances. This is mainly achieved because the software fits the full spin system of each compound rather than a single resonance and with some experience and knowledge of the pitfalls we mentioned above, almost a full spectrum coverage can be achieved. This increases the metabolic coverage significantly comparing to other available options, and makes NMR an attractive alternative in studies of mammalian cells metabolism.

Besides Chenomx, MestreNova (MNova) software (MestreLab Research SL) is also gaining popularity in the analysis of complex mixtures. Unfortunately, we do not have any experience with MNova, and thus, we cannot provide any recommendations, but the interested reader can look at the documentation provided online. Two other methods, BATMAN [77,78] and BQuant [79], have the advantage that they are freely available and can be used through the R statistical environment (http://www.R-project.org/). Both approaches are based on a Bayesian model to develop the most probable fitting of a peak based on specific input data. BQuant requires prior alignment and peak picking of the spectra. On the contrary, the BATMAN (Bayesian automated metabolite analyser for NMR) R package, fits NMR peaks on the basis of user-defined templates of chemical shifts, *J*-couplings, multiplicity of peaks and relative peak intensities (i.e. number of protons). An extensive template file is freely available to download from the HMDB database with approximately ~2500 peak patterns, corresponding to about 600 metabolites. The template is fully customizable, allowing the users to change the parameters based on their own experimental values. Based on the template info, the software renders an ideal peak and subsequently tries to fit it to the experimental signal by minimizing a mathematical function. In contrast to Chenomx which fits the full spin system of a compound, BATMAN allows the user to decide upon which resonance to fit. Apparently, a priority should be given to the most isolated ones before trying to fit the more complex peaks. The output file is the area calculated for each proton, i.e. it can be directly used for comparison of compounds' levels across multiple spectra with no further correction. However, if an internal standard is used then the output can be converted to relative concentration or to absolute concentration after T_1 corrections, as we mentioned in section 4.2.

While BQuant has to our knowledge not yet been applied to cellular extracts and culture media, BATMAN has been successfully used for the quantification of 25 extracellular metabolites from 86 single-gene transposon insertion mutant strains of the pathogen *Pseudomonas aeruginosa* [80]. The samples of this study exhibited large pH variations and one extra feature of BATMAN was particularly useful in dealing with severe shifting of peaks across the spectra: the ability to sort spectra based on the shifting of a target peak and extract the chemical shift information in order to use it for the template file. This is achieved by the splineFitBATMAN interactive tool [81], which runs in Matlab (The Mathworks Inc.). Furthermore, the authors found good agreement of the quantitation results from BATMAN in comparison to a sample which was profiled with Chenomx. The limitations of BATMAN are the required computational power, which slows down the process in case of large spectra numbers and the poorer fitting in case of more complex multiplets or severely overlapped peaks, when compared to Chenomx. This results in a reduced number of quantifiable compounds in a mixture but nevertheless, in our opinion it is still an excellent choice for quantification of NMR data, if Chenomx is not an option. For the purposes of this tutorial, in section 5.2.2 we provide the results of a comparative analysis for a subset of metabolites using BATMAN and Chenomx. For further details about how to construct the BATMAN template files, load the spectra and use the splineFitBATMAN tool in Matlab, the interested reader is encouraged to look at the online documentation of the package at http://batman.r-forge.r-project.org, as well as the provided reference [78].

4.2.2. Correction and evaluation of the quantitative data

As cell cultures can typically consist of different cell numbers

Table 1
Intracellular and extracellular metabolites identified by NMR based targeted profiling. The listed chemical shifts for each compound, were used for quantification via fitting with its reference spectrum from the library of ChenomX NMR suite. The mean concentration and CV (%) is also shown for the intracellular metabolites from a set of 8 cell extracts from the BHP2-7 cell line. For a limited number of metabolites, the quantification was repeated with BATMAN and the results are shown in parentheses.

