Nutrimetabolomics:
An Integrative Action for Metabolomic Analyses in Human Nutritional Studies

Appendix 3

Chapter 6 - Data Acquisition

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6. Data acquisition

6.1. Mass spectrometry

The basic principle of MS is to generate ions from either inorganic or organic compounds by a suitable method, which would be able to separate these ions by their mass-to-charge ratio (*m/z*) and to detect them qualitatively and quantitatively by their respective *m/z* and abundance. To achieve this objective, there is a simple basic scheme that all mass spectrometers follow. A mass spectrometer consists of an ion source, a mass analyzer, and a detector which are operated under high vacuum conditions. Section 6.1 will mostly review the first two components.

6.1.1. Ionization Modes

Eluted metabolites, separated by GC and LC, have to be ionized and transferred from the liquid phase into the gas phase. For example, coupling of LC to MS has led to a wide variety of interfaces, such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), or atmospheric pressure photoionization (APPI). These techniques are suitable for LC-MS, their selection depending on sample properties like molecular mass and polarity. However, APCI and ESI are predominant in metabolomic strategies. These interfaces are based on the same principle: spraying the sample through a capillary and bypassing a gas stream. In the majority of LC-MS metabolomic studies, the ESI interface has been widely used because it is more universal in terms of the number of ionizing compounds than APCI. By contrast, electron ionization (EI) or chemical ionization (CI) are the most commonly ionization modes in GC-MS.

Electron Ionization (Ei)

GC coupled to MS shows selectivity, specificity and robustness for metabolomic approaches, since available databases containing mass spectra and retention indices can tentatively identify metabolites due to the extensive and reproducible fragmentation pattern obtained in full-scan mode using EI. However, fragment ions are often less specific and fragmentation is usually rather extensive which complicates interpretation of the spectra of completely unknown compounds.

Nowadays the most widely used ionization technique for GC-MS based metabolomics is EI. This mode of ionization takes place in an ion source maintained at high vacuum (10^{-7} to 10^{-5} mbar) and a source temperature of about 200-230°C. The analyte molecules in the diluted gas phase are bombarded with electrons of 70 eV kinetic energy.[1] As the ionization potential of most organic compounds is about 10-15 eV, virtually any molecule can be ionized under standard EI conditions.[2] Some studies have also attempted to use low energy EI (12 eV – 20 eV) for analysis of aliphatic amides,[3] monosaccharides,[4] etc., citing advantages such as easier identification of the molecular ion and simpler appearance of the spectrum due to low fragmentation.[5] However, this comes at the cost of lower ionization efficiency and a marked loss in sensitivity. Therefore, the use of standardized ionization energy (i.e. 70 eV) is preferred as it ensures efficient ionization and comparability of the spectra generated with different instruments in different labs – a prerequisite for the reliable identification of unknown molecules by library matching.[6, 7]

Chemical Ionization (CI)

CI is a soft ionization technique. The electron beam first ionizes molecules of a reagent gas - usually methane, ammonia, or isobutane – which in turn react with the analyte molecules. Compared to EI, a lower amount of energy is thus transferred to the analyte molecule, leading to the formation of stable quasimolecular ions and lower fragmentation. CI and APCI can be a more favorable alternative due to the production of protonated molecular ions and reduced fragmentation. CI can be performed in either positive or negative mode, depending on the analysis of compounds of interest. The 'soft' reactions between the ionized reagent gas and the analytes usually lead to a protonation of the analyte molecules (especially in positive CI), but also deprotonation, hydride abstraction, charge exchange, adduct formation, reagent ion capture, and electron capture are possible. The value of proton affinity difference between the molecule and the gas determines whether a particular analyte can be protonated by a certain reactant ion and how exothermic the protonation will be. Therefore, the choice of the reagent gas can control the extent of ionization and fragmentation: Typically, methane causes stronger fragmentation than isobutane and ammonia.[2] Isobutane is an especially versatile reagent gas, because i) it provides low fragmentation positive CI spectra of most of unpolar analytes; ii) it gives almost exclusively one well-defined adduct; and iii) it can also be employed for electron capture. To date, CI has mostly been used in conjunction with EI data to confirm molecular weight of compounds of interest. CI can further be applied to characterize unknowns for low scoring EI hits. CI is not widely used across metabolomic studies and only few studies used this ionization mode.[8-11] CI can also be used in conjugation with GC/time of flight (TOF)-MS which allows for determination of molecular ions as well as elemental composition based on high mass accuracy and isotope fidelity for identification of unknown molecules[12] and also with triple-quadrupole (QqQ) GC-MS and quadrupole TOF (QTOF) GC-MS instruments to generate collision-induced dissociation (CID) fragmentation spectra and complementary structural information. One of the main problems with CI is that the constant infusion of reagent gas increases the pressure in the ion source and renders overall ionization less effective. In principle, the high intensity of the quasimolecular ion and the limited fragmentation enable low detection limits, but this can only be fully exploited with the help of selected-ion monitoring (SIM) qMS or selected reaction monitoring (SRM) and multiple reaction monitoring (MRM) measurements. Untargeted GC-CI-MS analyses performed in full-scan mode suffer from low overall sensitivity caused by a high baseline noise due to the reagent gas. Further, due to the many, in part instrument-specific factors influencing the fragmentation process, CI spectra are much less comparable than EI spectra, which hampers the development and the applicability of spectral libraries. One major limitation for the broad use of CI in metabolomics is the limited comparability of the spectra and thus the lack of reliable spectral libraries.

Electrospray Ionization (ESI)

This source is widely used and can be interfaced successfully with various mass analysers for LC-MS instrumentation. It is

a soft ionization technique that results in no or little fragmentation. The LC eluent is directly coupled to a metal capillary in the ionization source held at a high electric potential under atmospheric pressure (3 - 5 KV). The charged droplets formed exit the capillary and disappear as the solvent vaporizes leaving highly charged ions. The technique allows for desorption of cold intact molecules for analysis by mass spectrometer.[13, 14] The ion source can be operated to detect either positive or negative ions depending on the mode of application and applied polarity. The analytical runs can be performed by switching between either positive or negative mode. The factors that could affect the sensitivity of ESI include pH of the solution, solvent choice, adduct formation, ion suppression from matrix, nebulization and desolvation gas temperature and flow, and fragmentation voltage. ESI finds applications with highly and moderately polar compounds and is hence suitable for the analysis of various metabolites, proteins, xenobiotics, etc. Routinely used ESI parameters are shown in **Table S6.1**.

Atmospheric Pressure Chemical Ionization (APCI)

Besides CI, the use of APCI as a soft ionization technique is currently increasing in prominence across various metabolomics studies.[7, 15-18] However, some differences between GC-APCI-MS and LC-APCI-MS are noteworthy.

In LC-MS, APCI is considered as a complementary technique to ESI and its mechanism is similar to common CI. APCI uses corona discharge from a very fine needle for ionization of analytes and mobile phase and only single charged quasi molecular ions are typically produced in this way. One of the main advantages of APCI over ESI is its lower susceptibility to sample matrix interferences and APCI is thus less prone to ion suppression.[19] Although APCI is less dependent on parameters such as solvent choice, flow rates, and additives, other key parameters such as nebulizer temperature needs to be controlled in order to vaporize the sample. Care should be taken as higher vaporizer temperatures can lead to sample decomposition and hence it is not an ideal platform for thermolabile compounds. Traditionally, this technique is considered as more suitable for analysing compounds with moderate or low polarity such as steroids, lipids, and fat soluble vitamins[20, 21] but recent reports indicate that also strongly polar metabolites can also be ionized efficiently using APCI.[18] Likewise, in general, GC-APCI-MS has proven to enable sensitive detection and can provide additional information regarding compound identification.[7, 22] However, the molecular ion region of GC-APCI spectra may be highly informative but also complex at the same time. Besides the expected protonated molecular ions, silylation adducts[22] as well as other types of adducts may be observed, depending on analyte structure. Practical experience further shows that the presence of a heteroatom in the molecule renders ionization via APCI efficient; in contrast, pure hydrocarbons like alkanes are difficult to ionize and may produce uncommon molecular ions. For this reason, the use of FAMEs as RI markers for GC-APCI applications is recommended. Likewise, ion suppression may occur in vicinity of overloaded peaks (J. Kopka, unpublished data).Other ionization modes include cold EI, field ionization, and photo ionization but are rarely, if at all, used in metabolite profiling.[7]

6.1.2. Mass Analyzers

As mentioned above, ions formed are electrostatically directed into the mass analyzer where they are separated according to their *m/z*, and finally detected. Several types of mass spectrometers are available for hyphenation with GC and LC. In principle, all MS analyzers can be used for untargeted metabolomics but their specific characteristics must be kept in mind when defining the desired application and performance. For untargeted metabolomics, characteristic features such as mass resolving power, mass range, mass accuracy, and data acquisition frequency should be considered, while for targeted metabolomics, features such as dynamic range, scan cycle time, and scan speed needs to be taken into account.[23] Focusing on mass resolving power and mass accuracy, low- (LRMS) and high-resolution mass spectrometry (HRMS) can be differentiated. In the following section, a brief overview of the different types of mass spectrometers used for metabolomic applications will be provided.