Metabolite	δ ^{1}H (ppm)[a]	Conc.[d,e]	CV (%)	Metabolite	δ ^{1}H (ppm)[a]	Conc.[d,e]	CV (%)
1-Methyl-nicotinamide	4.47, 8.17, 8.89, 8.96, 9.27	0.62	4.37	Malate	2.34, 2.65, 4.29	2.20	12.49
α-ketoglutarate	2.42, 2.99	0.78	8.26	Methionine	2.11, 2.12, 2.18, 2.63, 3.84	0.96	23.15
2-Oxo-isocaproate	0.92, 2.60	0.34	34.14	Methyl-malonate[f]	1.23, 3.16		
3-methyl-2-oxovalerate[b]	0.88, 1.08, 1.44, 1.68, 2.91			Methyl-succinate[f]	1.07, 2.11, 2.51, 2.61		
ATP	4.25, 4.39, 4.59, 6.13, 8.26, 8.52	1.70 (3.05)	14.82 (14.87)	*N*,*N*-Dimethyl-glycine[c]	2.90	0.11	3.96
Acetate[c]	1.90	2.32	6.81	*N*-Acetyl-aspartate	2.00, 2.48, 2.67, 4.38	1.28	7.54
Alanine	1.47, 3.77	4.77 (6.06)	10.19 (15.83)	*N*-Acetyl-glutamine	1.91, 2.02, 2.31, 4.15		
Arginine	1.64, 1.72, 1.91, 3.23, 3.76	2.56	31.28	NAD+	4.22, 4.39, 4.53, 6.02, 6.08, 8.16, 8.18, 8.41, 8.82, 9.13, 9.32	0.39 (0.44)	8.38 (12.33)
Asparagine	2.84, 2.94, 3.98	0.35	45.06	Nicotinate adenine dinucleotide	4.40, 4.51, 6.03, 8.04, 8.14, 8.42, 8.73, 8.99, 9.12		
Aspartate	2.67, 2.80, 3.88	2.48	7.74	*O*-Phospho-choline	3.20, 3.58, 4.15	5.99 (8.05)	2.71 (9.54)
Betaine	3.25, 3.89	0.34	16.65	Ornithine	1.78, 1.92, 3.04, 3.77	0.75	40.66
Choline	3.19, 3.50, 4.05	0.59	29.82	Pantothenate	0.88, 0.92, 2.40, 3.97	0.42	13.45
Citrate	2.53, 2.64	0.98	9.54	Phenylalanine	3.12, 3.27, 3.98, 7.32, 7.36, 7.41	1.73	22.66
Creatine	3.02, 3.91	1.40	5.94	Proline	1.98, 2.05, 2.34, 3.33, 3.40, 4.12	6.29	6.84
Phospho-creatine	3.03, 3.93	2.54	5.53	Pyroglutamate[c]	2.02, 2.39, 2.49, 4.16	5.45	32.61
Formate[c]	8.44	0.74	23.01	Pyruvate[g]	2.35	0.27	31.17
Fructose[c]	3.55, 3.66, 3.69, 3.79, 3.88, 3.98, 4.01, 4.10	4.91	29.02	Serine	3.83, 3.93, 3.98	1.89	17.26
Fumarate	6.50	0.21 (0.17)	7.33 (13.17)	Succinate[c]	2.38	0.54 (1.60)	27.08 (41.83)
GTP	5.93, 8.13	0.14	9.88	Taurine	3.25, 3.41	10.76	4.41
Galactitol[f]	3.68, 3.97			Threonine	1.31, 3.57, 4.24	4.97	18.54
Galactose[f]	3.48, 3.63, 3.69, 3.72, 3.79, 3.84, 3.92, 3.98, 4.07, 4.58, 5.26			Tryptophan	4.05, 7.19, 7.27, 7.31, 7.53, 7.72	0.38	16.56
Glucose	3.23, 3.39, 3.45, 3.50, 3.71, 3.81, 3.88, 4.63, 5.22	61.86	35.21	Tyrosine	3.05, 3.18, 3.92, 6.89, 7.18	2.21	23.38
Glutamate	2.04, 2.11, 2.34, 3.74	36.69	3.17	UDP-Gluc-NAc	2.06, 3.54, 4.27, 4.36, 5.50, 5.96, 7.93	0.63	10.41
Glutamine	2.13, 2.44, 3.76	12.39	18.60	UDP-glucuronate	4.38, 5.62, 5.97, 7.93		
Glutathione (reduced)	2.15, 2.54, 2.94, 3.76, 4.55	7.86 (10.58)	13.15 (17.93)	UDP-Glucose[f]	4.19, 4.23, 4.27, 4.36, 5.59, 5.97, 7.94		
Glycine	3.55	6.23	8.39	UDP-Galactose[f]	4.27, 4.35, 5.63, 5.97, 7.95		
Histidine	3.12, 3.22, 3.98, 7.08, 7.87	0.94	22.55	UMP[f]	4.34, 4.41, 5.98, 8.09		
Hypotaurine	2.63, 3.34	4.21	6.33	Valine	0.98, 1.03, 2.26, 3.59	3.87	23.60
Isoleucine	0.93, 1.00, 1.25, 1.46, 1.97, 3.66	3.36 (3.43)	24.09 (22.64)	Myoinositol	3.26, 3.52, 3.61, 4.05	9.33 (14.71)	7.27 (14.19)
Lactate[d]	1.31, 4.09	71.06 (58.85)	35.72 (39.92)	*sn*-Glycero-3-phosphocholine	3.22, 3.60, 3.64, 4.31	0.89	6.21
Leucine	0.94, 0.95, 1.70, 3.73	3.10	24.75	β-Alanine	2.54, 3.16	3.00	5.81
Lysine	1.46, 1.72, 1.89, 3.01, 3.74	2.52	36.88	CoA	0.72, 0.84, 2.60	0.25	23.53
Cystine[b]	3.18, 3.38, 4.08			2-hydroxy-isobutyrate[c]	1.35		
Hypoxanthine[b]	8.18, 8.20			3-hydroxy butyrate[c]	1.19, 2.30, 2.40, 4.14		
Pyridoxine[b]	2.45, 7.64			Niacinamide[b]	7.58, 8.24, 8.70, 8.93		
trans-4-hydroxy-L-proline[c]	2.14, 2.42, 3.35, 3.47, 4.33			Galactose-1-phosphate[f]	3.72, 3.76, 3.90, 3.99, 5.48		