Table S6.1. ESI parameters.

Drying gas temperature	To set the temperature (300°C – 350°C is generally used) of the N_2 drying gas and is used to evaporate the LC effluent. The temperature value is dependent on the stability of the sample, LC and drying gas flow rate
Drying gas flow	To set the flow rate of N_2 drying gas. The typical flow rate value used is 8-10 $Lmin^{-1}$ and is dependent on LC flow rate and drying gas temperature
Nebulizer pressure	To set the nebulizing gas (N_2) pressure. The value is dependent on LC flow rate and for LC flows around 400 $\mu Lmin^{-1}$, 45 psig is considered adequate
Sheath gas temperature and flow	Sheath gas confines the nebulizer spray to more effectively concentrate the ions in front of the capillary more effectively. The temperature value will be dependent on the sheath gas flow provided.
Capillary voltage	To set the voltage applied at the entrance of the capillary. Although the optimum voltage can be set for the analysis of different compound classes analysis, a default value of 3000-3500V can be generally used
Nozzle voltage	To set the voltage applied at the nozzle. The value is very much compound and polarity dependent and ranges between 0-2000V
Fragmentor voltage	To set the voltage applied at the end of capillary. The value is highly specific for the analysis of interest. For small molecule analysis, a default value of 100V can be typically used and can be optimized accordingly

Low Resolution Mass Spectrometry (LRMS)

The low-resolution mass spectrometers can separate sufficiently two ions differing by one mass unit along the whole mass range scanned. The typical resolving power for low-resolution mass analysers, such as QqQ and quadrupole linear ion trap (QqLIT), are below 9000 full width at half maximum (FWHM) of the mass peak. In this section we will comment the most common instruments such as Quadrupole Mass Spectrometry (qMS) and Quadrupole ion-trap.

Quadrupole Mass Spectrometry (qMS): qMS instruments are widely used due to their comparatively affordable costs and their robustness. Briefly, the design of a single quadrupole mass spectrometer includes, a mass filter consisting of four parallel metal rods as well as an electron multiplier combined with the detector. Direct and alternating voltages are applied to the rods in a way that, for a certain time period, only ions of a limited *m/z* range can pass through the quadrupole analyzer on a stable trajectory. By changing these voltages over time, the ions within a predefined *m/z* range can be detected one after the other, i.e. in a step-wise scanning procedure. This means that the overall data acquisition rate of a qMS instrument depends on the scan speed as well as the selected mass range. In contrast to this 'scan mode' which is suitable for untargeted analysis, the SIM mode is an alternative mode of operation suitable for targeted quantification. Although modern qMS instruments achieve a maximum scan speed of up to 20,000 u/s,[24] their data acquisition frequency is typically limited to about 25-50 Hz (depending on the scan range). This is sufficient for one-dimensional GC-MS metabolomics where peak base widths are in the range of seconds. In contrast, for adequate reconstruction of the narrow GC×GC peaks (see section 5.4), fast-scanning qMS instruments are required and have to be operated at maximum scan speed. Recently, the applicability of GC×GC instruments with a fast scanning qMS detector was demonstrated in an untargeted as well as targeted metabolomic studies.[24, 25] In practice, qMS instruments reach only nominal mass resolution although is it possible to increase mass resolution by applying polynomial recalibration of the raw MS data.[26] In contrast, the linear dynamic range of qMS systems is typically larger than that of many high-resolution instruments,[27] which underlines their suitability for quantification.

In summary, despite their limitations concerning data acquisition rate and mass resolution, qMS instruments are cost-efficient, robust, reliable, and sensitive enough for many applications and therefore still used in the metabolomic community.[28-32]

Triple quadrupole instruments are advanced qMS systems, which combine improved quantification abilities with greater flexibility. They are ideal for targeted quantification of biomarkers. Beyond that, they enable the analyst to perform experiments that can help to elucidate the structure of unknown compounds. As the term 'triple quadrupole' implies, the analyzer of these instruments consists of three quadrupoles, of which the first (Q1) and the third (Q3) are operated in the same way as in single quadrupole systems (scan or SIM mode) while the second (Q2) acts as a cell for molecular dissociation by CID. This setup enables in principle four modes of operation (**Table S6.2** below). Of these four modes, the SRM mode is the method of choice for targeted quantification. By measuring specific transitions instead of single ions (as in SIM measurements with single quadrupole instruments), a higher selectivity, a wider linear dynamic range, a better signal to noise (S/N) ratio and thus lower detection limits are achieved.[33] In contrast, experiments using the other MS/MS modes can provide helpful information about the structure of unknown compounds, especially in CI ionization. It has to be noted that the MS/MS quantification with a triple quadrupole instrument is especially effective if only a limited fragmentation of the target compounds occurs in the ion source, because the aim is to create specific fragments later in the CID cell (Q2). However, many of the TMS or methoxime/TMS derivatives of metabolites detected in a metabolomic analysis undergo a strong fragmentation directly after electron impact ionization. For this reason, soft ionization techniques like CI are a relevant alternative especially for targeted quantification.

Quadrupole ion-trap: These instruments work with a similar principle as that of single qMS. The applied radiofrequency potentials determine the confining capacity of the ions in the ion trap with higher *m/z* ions becoming more stabilized and detected, while the lower *m/z* ions become less stabilized and not detected.[34] There are some reports of the use of quadrupole ion-trap in the determination of dioxins, furans, and polychlorinated biphenyls.[34-36] However, with the advent of new advanced instrumentation, quadrupole ion traps are not as widely used due to low mass accuracy and resolution.[37]

High-Resolution Mass Spectrometry (HRMS)

The mass resolving power is the capacity of a mass spectrometer to separate ions of close *m/z* ratios. It is defined as the ratio of the measured mass "m" to "Δm", the full width of the peak at half its maximum (i.e. m/Δm, FWHM). The resolving power of high-resolution mass instruments is usually between 5-10 times higher compared to LRMS and allows discrimination between isobaric interferences and ions of interest, even with in complex matrices such as plasma, serum or urine. High resolution instruments provide mass accuracy level below 5 ppm, and a mass resolution above 10,000 (m/Δm, FWHM). The high mass accuracy, in combination with high spectral accuracy (i.e. the accurate measurement of isotopic distributions) is a fundamental requisite in untargeted metabolomics and can only be achieved by using high resolution mass spectrometers, such as TOF-MS and Fourier transform MS, including Fourier transform ion cyclotron resonance (FT-ICR) and Orbitrap technologies. The mass resolution generally increases from TOF-MS to Orbitrap to FT-ICR-MS, but there are pros and cons to each technique (i.e. acquisition rates, mass accuracy stability). On the one hand, the time-of-flight (TOF) mass analysers, measuring the flight time of accelerated ions, have the resolving power usually in the range of 12,000 100,000 FWHM. On the other hand, Orbitrap measuring the ion-oscillation frequencies, usually reach even higher values (up to 500,000 FWHM).[38] ToF mass spectrometers are available from Agilent Technologies, Bruker Daltonics, AB SCIEX, and Waters, while Orbitrap technology is available exclusively from Thermo Fisher Scientific.

Recently, hybrid and tribrid instruments combining ion traps and/or quadrupoles with the Orbitrap Fourier transform-based mass analyzer or IRC or TOF have emerged as ideal platforms for metabolomics and also proteomics both applied in different research areas. Trade names for these increasingly common instruments include the Q-Exactive (quadrupole-Orbitrap, Thermo Fisher Scientific), Orbitrap Elite (LIT-Orbitrap, Thermo Fisher Scientific),

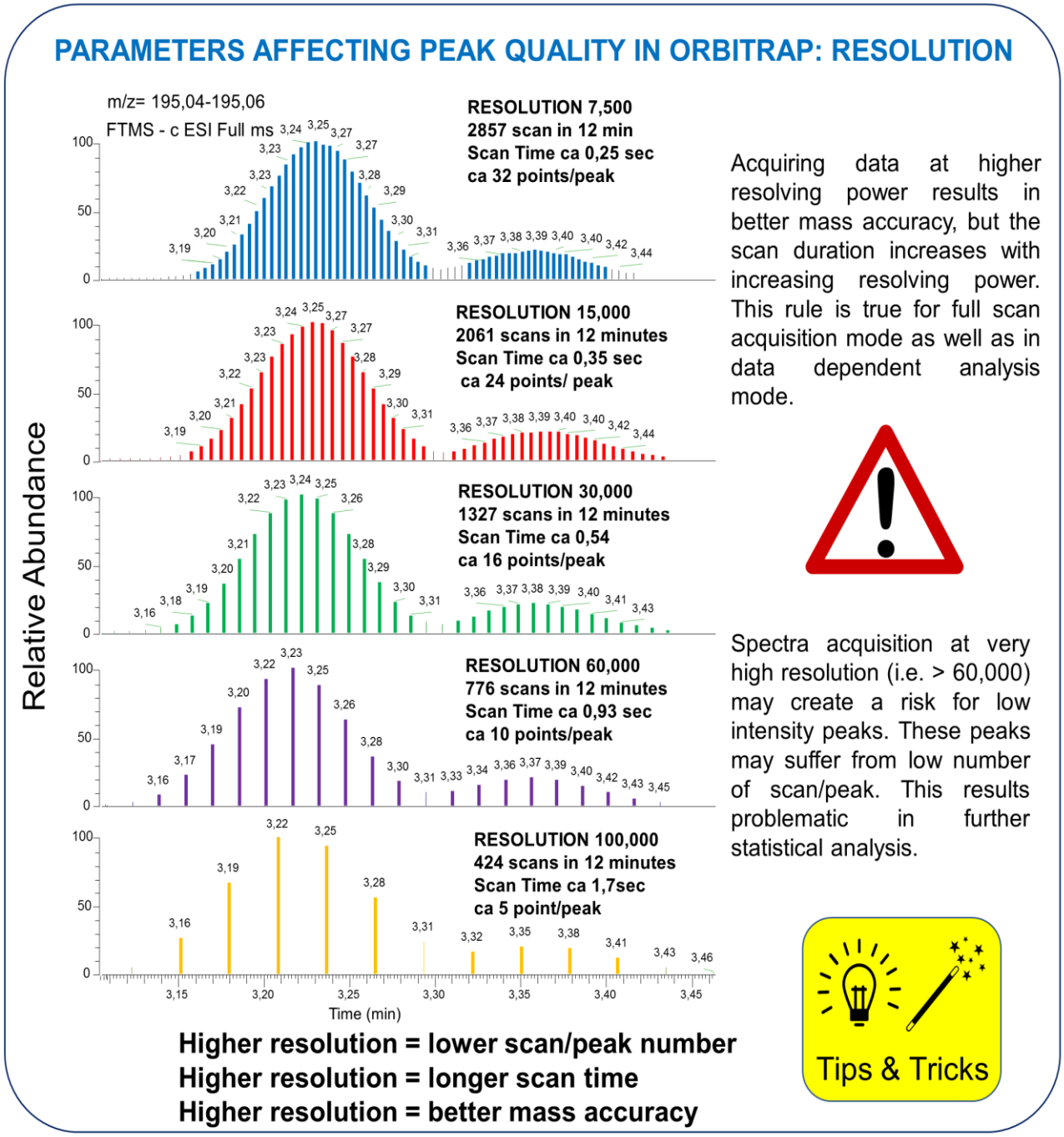
Table S6.2. Analysis modes of a triple quadrupole instrument.

Mode	Q1 mode	Q3 mode	Use
Product scan	SIM	Scan	Acquisition of complete CID spectra of molecules with a specific *m/z* ratio
Precursor scan	Scan	SIM	Identification of compounds which form a specific fragment upon CID
Neutral loss scan	Scan	Scan (with constant mass offset to Q1)	Identification of compounds from which a specific neutral molecule is eliminated during CID
Selected or multiple reaction monitoring (SRM/MRM)	SIM	SIM	Selection of a precursor in Q1 and one (SRM) or several (MRM) fragments in Q3 for highly selective targeted quantification

Fusion Tribrid (quadrupole-LIT-Orbitrap, Thermo Fisher Scientific), TripleTOF® (a quadrupole TOF), TOF/TOF ™ (AB Sciex), impact II™, maXis II™ ESI–QTOF Instant Expertise™ (Bruker), SYNAPT G2-Si (Waters), or 6550 QToF, Agilent Technologies. All instruments are very popular in metabolomics including application in nutritional sciences, while selection of one of them depends on economic status of customer. Several excellent reviews of existing high resolution mass spectrometers and their applications were published recently.[38-42] In this section we will focus on following high resolution mass spectrometers: Orbitrap, Time of flight (TOF)/Q-TOF, Fourier transform ion cyclotron resonance (FTICR), and finally an ion mobility spectrometry (IMS).

Orbitrap: In FT-based analyzers mass-to-charge is measured as a function of ion frequency during oscillatory motion. The Orbitrap mass analyzer uses electrostatic fields to trap and analyze ions and consists of one central spindle and one outer barrel-like electrode. Ions injected into the Orbitrap are electrostatically trapped while rotating and oscillating along the central electrode. Oscillating ions induce an image current signal on the outer electrodes. Image current signals are converted into frequencies by Fourier transformation. The frequencies, which are characteristic of each ion *m/z* value, are finally converted into a mass spectrum.[39, 42-46] The Orbitrap provides a very high resolution of up to 500,000 (FWHM), high mass accuracy of <1 ppm, and high sensitivity analyte detection.[47] These parameters could result in confident compound annotation and quantification leading to increased application in the field of targeted studies.[47, 48] The GC-Q-Orbitrap has filled a gap in high-resolution instrumentation that exists today in GC-HRMS: the identification of unknown molecules in the analysis of complex samples. This newly introduced GC-Q-Orbitrap technology has introduced a molecular-ion-directed acquisition (MIDA) approach for MS/MS, providing information on MS/MS spectra for intact, or nearly intact, ionic species. This new approach in combination with standard methods of chromatographic deconvolution, retention index correlation and spectral database searching can open new horizons to the GC-HRMS metabolomic community.[48] There are several parameters that influence the quality of scan data during acquisition, such as mass resolving power or number of microscans per scan. As shown on **Box S6.1** acquiring data at higher resolving power results in better mass accuracy, but the scan duration increases with increasing resolving power, thus diminish number of scan/peak. As showed on **Box S6.2** an increasing number of microscan increases the scan cycle time resulting in the collection of less MS spectra, thus lower number of scan per peak. At the same time increasing number of microscans improves the signal to noise ratio. There are two additional parameters controlling acquisition of data: the maximum ion injection time and automatic gain control value, both correlated with each other. The maximum ion injection time is the maximum time that ions are allowed to accumulate in the linear ion trap or the C-trap, before being transferred to the Orbitrap (i.e 100 ms, 50 ms), The AGC regulates the number of ions in the mass analyzer in order to avoid or minimize space charge effects to improve mass accuracy (i.e. $1x10^4$) The MS and MS/MS events are controlled by either the AGC target value or the maximum ion injection time depending on which parameter is reached first. Setting the ion injection time very high (e.g. > 1000 ms) can result in significantly longer scan cycle times in cases of weak or no signals (samples with metabolites at low concentration)

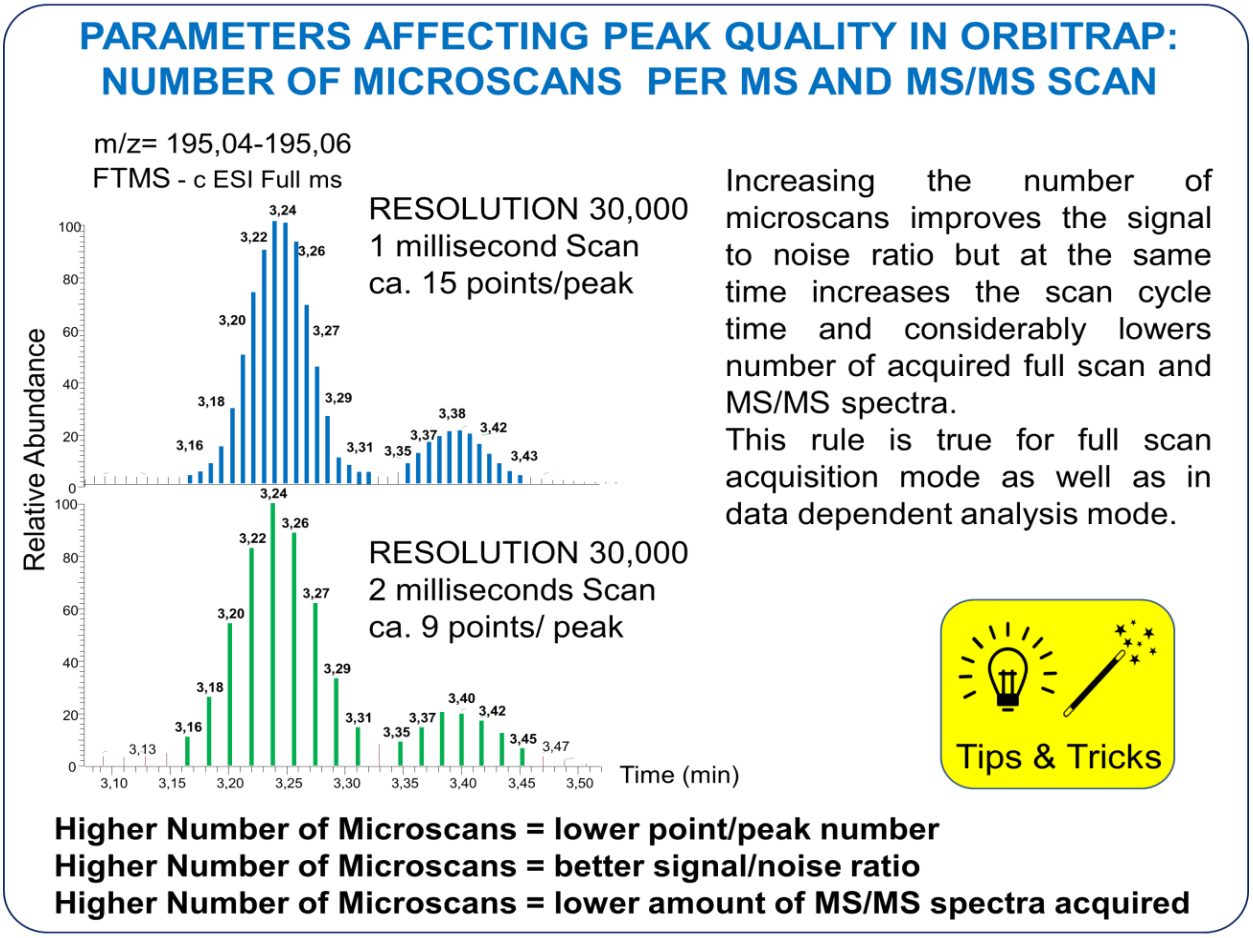
Time of flight (TOF)/Q-TOF: ToF is a temporally dispersive mass analyzer using the differences in transit time through a drift region to separate ions of different masses. In other words, the TOF mass spectrometer separates the ions based on the time it takes for the ions of different masses to move from the ion source to the detector.[49] The potential of each ion accelerates it into the flight tube and because all similarly charged ions share the same kinetic energy, those with lower masses have greater velocity and strike the detector first. TOF/QTOF are widely used in untargeted metabolomic studies where data is acquired over a wide mass range with higher mass accuracy and scan rates.[50-54] TOF instruments often come with several advantages such as faster scan speeds (up to 20 scans/sec (LC-TOF-MS instruments) or even up to 200 scans/sec in case of nominal mass GC×GC-TOF-MS instruments[55] which potentially offer more spectra for deconvolution, high mass resolution (up to 40,000), and accuracy (<1 ppm).[56, 57] The recent developments in hybrid instruments such as quadrupole mass filters coupled to TOF analyzers (Q-TOF) led to acquisitions recorded at different collision energies in a single chromatographic run, thus allowing for data dependent and MS/MS experiments for accurate compound identification. The Q-TOF-MS instruments are increasingly being used in untargeted "omics" approaches where they can serve the purpose of exploratory analysis over a wide dynamic mass range as well as structural elucidation and quantification of the markers of interest (targeted approach).[58]