[a] All chemical shifts are based on the TSP calibrated at δ −0.0159 ppm.
[b] Present only in the culture medium.
[c] Not listed in culture medium formulation but identified by NMR in cells-free medium samples.
[d] Concentration (Conc.) is expressed as μM/10^6 cells after, in total ~ 24.5 millions cells were extracted per sample; CV (%): coefficient of variation; calculated from a set of 8 cell extracts used for this tutorial as (std/mean)*100.
[e] Quantification was performed with Chenomx NMR Suite 8.1. In parentheses are the results of quantification with BATMAN for a subset of the metabolites.
[f] These metabolites were identified in BHP2-7 cell lines in a separate experiment after specific treatment (ongoing study).
[g] Pyruvate and oxaloacetate have the same chemical shift and cannot be quantified separately in proton NMR.

and total biomass, a further normalization step of quantitative data is needed. Such is necessary in order to make the extracted concentrations comparable between different experiments, cell lines and culture media. Although this topic is not an integral part of the here described quantitative NMR method, it is important for the user to consider corrections taking different cell numbers, biomass or even cellular volume into account. The latter is particularly important if the intracellular metabolomes from different cell types are to be compared. In our experience, when comparing cells from a single experiment or rather similar cell types, correction for total protein has been proven to be the most straight-forward approach. We usually re-dissolve protein pellets obtained after the precipitation step in 10% sodium dodecyl sulphate for subsequent BCA based protein quantification. Also, corrections based on cell numbers are frequently used and have proven their feasibility.

An additional point that requires evaluation is the starting composition of the culture medium. Although the manufactures often provide a detailed list of nutrients in the medium, an evaluation of the actual composition used in each study is strongly recommended. Especially for media with FBS, it is often the case

that some metabolites will present deviating concentrations at starting conditions. Such differences can vary between batches of culture medium or FBS and may prove significant when absolute concentrations are used throughout several experiments, particularly when studying metabolite uptake and release. Hence, close monitoring of culture media and batch control are mandatory. In our laboratory, we routinely measure cell free study medium in order to obtain metabolite starting concentrations.

5. Case study: the BHP2-7 cell line

In this tutorial, the BHP2-7 cell line is used as an example for quantitative metabolome analysis. BHP2-7 is a cell line derived from a papillary thyroid carcinoma (PTC), a differentiated type of thyroid cancer [82]. PTC is the most common type of thyroid cancer, around 70–80% of all thyroid cancers are of the PTC subtype with an incidence rate of around 63,000 estimated new cases in the United States for 2016 [83]. Genetic alteration in the Mitogen-Associated Protein Kinase (MAPK) pathway are often found in PTCs and are believed to be important events for tumor initiation, progression and clinical outcome [84]. The most prevalent genetic alteration in classic PTC is the V600E hotspot pathogenic variation of *BRAF* followed by one of the (at least) 12 possible gene fusions targeting the *RET* proto-oncogene (comprising. *RET/PTC1-12*). BRAF and RET/PTC fusions are mutually exclusive [85]. The BHP2-7 thyroid cancer cell line is known to harbour a *RET/PTC1* rearrangement that subsequently leads to constitutively active MAPK signalling, which in turn leads to metabolic changes that have been implicated in metabolic syndromes such as type 2 diabetes and cancer [86].

5.1. Sampling BHP2-7 cells

BHP2-7 were cultured in 1:1 DMEM/F:12 medium, supplemented with 10% FBS. For these experiments, culturing was performed without the addition of antibiotics. Furthermore, a HEPES-free medium was acquired for quantitative NMR experiments. Cell cultures were kept in T75 culture flasks and incubated at 37 °C under 5% CO_2. Cultures were passed on when reaching around 80–90% confluence, which occurred roughly every three to four days. Cultures were taken from the incubator and medium was aspirated. Cultures were washed with 5 mL PBS at room temperature and 1 mL trypsin/EDTA was added and flasks were incubated for 10 min to cause the cells to detach. Trypsin was neutralised by the addition of 9 mL of fresh culture medium and cells were passed on in a dilution of 1:20, meaning 9 mL of medium was added to the flasks of which 0.5 mL was transferred to a 15 mL tube and spun down (300 × g, 3 min). The supernatant was removed from the cell pellet and cells were re-suspended in 10 mL culture medium and plated in a fresh T75 flask.

To perform the quantitative NMR study, cells were plated in 10 cm petri dishes instead of T75 culture flasks as they allow for easier handling of the cell cultures for quenching and extraction. As the surface area of the petri dish (78.54 cm^2) is almost equal to that of the T75 culture flasks (75 cm^2), the same dilution of 1:20 was used to plate the cells. The chosen dilution differs for each specific cell line as each has a unique growth rate, but the used dilution aims to generate a culture that shows 80–90% confluency after 72 h in culture to circumvent any metabolic changes caused by decreased proliferation due to contact inhibition. Optimal dilutions should be determined experimentally for each individual cell line before starting a large-scale experiment. Briefly, cells were detached and trypsin neutralised by the addition of 9 mL of culture medium. Cells were spun down (300 × g for 3 min) and supernatant was removed after which a fresh cell suspension was made by the addition of 10 mL culture medium. The suspension was carefully homogenised and 0.5 mL pipetted in each petri dish. Petri dishes were supplemented with 9.5 mL culture medium and the cells were allowed to settle at room temperature for one hour before placing them in the incubator.

5.1.1. Quenching and extraction of BHP2-7

0.3 mL of culture medium was aspirated from each sample and mixed with 0.6 mL of 100% LC-grade cold MeOH (−80 °C) in a microcentrifuge tube. The samples were then temporarily stored at (−80 °C) until NMR analysis. The remaining culture medium in the dish was quickly aspirated and cells were washed with 5 mL of warm PBS (37 °C). Culture medium removal and washing occurred in less than 5 s. We subsequently quenched metabolism by snap-freezing the cells with approximately 10 mL of lN_2. After about 30 s when most of the lN_2 had been evaporated, the culture dish was placed on dry-ice until all remaining samples were quenched.

For the extraction of intracellular metabolites, 1.5 mL of cold aqueous 90% MeOH/$CHCl_3$, 9:1 (v/v) solution was added to each culture dish and the cells were scraped using a cell lifter and transferred to a microcentrifuge tube. The extraction mixtures were then centrifuged at 16000×*g* at 4 °C for 10 min. The supernatants were collected in cryo-vials and stored at −80 °C until analysis. The extraction solvent was kept on dry-ice during the extraction of all samples and the whole procedure was performed in a cold room (4 °C).

Although we usually recommend to carry out correction based on protein content, we have found that cell numbers and protein content correlate linearly. In turn, in some cases cell numbers can also be used as correction parameter, with protein content being preferred. However, the most important point in this context is consistency. For this experiment cell numbers per sample were estimated from cells cultured in parallel to the experiment. Cells were collected after trypsin detachment and 10% of the cells were taken up in 0.5 mL PBS and counted in a 20-fold dilution using a fluorescence based Muse automated cell analyser. Approximately 2.45×10^7 cells per sample were extracted for this study.

5.1.2. NMR sample preparation

At the day of NMR analysis, culture medium samples were centrifuged at 16000×*g* at 4 °C for 20 min and the protein-free supernatants were collected. The same protocol was repeated for cells-free culture medium in order to assess the composition of nutrients. Since FBS can induce some variation to the composition of the medium with regard to the one provided by the supplier, we have also measured FBS-free DMEM/F12samples. Organic solvents after extraction were removed under a gentle stream of nitrogen (~2 h). Dried material was reconstituted with 250 μL of 0.15 M phosphate buffer (pH = 7.4; 0.15 M K_2HPO_4, 0.2 mM NaN_3, and 0.4 mM TSP-d_4) in D_2O. Each sample was vortexed for 30 s and transferred to a 96 well-plate. 165 μL of each sample was then transferred to 3 mm NMR tubes using a Gilson 215 liquid-handler. The NMR tubes were subsequently placed in a SampleJet with cooling racks (6 °C) and measured within the same day.

Leftovers (30 μL) from each sample after the addition of buffer and transfer to NMR tubes, were pooled and aliquoted to cryo-vials for temporary storage at −29 °C. These pool samples were later used for 2D NMR experiments and spectrometer optimization as described in section 3.1.

5.1.3. NMR experiments

^{1}H NMR data were obtained using a Bruker 600 MHz AVANCE II spectrometer equipped with a 5 mm TCI cryo probe. The same set of experiments were acquired for both cells extracts and culture media samples. All ^{1}H NMR spectra were recorded at 300 K with presaturation ($\gamma B_{1/2\pi} = 50$ Hz). Duration of 90° proton pulses was

automatically calibrated for each sample using a homonuclear-gated nutation experiment [87] on the locked and shimmed samples after automatic tuning and matching of the probe head. For the 1D NOESY experiment, we collected 128 scans of 65536 points covering 12335 Hz, using a relaxation delay of 4 s, acquisition time of 2.72 s and mixing time of 10 ms. The free induction decays (FIDs) were zero filled by a factor of two and Fourier transformed. All spectra were automatically phase and baseline corrected and referenced to the internal standard (TSP; δ −0.0159 ppm, for import in Chenomx, see 4.2.1). 2D *J*res were acquired with presaturation, using the same pulse as for 1D NOESY. The relaxation delay was set at 2 s and the spectral width was 16.66 ppm (12288 Hz) for the direct dimension and 78 Hz for the indirect one and 4 scans were acquired over 40 increments. The FIDs were automatically processed with Fourier transformation, tilted by 45°, symmetrized around the direct dimension (F2) and calibrated using the TSP signal at δ −0.0159 ppm in the F2 dimension and at 0.0 Hz in the indirect (F1) dimension. The total time of measurement was 24 min per sample, including 5 min for temperature stabilization, automation routines for tuning, matching, locking to deuterium frequency, shimming and pulse calibration and the 1D NOESY and *J*res acquisition.