Box S6.1. Influence of selected resolution on peak quality in Orbitrap

Fourier transform ion cyclotron resonance (FTICR): FTICR is reported as a high-resolution mass analyzer capable of obtaining high accuracy and resolving power with sub-parts-per-million mass accuracy[59] and hence can be useful for empirical formula calculations and compound identification.[56] It principally utilizes cyclotron frequency in a fixed magnetic field to determine the *m/z* ratio of the ions. The instrument however is relatively expensive and has the disadvantage of slow acquisition rates.[60] There are a few studies reported where GC-FTICR-MS has been used for the identification of metabolites such as dioxans.[61]

Ion mobility spectrometry (IMS): Nutritional metabolomics based on mass spectrometry is exponentially growing, and new analytical approaches may improve the performance of metabolomic analyses, since significant challenges associated with throughput, metabolome coverage, and compound identification still exist. In this context, ion mobility mass spectrometry offers great potential for improving throughput and depth of coverage in metabolomic experiments. IMS is a gas-phase electrophoretic technique that provides a third dimension of separation based on size, shape, and charge of ions. The new generation of ion mobility spectrometers is coupled to high



Box S6.2. Influence of number of microscans on peak quality in Orbitrap.

resolution mass spectrometry instruments, adding a mobility region. So far, eight different types of IMS have been employed.[62] IMS is a promising approach that can overcome HRMS limitations, such as biomarker identification and separation of structurally closely related isomers. As an example, IMS has been applied to lipid analysis, improving structural separations and providing class identifications from mobility mass correlations: Kyle et al.[63] demonstrated that sn-backbone positions and cis versus trans double bond orientations can be distinguished using reverse phase LC coupled with IMS-QTOF.

6.1.3. Data Acquisition Mode

Data acquisition parameters play a critical part in the overall raw data quality. In untargeted metabolomics, independently of its application, mass spectrometry data must be acquired in two ways, first data obtained from full scan spectra, which are further submitted to statistical analysis, and in a later stage MS^2 or MS^n once statistically significant *m/z* features have been identified by statistical analysis. This means that samples should be injected more than once. Depending on the mass spectrometer type and its performance, analysis in the positive and negative ion modes can be performed separately with two consecutive injections or in one injection with the use of a polarity switching mode. As mentioned previously, the reinjection of a sample is necessary; in order to obtain fragmentation information for *m/z* features that gave statistically significant results. Acquisition of MS^n spectra can be performed by using one of several methods such as SIM, MRM, MS/MS (data dependent acquisition (DDA); data independent acquisition (DIA); Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH); MS^{ALL}, another data-independent acquisition method). Modalities DDA and DIA have an advantage of an automatic fragmentation of *m/z* ions basing only on their intensity, and not on their *m/z*. This is particularly useful when time between statistical analysis and reinjection can take several days up to few weeks. Condition of mass spectrometer can change during that time. At that point it is advisable to perform injections for MS^n acquisition in an automatic modality DIA or DDA immediately after acquisition of full scan data. Some scientist select only a part of dataset for this automatic reinjections, mainly due to the cost, however it is highly advisable to reinject full dataset with DDA or DIA modalities, for an extraordinary possibilities of a retrospective analysis of full dataset in the future. Details on each of mentioned here data acquisition modes are reported below.

Full Scan Mode: In untargeted metabolomics, MS data acquisition is typically performed as a full scan over a *m/z* range that depends on the specific application. In GC-MS the *m/z* range varies approximately from 45-60 to 550-750 Da.[64] In case of derivatized primary metabolites, ions below *m/z* 45 are usually less specific and can be excluded, especially because some of them may contribute substantially to baseline noise like *m/z* 44 (CO_2). On the other hand, most derivatized primary metabolites have a

molecular mass below 600 Da or, if they are larger like disaccharides, fragment strongly so that large ions are not observed.For this reason, an upper scan range limit of ca. *m/z* 600 is usually sufficient, especially in case of qMS instruments where the time needed per scan is a critical parameter that determines the overall data acquisition rate. The scan time will depend on the mass range specified i.e. the higher the mass range, the lower the number of scans per second. In LC-MS, the full scan *m/z* range varies depending on the particular application and/or compounds of interest. The LC-MS lipidomic analysis requires a wider range of *m/z* values starting from relatively low masses of ca. 100 up to 2000 Da, allowing to monitor some complex endogenous lipids, for instance glycerophospholipids or cardiolipins. When the main interest of the nutritional study focuses on so called small molecules, for example plant compounds that undergo human metabolism, the *m/z* range should be flexible enough to detect, on the lower side of this range, molecules such as glycine and/or sulphate derived from neutral losses of conjugated metabolites, up to more complexed glucuronide conjugates on the upper side. Thus, the lower *m/z* range can start from 70-80 Da, while the upper limit could reach up to 700-800 Da. This upper range allows not only for the monitoring of food/plant-derived metabolites but also for endogenous compounds such as glucuronidated fatty acids, which are frequently found in plasma/serum samples after nutritional intervention. As mentioned above, also LC-MS analysis suffer from noise derived from baseline and/or solvents and blanks (i.e. 59.060 from $[CH_3CN+NH_3]^+$, 44.998 $[HCOOH]^-$ or 112.98563 in negative mode from formic acid dimer commonly used in mobile phases). For this reason, masses lower than 70 Da should be avoided. For a full list of contaminants and artifacts, see Keller et al.[65]

Selected Ion Monitoring (SIM). SIM allows to monitor a particular *m/z* value resulting in detection of specific compounds of interest with increased sensitivity compared to full scan mode. It allows for more points across a chromatographic peak, thus increasing the accuracy and precision of the results obtained. In a single scan cycle, no more than eight *m/z* values need to be entered in order for the SIM method to be effective. The amount of time to spend sampling a specific ion (dwell time) is usually 100 ms for 2-3 ions. For more ions, shorter dwell times can ensure enough data points. SIM is mainly used in targeted metabolomics to identify analytes especially with lower detection limits, thus allowing for quantification of markers of interest.[66, 67]

Combined Scan/SIM approach. The simultaneous data acquisition in scan and SIM mode allows for a reduction in sample analysis time and eliminates injection port bias since the data is acquired from the single run and minimizes uncertainty related to compound identification. For an effective chromatographic integration in a combined mode, it is suggested to increase the scan speed, or decrease the SIM dwell time, to achieve the desired number of data points.