Additional 2D NMR spectra were recorded to aid the assignment of metabolites using pool samples. The set of 2D experiments included ^{1}H–^{1}H COSY, ^{1}H–^{1}H TOCSY, ^{1}H–^{13}C HSQC (edited to provide information of carbon multiplicity) and ^{1}H–^{13}C HMBC using a low pass filter for one bond couplings. Presaturation for water suppression ($\gamma B_{1/2\pi} = 50$ Hz) was applied in all experiments. The standard parameters implemented in Topspin 3.0 (Bruker Biospin, Ltd.) were used for all experiments. All parameters used for this study are provided in the supplementary material.

Prior to the automatic measurements of all samples, one pool sample was used to optimize the instrumental parameters and assess stability. The standard set of experiments was acquired right after sample preparation, after 24 h left standing in the cooling rack (6 °C) of the SampleJet and after storage at −29 °C for one week.

An additional set of measurements was performed, with samples from cells-free DMEM/F12 medium (without FBS). In this set, each sample was measured with the experimental parameters described above for 1D NOESY as well as with the METNOESY pulse sequence and parameters suggested by Chenomx (supplementary information). Briefly, METNOESY does not use pulse-field gradients and the repetition time is 5 s, split in 1 s of delay and presaturation and 4 s of acquisition. The same receiver gain RG and 90° pulse was used in both experiments.

5.2. Analysis of NMR spectra

The 1D NOESY NMR spectra of BHP2-7 cell extracts exhibited a reproducible profile, as seen confirmed by visual inspection of an overlay of all spectra. This was also true for the TSP peak which was identical in all spectra. We have also evaluated the stability of the samples during 24 h kept at 6 °C using multiple measurements of the pool sample, immediately after sample preparation and after 24 h. These findings, indicate that sampling was consistent among all samples and that samples are stable within a 24 h time window when left standing at 6 °C. Hence, we can assume that following this experimental setting for more cell lines, the differences in metabolic profiles can be attributed to true biological variation.

5.2.1. Spectra annotation

Metabolites were annotated with combined use of Bruker's bbiorefcode and Chenomx NMR databases and analysis of the 2D NMR spectra (Fig. 7). The IDs and exact chemical shifts of UDP-*N*-acetyl glucosamine, UDP-glucuronate, UDP-glucose, *N*-acetylaspartate, and ATP were confirmed with spiking experiments of the pure compounds. In total 65 intracellular metabolites were identified in the cellular extracts of BHP2-7 and 45 metabolites in the culture medium (Table 1). Fig. 7 depicts some characteristic peaks from the majority of the intracellular metabolites (62 out of 65). The panel of identified compounds covers several classes like amino acids, carbohydrates, nucleotides, amines, intermediates of TCA cycle and other organic acids and co-factors. With regard to culture medium analysis, we identified the majority of nutrients provided by the manufacturer, with the exception of inorganic salts and macromolecules. Moreover, additional compounds that were excreted by the cells during growth, were also found (Table 1).

5.2.2. Quantification of metabolites

All identified molecules were quantified with Chenomx NMR suite. We managed to profile the majority of the NMR resonances in the proton spectra of BHP2-7 with only a few exceptions of unknown peaks that needed further verification. Although there is not any theoretical or reference composition for cells extracts for us to compare with our results, the experiment was performed with 8 biological replicates of one cell line. Therefore, if all steps (Fig. 1), from cell culture, quenching and extraction, to the NMR measurement and the quantification, had occurred perfectly, we should obtain the exact same results for each extract. We used the coefficient of variation as a measure of the method precision. Overall, we obtained an average CV of 17.34% (median CV = 14.82%). Considering all steps of the workflow where an error could have occurred, we conclude that the 8 extracts were overall a homogeneous group. Among all intracellular metabolites, asparagine, ornithine, lysine, arginine, pyruvate, 2-oxoisocaproate, pyroglutamate, lactate and D-glucose had the largest CV (>30%). D-glucose occurs in two forms in solution (α and β), but Chenomx uses a mixture of the two and, at least in our hands, the software cannot fit all peaks correctly. On the other hand, we don't have an obvious explanation for the large CV of lactate. The overlapped resonance of threonine at δ 1.33 ppm (Table 1) could be one reason, but it was quantified with a lower CV (19.54%). The large variation for asparagine and pyruvate could be a combination of the low level and the overlap with other larger peaks, glutamate and glutathione, respectively. Finally, for the other compounds with high CV, some could be due to overlapped complex resonances and low levels (ornithine, pyroglutamate and 2-oxoisocaproate) and due to overlap with each other (lysine and arginine). On the other hand, we can also see that several compounds quantified in the low μM range (2.7–~20), like fumarate, *N,N*-dimethylglycine, NAD^{+}, GTP, 1-methylnicotinimide and UDP-*N*-acetylglucosamine exhibited low CV (<10%) which means that the LOQ can be set to a low S/N, e.g. the fumarate singlet had an average S/N of 6, in those cases of better resolved resonances. It is also noteworthy, that ATP, which is known to have fast turnover rates [33,34], was quantified with a CV of 14.82%, indicating a robust sampling procedure.