Multiple Reaction Monitoring (MRM). With SIM there is the possibility that ions of the same mass co-elute in highly complex matrices such as plasma. MRM allows for selection of precursor ions from the full scan mode, which are fragmented in the collision-induced cell. The ions produced by this fragmentation are then selected based on the abundance of the transition from precursor- to product ion. MRM detection limits are 5-10 times lower than the SIM detection limits. The difficulty in applying the MRM method in untargeted workflow is the *a priori* identity of the compound under investigation. Nevertheless, when this technique is applied on tandem MS, it eliminates matrix interferences, making it sensitive and selective and thus ideal for the identification and quantification of biomarkers.[68]

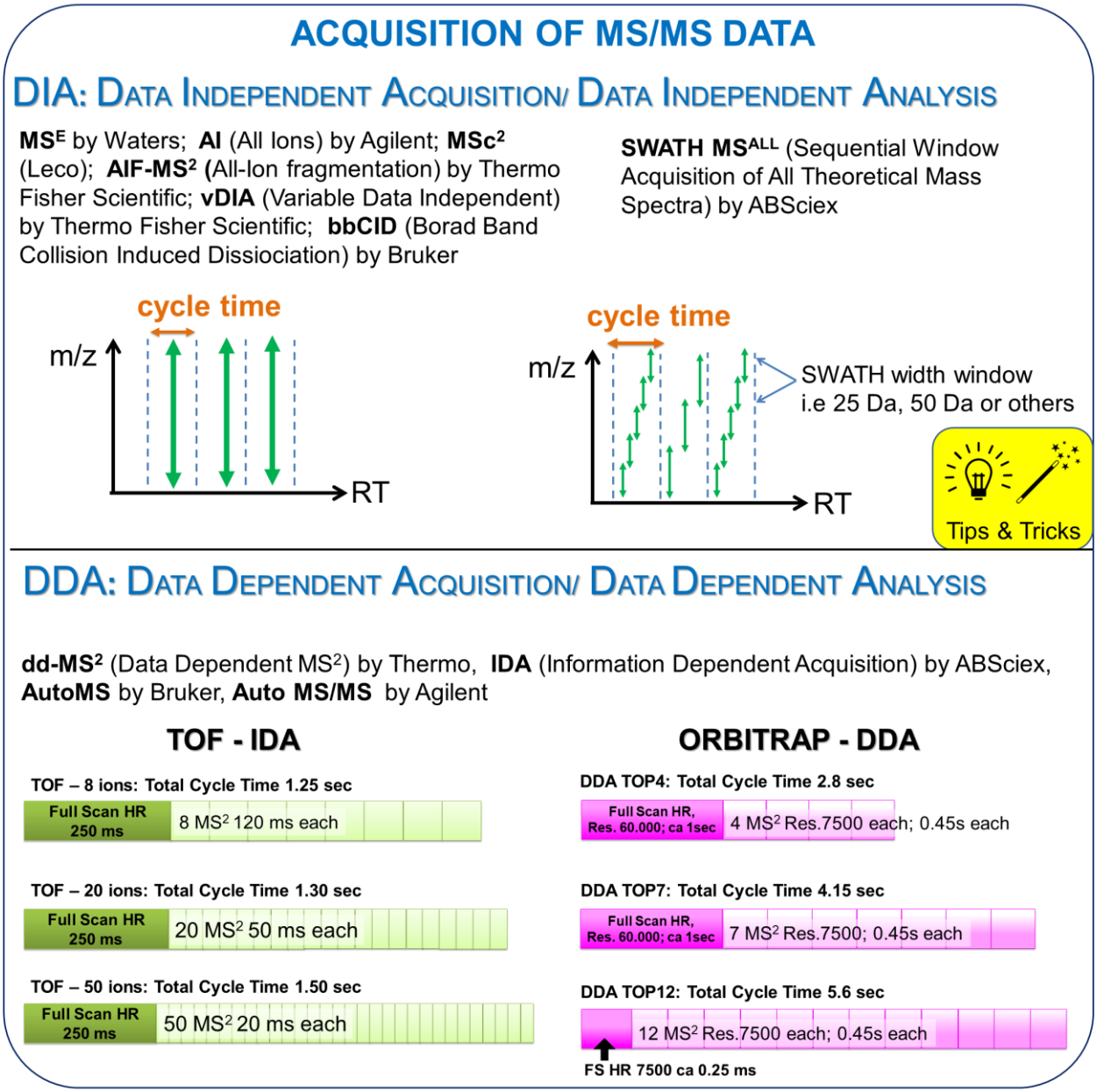
MS/MS. The MS/MS mode allows the production of fragmentation spectra for structure elucidation using one of the two methods for data acquisition strategies: DDA (Data Dependent Acquisition) and DIA (Data Independent Acquisition).[69] In both methods the quadrupole isolates the desired ion, which is fragmented in the collision cell. Differences between the two acquisition modes are related to the way that the precursor ion is selected. See **Boxes S6.3 and S6.4** for a visualization of the concept. Finally, the third analyzer (i.e. either the quadrupole Orbitrap or TOF) is measuring the fragment ions. Both DDA and DIA methods collect information about full scan and MS/MS fragments. Of note, the collision energy can be optimized during MS/MS. to modulate the number of fragments and the acquisition time (modulating the speed at which the spectra can be recorded) can be adjusted to perform simultaneously MS and MS/MS events . The DDA mode (data-directed acquisition, data directed analysis or data dependent acquisition) produces MS^n spectra selecting precursor ions based on their intensity. The operator can select how many ions from each scan can be exposed to fragmentation: top 3 (automatic MS/MS analysis of three most intensive ions per scan), top 5 (automatic MS^2 analysis of five most intensive ions per scan), top 10, top 20 etc. The DDA mode produces MS^2 and pseudo MS^n spectra with minimal interference since only ions within the selected narrow *m/z* window, typically 1-3 Da wide, are transferred to the collision cell to generate the product ions. Fragment ions in MS^2 spectra can be acquired at high or low resolution.

High resolution is recommended as it allows for better structure elucidation and further compound annotation. Collision energy used for fragmentation is usually a compromise as only one collision energy can be used in an MS/MS scan event (i.e. 35 eV in first scan event, or 45 eV in the second scan event). For this reason, a collision energy suitable for most of metabolites present in the sample should be selected (see **Boxes S6.3 and S6.4**). The DDA mode in a Q-ToOF equipment's are conducted with a fixed scan rate, increasing the number of ions submitted to fragmentation, diminishing drastically the points per peak. The DDA mode in an Orbitrap is performed with a fixed resolution, increasing the number of ions submitted to fragmentation, extending scan times significantly, and diminishing the points per peak (see **Box S6.3**). In both cases, an automatic acquisition of MS/MS spectra in mode top 7, top 15, or more, allows for the collection of a high number of spectra, which can be used at any time, for instance in future retrospective analysis. **Box S6.3** shows an Orbitrap DDA method scheme, where duration of full scan and MS/MS events depends on the resolution selected by the operator. As such, a full scan event at a resolution of 60,000 FWHM takes 1 s, followed by 4 (top 4) or 7 (top 7) MS/MS scan events each with a resolution of 7500 FWHM and duration 0.45 s. Such settings give a total cycle time of 2.8 s for the top 4 method, and 4.15 s for TOP7 method.

In the same **Box S6.3** a scheme of TOF – IDA method reports Full Scan event duration of 250 ms, followed by consecutive eight MS/MS scan events of duration 120 ms each, for total cycle time duration of 1.25 seconds. Of course, additional MS/MS scans will extend the total cycle time to 1.5 s or more. To some extent, DDA spectrometry methods

can be used as unique way of acquisition of MS and MS/MS spectra in one injection. However, a very important aspect of such analysis must be considered, namely, the quality of MS scan spectra (number of points per peak) that must serve for further statistical analysis. When combining, in one injection two types of scans, the number of scans per peak (points per peak) can diminish drastically. In DDA methods duration of full scan and MS/MS scan is set by Operator and must consider chromatographic peak duration. In an ideal situation total cycle time should be much shorter than duration of a chromatographic peak, allowing for at least 12 to 15 scans per peak. Still such settings do not guarantee good quality of low intensity peaks. Indeed low number of point per peak can have a negative impact on low abundant metabolites, or down-regulated endogenous metabolites that tend to have low intensity. In consequence the Gaussian curve of a low intensity metabolite cannot be correctly assessed during data pre-processing. As a consequence, the peak area of these metabolites suffers from under- or overestimation during data processing. More details regarding an example of Orbitrap LTQ-XL different acquisition modes are presented in **Box S6.4.** It is advisable to perform two injections of each sample, in order to obtain the highest quality data set: one injection dedicated to the full scan mode, and the second injection dedicated to the MS^2/MS/MS mode.

DDA methods are an excellent way of acquiring an elevated number of MS^2 and pseudo MS^n spectra. Nonetheless, still DDA suffers from limitations. The situation becomes problematic, when low intensity metabolites are



Box S6.3. Different DIA and DDA data acquisition modes.

not included in the top 3, top 7, etc. lists, and thus are not fragmented. In this context, two interesting tools can be used with DDA or IDA acquisition methods improving the MS/MS acquisition performance. Both Q-ToFs and Orbitrap have an option to introduce a list of compounds to be included in the fragmentation. This option can be illustrated with the following example: in a study focused on urine biomarkers of apple intake, the operator will be interested in the automatic acquisition of phloretin metabolites. A list of theoretical masses of phloretin metabolites (i.e. phloretin sulfate or glucuronide) can be introduced in the so called "Inclusion List" and the mass spectrometer will automatically perform MS^2 analysis, even if these ions are of low intensity and do not belong to top 3 or top 20 most intensive ions per scan. This additional tool guarantees that compounds of particular interest will have MS^2 or MS^n spectra that are acquired automatically.