In order to assess the performance of BATMAN, we have also quantified a subset of metabolites (Table 1; BATMAN values are shown in parentheses). Among these metabolites, only isoleucine lactate and ATP had a similar CV compared with Chenomx results. With regard to the average concentrations, both methods were in agreement for α-ketoglutarate, NAD^{+}, isoleucine and fumarate, but in all other cases, there were noticeable differences. To our view, some compromises have to be made when working with BATMAN, especially for those peaks that are partially masked by larger ones, or those masking other smaller ones. Apart from that, the BATMAN method can still provide quantitative information for a smaller set of metabolites albeit with a lower precision.

As already mentioned, knowing the composition of the culture media can be also of great importance. In some cases, the uptake

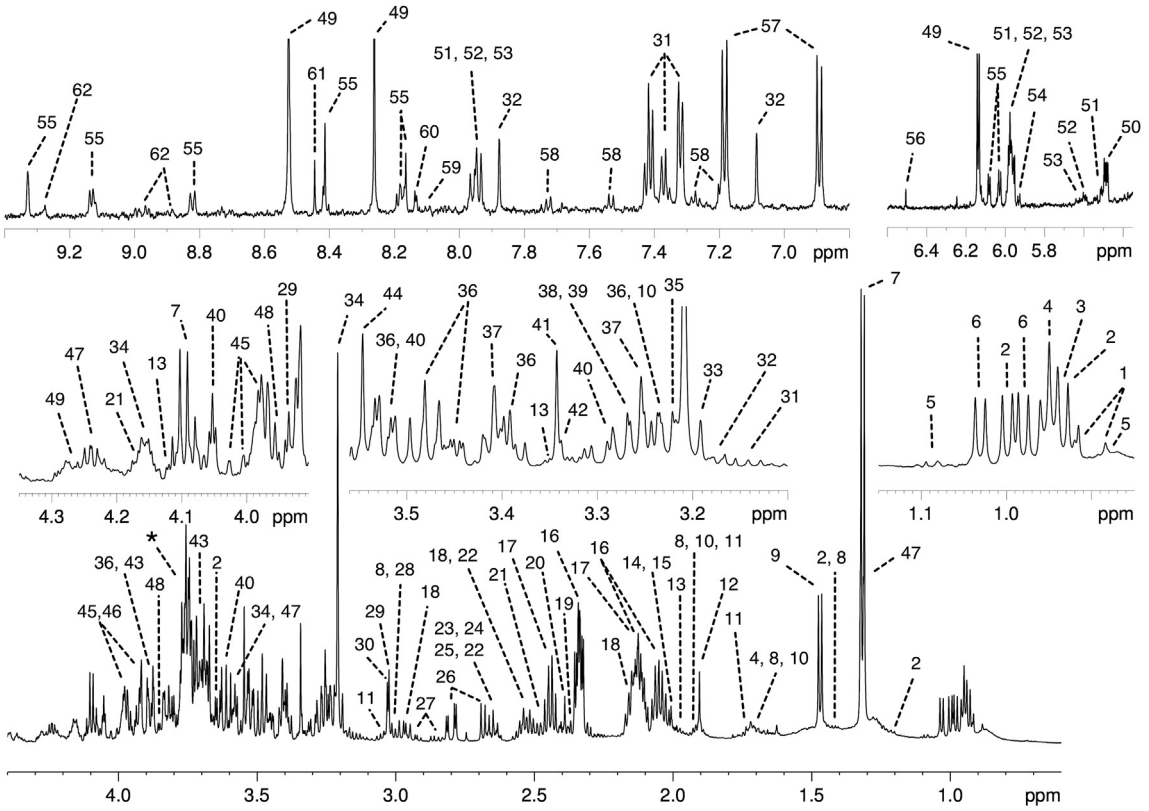


Fig. 7. Regions of the 600 MHz ^{1}H NMR of BHP2-7 cells extract dissolved in deuterated phosphate buffer. The characteristic peaks from most of the identified metabolites (in total, 65) for this cell line are annotated with numbers. Keys: 1: Pantothenate; 2: Isoleucine; 3: 2-oxoisocaproate; 4: Leucine; 5: 3-methyl-2-oxovalerate; 6: Valine; 7: Lactate; 8: Lysine; 9: Alanine; 10: Arginine; 11: Ornithine; 12: Acetate; 13: Proline; 14: *N*-acetylaspartate; 15: *N*-acetylglutamine; 16: Glutamate; 17: Glutamine; 18: Glutathione (GSH); 19: Pyruvate (Oxalacetate); 20: Succinate; 21: Pyroglutamate; 22: Citrate; 23: Methionine; 24: Hypotaurine; 25: Malate; 26: Aspartate; 27: Asparagine; 28: α-ketoglutarate; 29: Creatine; 30: Phosphocreatine; 31: Phenylalanine; 32: Histidine; 33: Choline; 34: *O*-phosphocholine; 35: *sn*-glycerophosphocholine; 36: D-glucose; 37: Taurine; 38: Betaine; 39: Trimethylamine-*N*-oxide; 40: Myoinositol; 41: Methanol (residual solvent); 42: Hypotaurine; 43: Galactitol; 44: Glycine; 45: Fructose; 46: Galactose; 47: Threonine; 48: Serine; 49: ATP; 50: Galactose-1-phosphate; 51: UDP-*N*-acetylglucosamine; 52: UDP-glucose; 53: UDP-glucuronate; 54: UMP; 55: NAD^+; 56: Fumarate; 57: Tyrosine; 58: Tryptophan; 59: GTP; 60: Nicotinate adenine dinucleotide; 61: Formate; 62: 1-methylnicotinamide; * overlapped peaks from D-glucose, D-galactose, Fructose, GSH and several amino acids (-C_αH-).

and release of nutrients is needed, in order to get insights on cells growth and physiology. To do this, the exact composition of the starting culture medium has to be measured. Although, frequently this information is provided by the supplier, it is strongly recommended to perform in house analysis on the same platform used for the other samples. In order to address these issues, we quantified the DMEM/F12 medium, with and without 10% FBS. Our findings are shown in Table 2. For this experiment, we have used the same pulse sequence as the one for cell extracts (Prof-1, Table 2). Except for the metabolites that were not listed but were detected and quantified in our samples, it is apparent that the common nutrients exhibited some rather large differences. For example, alanine and glutamate were found to be almost 4 times more abundant than the supplier indicated, pantophenate 2.7 times and myo-inositol 2 times increased. On the contrary, arginine, histidine, hypoxanthine, lysine, proline and pyruvate were found at lower levels than expected. These measurements were accompanied by an average CV of 14.01%. Aiming to evaluate where this variation comes from, the same medium was measured under the same condition but without FBS. Overall, the CV was significantly reduced to 4.55%, with only glutamate displaying a relatively large CV of 22.97%, but some differences between the listed values and our data of the quantification still remained. Although the deconvolution error for some cases cannot be excluded, the relatively good precision of the measurements, indicate a different type of error.

It was mentioned in section 3.2 that, at least according to the developer, if Chenomx is used for quantification, then for the best accuracy, the data should be collected with the METNOESY experiment. In fact, no need for T_1 relaxation times is needed in this case and the absolute concentrations can be extracted. In order to evaluate whether our 1D NOESY experiment provided data that cannot be accurately fitted by Chenomx, we measured and quantified the same samples using the METNOESY experiments (Prof-2). Overall, we obtained an average CV of 4.24% which was comparable with Prof-1. More importantly, the two methods resulted in the same results with only minor variation as it can be seen by the ratios of Prof-1/Prof-2 in the last column of Table 2. We can safely exclude the possibility of incorrect internal standard addition, if this was the case, we should observe systematic variation between the quantified and the listed values. However, some compounds quantified by both methods were in agreement with the listed values (e.g. arginine, glutamate (prof-2), glycine, histidine and even the less concentrated hypoxanthine, among others). Based on these findings, we conclude that the real composition of the culture medium was to some extent different from the values given by the supplier.

As a final note, we can conclude that the 1D NOESY-based profiling, described in this tutorial, can be used with the Chenomx method for quantification. The data from the DMEM/F12 analysis show that only small variations should be anticipated between the two profiling experiments. Furthermore, due to the gradients applied in the pulse sequence of the 1D NOESY

Table 2
Quantification of nutrients (mM) in DMEM, with and without 10% FBS and comparison with values given by the supplier. For the latter, the comparison of the two suggested NMR profiling experiments is shown together with the CV (%). Prof-1 is the NOESY experiment we routinely use for metabolomics studies and Prof-2 is the METNOESY experiment suggested by Chenomx NMR Suite. Supplier indicates the concentrations given by the supplier.