Besides, both types of mass spectrometer have a parameter defining a minimum ion intensity to be considered for fragmentation. Moreover, the Orbitrap has another tool called the "Dynamic Exclusion List", a second useful tool that avoids multiple fragmentation of the same *m/z* and in consequence acquisition of the same MS^n spectra. In this tool, a maximum number of repeated MS^2 spectra can be defined, so the MS will fragment an ion only two or three times. Important parameters in the "Dynamic Exclusion List" are:

- Maximum number of repeated MS2 scans for *m/z* ion: i.e. 2. Note that one single scan could result in low quality MS2 spectra, especially if the fragmentation occurs at the beginning of the Gaussian curve of the peak)
- Exclusion list duration: i.e. 18 sec. Note that the selected duration time must allow for the fragmentation of eventual isomers with similar t_R. This is particularly critical for sulfates or glucuronides in nutritional studies as these metabolites usually appear in several conjugated forms.

The second abovementioned MS/MS mode for automatic spectra acquisition is DIA. In DIA (data-independent analysis or data-independent acquisition), all precursor ions from the full scan mode are sent to the collision cell for fragmentation. The following technologies using this acquisition mode are available: MS^{ALL} SWATH by ABSciex, MS^E by Waters; AI (All Ions) by Agilent, AIF-MS^2 (All-Ion fragmentation) by Thermo Fisher Scientific, MSc2 by Leco, vDIA (Variable Data Independent) by Thermo Fisher Scientific, and bbCID (Borad Band Collision Induced Dissociation) by Bruker. In essence, DIA allows a complete and permanent recording of all fragment ions of the detectable metabolite precursors present in a biological sample. In contrast to DDA, DIA neither selects a priori the precursor ions to trigger the acquisition of fragment ion spectra nor provides information on their intensity. Instead, either all of the precursors contained in the entire mass range (MS^E, All Ions MS/MS, AIF, MSc^2) or all of the precursors contained in a number of predefined isolation windows (SWATH) are fragmented together (see **Box S6.3)**. In the SWATH acquisition set-up, the mass spectrometer steps, within 2-4 s cycle time, through a set of precursor acquisition windows. These windows cover the mass range readily covered by a quadrupole mass analyzer (i.e. *m/z* 70-800 Da) and fall within the mass range of most of the small metabolites in living organisms. During each cycle, the mass spectrometer fragments all precursors from the quadrupole isolation window (e.g. *m/z* 475-500 Da for a 25 Da-wide window) and records a complete, high accuracy fragment ion spectrum of all precursors selected in that isolation window. For each cycle, the same precursor isolation window is repeatedly fragmented during the entire chromatographic separation, thus providing a time-resolved recording of the fragment ions of all metabolites precursors that elute during the chromatography.[70-72]

To conclude, excellent comparisons of different MS/MS mode of analysis were recently published.[39, 42, 69, 73] Also reviews on advanced applications of mass spectrometers are available from Bonner et al.[74], Gillet et al.[70], Zhang et al.,[71], Hopfgartner et al.,[72] and Roemmelt et al.[75]

Although the above MS/MS methods perform with different efficiencies, all certainly provide very high numbers of automatically acquired MS2 spectra, which support the annotation and structure elucidation of metabolites. In summary, we recommend to perform one injection dedicated to the full scan mode and one injection dedicated to the MS/MS mode in order to have a very high quality dataset that can be used for statistical analysis and structure elucidation. Moreover a combination of such injections, with use of DDA, IDA, SWATH, or MS^{ALL} modes allow for additional further explorative research parallel to the main goal of the study, as well as future retrospective analysis due to the high number of MSn data acquired for annotations.

Instrument Parameters in GC-MS

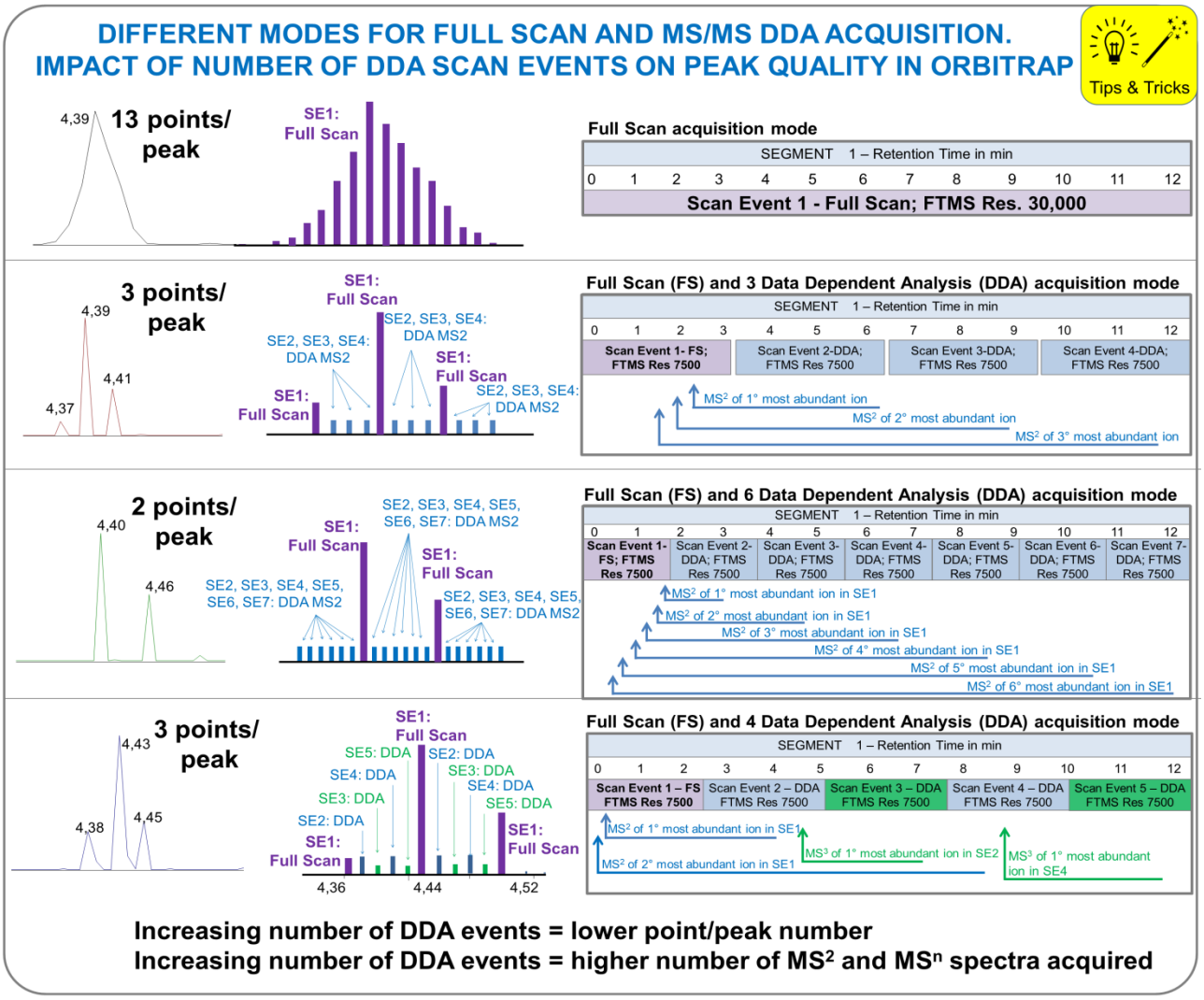
Transfer line temperature. A transfer line is used between the GC and ion source of the MS where the sample from the GC column needs to be transferred to MS ion source that is held under vacuum. An isothermal temperature limit at which the column film is stable for a long period of time with low column bleed needs to be maintained in the transfer line which ensures stable transfer of analytes without any condensation or thermal degradation.[2] Usually the transfer line temperatures are maintained at 300 (260/280)-340°C depending on the column specifications. However, it is suggested to use temperatures below 300°C to ensure stability of tubing (5% phenyl phases).

Ion source temperature. The ion source temperature is usually maintained at a default setting of 200-230°C, depending on the instrument and ionization mode. With increase of ion source temperature, higher intensities can be observed, while a decrease in intensity of the quant ion can be noticed. Moreover, increase in ion source temperature can also lead to contamination of the source/lens system

On the other hand, with insufficient heating, moisture can be found coated on the surface of the ion source and the lens system.[2] Hence ion source temperature needs to be maintained in such a way that can provide a compromise between peak shape and the ion/compound response.

Emission current. The emission current heats up the filaments causing it to emit electrons which then ionize the analyte molecules. On one hand, a higher emission current means that more electrons are produced; thus, more ions are produced and lower detection limits are achieved. On the other hand, a higher emission current decreases filament lifetime drastically. As a compromise, default emission currents in the range of 35 µA (Agilent) to 60 µA (Shimadzu) are typically recommended by instrument manufacturers. As a precaution, it is recommended to exchange the filaments after the recommended maximum number of operating hours.