Metabolite	supplier	Prof-1[a] (DMEM-FBS)			Prof-1 (DMEM)			Prof-2 (DMEM)			DMEM (1 vs 2)
	mM	mM	CV (%)	ratio[b]	mM	CV (%)	ratio[b]	mM	CV (%)	ratio[b]	ratio[c]
Alanine	0.050	0.190	15.751	3.809	0.084	0.384	1.675	0.086	1.481	1.724	0.972
Arginine	0.699	0.567	7.227	0.811	0.720	5.315	1.029	0.695	15.263	0.994	1.035
Asparagine	0.050	0.053	12.923	1.064	0.069	4.555	1.380	0.073	5.492	1.458	0.947
Aspartate	0.050	0.057	19.050	1.147	0.064	2.385	1.273	0.069	4.106	1.375	0.926
Choline	0.064	0.080	14.958	1.255	0.085	2.642	1.330	0.084	1.437	1.307	1.017
Cystine	0.100	0.131	15.471	1.313	0.130	4.736	1.298	0.133	4.295	1.328	0.977
Glucose	17.506	14.555	11.068	0.831	16.995	2.139	0.971	16.951	3.784	0.968	1.003
Glutamate	0.050	0.187	10.879	3.734	0.065	22.972	1.299	0.054	5.998	1.071	1.213
Glutamine	2.500	2.092	13.302	0.837	2.306	0.764	0.923	2.332	2.114	0.933	0.989
Glycine	0.250	0.265	16.237	1.060	0.279	0.866	1.114	0.274	2.856	1.094	1.018
Histidine	0.150	0.119	16.350	0.792	0.156	2.815	1.040	0.147	0.915	0.981	1.060
Hypoxanthine	0.015	0.013	19.645	0.876	0.013	5.303	0.878	0.012	3.632	0.798	1.100
Isoleucine	0.416	0.399	13.286	0.959	0.473	0.476	1.138	0.481	0.499	1.157	0.983
Leucine	0.451	0.410	16.326	0.909	0.445	0.810	0.988	0.439	1.675	0.973	1.015
Lysine	0.499	0.435	11.938	0.872	0.496	7.354	0.995	0.482	1.658	0.967	1.029
Methionine	0.116	0.123	14.811	1.060	0.159	2.574	1.377	0.164	1.837	1.416	0.973
Niacinamide	0.017	0.017	6.381	1.027	0.023	3.030	1.395	0.021	2.028	1.240	1.125
Pantothenate	0.005	0.013	12.154	2.700	0.013	4.020	2.719	0.013	1.754	2.804	0.970
Phenylalanine	0.215	0.192	16.275	0.892	0.242	1.864	1.125	0.238	1.913	1.108	1.015
Proline	0.150	0.126	13.818	0.839	0.141	2.865	0.942	0.143	2.787	0.954	0.988
Pyridoxine	0.010	nd	nd		0.003	11.945	0.297	0.003	5.149	0.304	0.978
Pyruvate	0.500	0.255	15.034	0.510	0.119	10.381	0.239	0.114	11.880	0.228	1.047
Serine	0.250	0.281	15.552	1.126	0.309	3.413	1.235	0.320	1.360	1.281	0.964
Threonine	0.449	0.436	14.535	0.970	0.518	2.528	1.153	0.520	2.574	1.158	0.996
Tryptophan	0.044	0.040	9.927	0.904	0.047	1.294	1.063	0.047	1.737	1.052	1.010
Tyrosine	0.214	0.200	13.588	0.935	0.252	1.810	1.180	0.248	1.310	1.161	1.016
Valine	0.452	0.450	14.374	0.995	0.499	0.992	1.105	0.491	1.054	1.087	1.017
myo-Inositol	0.070	0.158	16.972	2.252	0.076	6.610	1.082	0.073	2.849	1.044	1.036
Formate		0.025	11.843		0.005	7.188		0.005	8.479		1.105
Pyroglutamate		0.027	15.712		0.031	12.540		0.033	25.403		0.933
DMG		0.001	11.106								
Betaine		0.018	21.574								
Succinate		0.092	15.137								
Lactate		1.731	11.491								
Fructose		0.465	15.311								
Creatine		0.028	14.010								
Acetate		0.052	11.642								
2-Hydroxyisobutyrate		0.004	13.497								
3-Hydroxy-butyrate		0.010	13.788								
trans-4-hydroxy-L-proline		0.009	13.724								

nd: not detected.
[a] Prof-1: measured with the noesygppr1d; Prof-2: measured with the Chenomx suggested, METNOESY experiment.
[b] Ratio between quantified and supplier values.
[c] Ratio between prof-1 and prof-2 values.

(noesygppr1d, Bruker, Topspin 3.0), a better water suppression and S/N are achieved compared to the METNOESY experiment. When sensitivity is an issue, this is an important improvement to consider. Overall, since the library of compounds in Chenomx software was developed to result absolute concentrations with accurate fitting, following the recommendation described in this tutorial for sampling, analysis and quantification, it is feasible to obtain a close to accurate representation of the cellular metabolome.

6. Concluding remarks

Liquid chromatography and gas chromatography coupled to MS are the gold standard for targeted and untargeted metabolomics. Nevertheless, several pitfalls particularly related to absolute quantification (e.g. matrix effects, labelled standard materials, ionization suppression) make NMR spectroscopy an ideal alternative. While NMR spectroscopy cannot reach the sensitivity of the aforementioned techniques, its robustness and ease of obtaining (absolute) quantitative data without the need of having (labelled) standard materials at hand are two major advantages. With the here described protocol, more than 65 intra and extra-cellular metabolites from diverse chemical classes can be quantified. The quantitative analysis of small carboxylic acids, amino acids, TCA cycle intermediates and even bile acids would require several dedicated liquid chromatography and gas chromatography based methods [88] not to mention intrinsic problems of the methods such as ionization suppression for liquid based and metabolite degradation for gas based separation techniques [89]. In conclusion, if a NMR spectrometer and enough sample material are available and expected metabolite concentrations are high enough (μM range), in our experience, NMR spectroscopy is the most straightforward way for obtaining absolute quantitative intra- and extra-cellular metabolic data. Moreover, the now described protocols for quenching and extracting intra- and extra-cellular metabolites can be applied to any subsequent analysis technique, chromatography based, or not.

Acknowledgements

The authors are thankful to Dr. Liam McDonnell and Prof. Judith V. M. G. Bovée for valuable discussions during the optimization of our protocols.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2017.05.011.

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