Detector voltage. The voltage applied to the electron multiplier or a photomultiplier to adjust overall signal intensity is referred to as detector voltage. It can be



Box S6.4. Influence of number of DDA events in peak quality in Orbitrap. Different types of data acquisition.

manually adjusted to meet the analytical validation requirements such as linear range, and detection limits (maybe only in specific regions of the chromatogram), especially when the concentrations of the compounds of interest are highly different. Increasing the voltage raises the signal abundance, thus increasing the sensitivity, but at the same time decreasing the life span of the multiplier. Hence the multiplier needs to be operated at an optimized voltage that can provide adequate sensitivity. Photomultipliers allow a good sensitivity with a high amplification gain. For TOF and QTOF instruments, microchannel plate (MCP) detectors are the ones of choice because they provide a flat conversion surface with a large area, suitable for recording ion packets that are large in the y-dimension and thin along the z-axis.

Tuning. Tuning consists in adjusting source and analyzer parameters to achieve the desired signal intensity and resolution. MS tuning can be highly instrument and application specific and it is typically done through in automatic process defined by the instrument manufacturer ('autotuning') and triggered regularly by the operator. It is important to tune the MS frequently to achieve maximum spectral sensitivity and resolution to ensure exact identification of mass-to-charge ratios, but also to correct for increasing contamination of the ion source. The tuning also needs to be performed under following circumstances: i) When columns are changed; ii) When the mass spectrometer is vented; iii) When the ion source conditions or column flow is changed in the analytical run method; iv) Mass analyzers are generally tuned with highly stable perfluorotributylamine (PFTBA). The fragment ions with *m/z* values 69, 219 and 502 ions are used to calibrate over the entire range of the spectrum. However, manual tune can be performed to achieve maximum sensitivity for a narrower scan range. In case of decrease in signal intensity in QC samples. It should always be performed under the same conditions, especially concerning GC oven, injector, ion source, and transfer line temperature as well as column flow. After automatic or manual tuning, the tuning report needs to be inspected carefully. The tuning algorithm sets the parameters in the system in such a way to attain maximum mid-range and high end sensitivity. Although these reports are instrument- and manufacturer-specific, some general recommendations can be given (**Table S6.3**). A set of particularly useful information regarding several parameters of GC systems, as well as tuning performance are given in **Box S6.5**.

Instrument Parameters in LC-MS

Performing analysis in the untargeted mode, independently of its application, imply using quite general settings of ion

source that satisfy wide range of compounds in biological fluids (for example in plasma from small phenolic acids up to fatty acids). Ion source temperature settings should be high enough to let evaporation of arriving sample with mobile phase, and here a key importance is the water content. High content of water requires usually higher ion source (and capillary) temperatures, while application in lipidomics, where organic solvents are predominant lower temperature would be sufficient. Voltages applied in ions source should be high enough to ionize compounds, however not too high in order to avoid strong ion source fragmentation phenomena. Parameters of ions source depends very much on mass spectrometer, and vary from ESI, or APCI etc. For this reason only few examples of ions source parameters used in nutritional studies for TOF and Orbitrap are given in **Table S6.4** below.

Tuning. In LC/MS, the mass analyser is tuned using an electrospray calibrant called the tuning mix and either an autotune or manual tune can be performed in both the positive and negative ion mode. Typically, a tune mix consists of a set of perfluorinated compounds that are distributed over a mass range. Autotune is usually performed when the instrument is vented or when the signal intensities of the masses diminish over a period of time. However, a mass calibration check tune can be performed on a daily basis in order to ensure the mass accuracy of the instrument and the signal intensity of the masses can be evaluated. Similarly to tuning in GC, also in LC tuning should be performed when columns are changed, when the mass spectrometer is vented, when the ion source conditions are changed in the analytical run method etc.

6.2. Data Acquisition in NMR Spectrometry

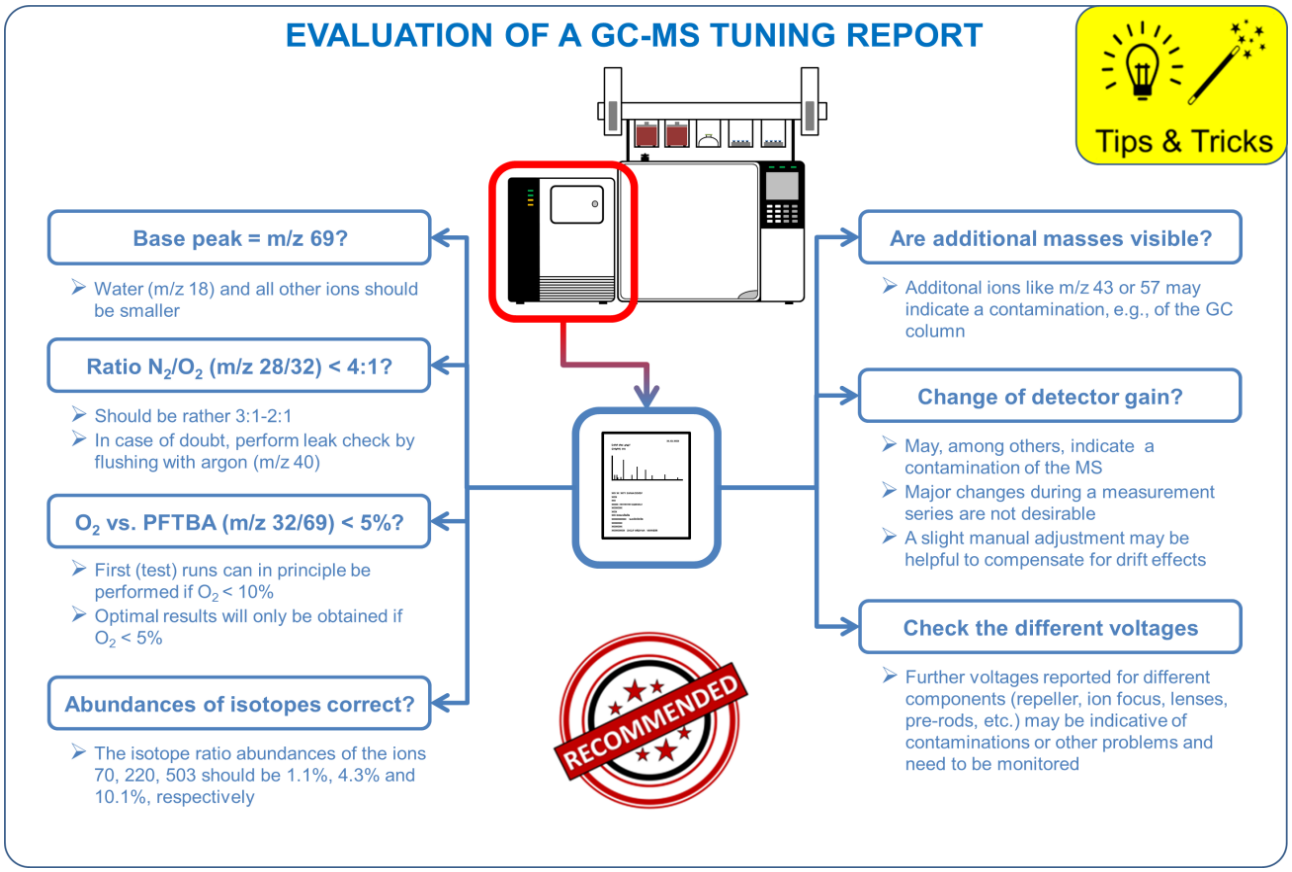
6.2.1. Pre-Acquisition Conditions

Temperature control must be calibrated once a week using, for example, a standard methanol sample. Experiments must be performed at constant temperature (i.e. 300 K or 298 K).

When the samples are loaded into the probe, they need to be allowed to equilibrate in the spectrometer: Generally, 5 min in the tube probe is recommended, although longer times may be required depending on the specific temperature controller efficiency and, thus, on the configuration of the available spectrometer. Each sample needs to be tuned and matched. The offset of the water signal (O1) is then determined by following an iterative procedure: a simple pulsed 1D experiment (i.e. "zg" on Bruker spectrometers) is ran with 1 scan, then the FID (free induction decay) is transformed, all signals resulting from the residual water peak are phased (without caring about the phase of the water) and the value of O1 is changed depending on the shape of the water signal. If this has a negative dip on the left side, then the O1 value is too big, whilst if the dip of the water signal is on the right side, the value of O1 is too small. This procedure is thus repeated until the optimal O1 value is found. When many samples with the same characteristics are analyzed by using an auto-sampler, the acquisition of the NMR spectra is automatized. It is advisable to optimize O1 on the first representative sample, then transfer automatically such parameters to the whole series. All other acquisition parameters (e.g. tuning, matching, shimming, 90° pulse length, and receiver gain) are automatically optimized on each sample prior to acquisition of the corresponding sample. Impact of parameters such as shimming procedure, calibration of frequency off set O1 and L4 loop counter on spectra quality is given in **Box S6.6**. Each acquired spectrum is then quality-checked (**Table S6.5**). If the spectrum does not meet the selected standards, the sample is re-analyzed and the spectrum is re-acquired. If feasible with the available instrument and not too time-consuming, another option is to optimize the spectral acquisition parameters for each sample.

Table S6.3. Parameters for the tuning and maintenance of GC-MS spectrometers.

Mass analyzers are generally tuned with highly stable perfluorotributylamine (PFTBA). The fragment ions with *m/z* values 69, 219 and 502 ions are used to calibrate over the entire range of the spectrum
m/z 69 should be the base peak; all others including the water peak (*m/z* 18) should be smaller
The ratio between *m/z* 28 (nitrogen) and 32 (oxygen), should not be identical or close to 4:1 (air leak), rather 2:1 to 3:1. In case of doubt, use argon gas for searching the leak (check especially the "door" of the MS, transfer line connection, column connector (GCxGC))
Check the ratios of the ions 219 to 69 which should be at least 50% and also the ions 502 to 69 should be less than 3%
The isotope ratio abundances of the ions 70, 220, 503 should be 1.1%, 4.3% and 10.1% respectively
The default values of ion source parameters such as repeller voltage, ion focus voltage, lens voltage, X-Ray voltage, Electron multiplier voltage needs to be monitored



Box S6.5. Evaluation of tuning in GC-MS.

Table S6.4. Selection of parameters of ions source in Orbitrap (Thermo Fisher Scientific), Triple TOF5600 (ABSciex), Qq-TOF (Bruker Daltonics); QTOF (Agilent), QTOF (Waters)

Parameter/ TOF model	**Triple TOF™ 5600 (AB SCIEX); ESI;**	**Triple TOF™ 5600 (AB SCIEX); ESI;**	**TripleTOF™ 5600+ (AB SCIEX); DuoSpray® Source**	**Triple TOF™ 5600 (AB SCIEX); ESI;**	**TripleTOF™ 5600+ (AB SCIEX); DuoSpray® Source**
	plasma metabolomics	urine metabolomics	shotgun plasma lipidomics	metabolomics of different biological fluids and tissues	metabolomics of different biological fluids and tissues
Ion spray voltage floating (V)	Pos 5500 Neg -5500	Pos 5500 Neg -4500	Pos 5000 Neg -4000	POS 4500	POS 5500
Temperature (°C)	500	550	300	500	700
curtain gas flow rate (ml/min)	25	35	20	25	
Ion source gas GS1 (au)	50	60	20	Not reported	25
Ion source gas GS2(au)	40	60	15	Not reported	60/70
Declustering potential (DP) (V)	Not reported	80	40	80	
Collision Energy (CE ±CES) (eV)	35 ±15	10	Not reported	40 ±15	20
Full Scan mode m/z range	70-1250	100-1000	200-1200		100-1000
Full Scan mode acquisition time (sec)	0.25	0.25	0.3		
Automatic MS/MS data colection mode	IDA: CE 35eV; acquisition time 0.17 s, m/z scan range 50-1250;	IDA: 35±15eV, acquisition time 0.1s, exclusion isotope within 4Da, mass tolerance 50 mDa, maximum number of candidate ions to monitor per cycle 8; m/z scan range 50-1000.	MS/MS^{ALL}: 1000 product ion experiments with 1000 precursors evenly spaced from m/z 200.051 to m/z 1,200.051, measuring across m/z 100–1,500, accumulated for 300 ms each. Collision energy for each MS/MS step was 50 ± 30 eV and −40 ± 30 eV	n.a	1. Information-dependent acquisition mode (cycle time 350 ms): HR TOF as survey scan (accumulation time 100 ms) and HR MS–MS as dependent scan with a CE range 20–50 eV (four MS–MS dependent experiments) (accumulation time 50 ms); 2. MS^{ALL} (cycle time 350ms): HR TOF MS at low energy (CE = 10 V) (accumulation time 150 ms) and HR TOF MS at high energy (CE=35 V) (accumulation time 150 ms); 3. Sequential window acquisition of all theoretical fragment ions spectra (SWATH) (cycle time 375ms): 15 PIS (Q1 20 u window) (accumulation time 25 ms each PIS, CE range 20–50 eV).
Solvents	Solv. A: water with 0.1% formic acid Solv. B: methanol with 0.1% formic acid.	Solv. A: Ultrapure water 0.1% formic acid (v/v) and 2mM ammonium formate (v/v) Solv. B: acetonitrile/methanol (1/1), with 0.1% formic acid (v/v) and 2mM ammonium formate (v/v)	Solv. A: methanol: isopropanol (3:1, v/v) with 5 mM ammonium acetate; Solv. B: 98% isopropanol and 2% methanol with 5 mM ammonium acetate	Solv. A: Water with 0.1% formic acid Solv. B: Methanol with 0.1% formic acid	Solv. A: Water with 0.1% formic acid Solv. B: Acetonitrile with 0.1% formic acid
Reference	[76]	[77]	[78]	[79]	[72]

Parameter/ Orbitrap model	Orbitrap Exactive (Thermo Fisher Scientific); ESI	Orbitrap LTQ-XL (Thermo Fisher Scientific); ESI	Orbitrap Q-Exactive (Thermo Fisher Scientific); HESI-II	Parameter/ TOF model	Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) (Agilent)
	plasma lipidomics	urine metabolomics	plasma metabolomics		plasma, serum metabolomics
Source voltage (V)	Pos 4500	Pos 5000; Neg 3500	Pos 3500; Neg 2500	**Capillary voltage (V)**	3500 both modes
Capillary voltage (V)	Pos 25	Pos 30; Neg -30		**Skimmer voltage (V)**	60
Capillary temp (°C)	275	320	250 (heated vaporizer 300)	**Fragment voltage (V)**	120
Sheath gas flow (au)	60	70	50 psi	**Source temperature (°C)**	350
Auxiliary gas flow (au)	25	30	10 psi	**m/z range**	50-1000
Microscans per scan	3	1	-	**Full scan mode**	2 spectra/s
Maximum injection time	250 msec	100 msec	100 msec	**MS/MS mode**	0.5 spectra/s
Automatic gain control	$1*10^6$	$1*10^6$	$1*10^6$	**Collision energy (V)**	20
Automatic MS/MS colection mode: dd-MS2	-	Res.7,500; auto gain control target under 1×10^5, a maximum isolation time of 50 ms, selected top 3 peaks, isolation window 1Da, collision energy of 35.	Res: 17,500, an auto gain control target under 1×10^5, a maximum isolation time of 50 msec, selected top 5 peaks, an isolation window of m/z 1.5, a normalized collision energy of 30, an under fill ratio of 5.00%, and an intensity threshold under 1×10^5		
Solvents	Solv. A: 20% isopropyl alcohol in acetonitrile; Solv. B: 20% IPA in ammonium formate (20 mM)	Solv. A: Milli-Q water with 0.1 % formic acid. Solv. B: acetonitrile with 0.1 % formic acid.	Solv. A: Milli-Q water with 0.1 % formic acid. Solv. B: acetonitrile with 0.1 % formic acid.	**Solvents**	Solv. A: Milli-Q water with 0.1 % formic acid. Solv. B: acetonitrile with 0.1 % formic acid.
Reference	[80]	[81]	[82]		[83]

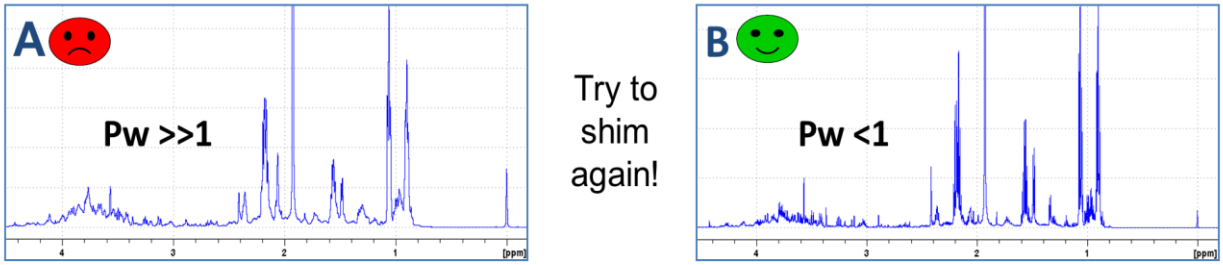
Parameter/ TOF model	ESI - Synapt G2-Si QTOF MS (Waters Corporation)	Quadrupole time-of-flight mass spectrometer; ESI (Premier QTOF, Waters Corporation)	Parameter/ TOF model	Ultra-High Resolution Qq-Time-Of-Flight (Bruker Daltonics)
	Plasma	Plasma , urine		Urine metabolomics
Cone Voltage (V)	10	30	**Plate offset voltage (V)**	500
Capillary voltage (V)	1500 both modes	Pos 3200, Neg 2800	**Capillary voltage (V)**	4500 pos; 3600 neg
Source temperature (°C)	120	Ion source gas 120; dissovation gas 400	**Dry gas flow Temp (°C)**	200
Nebulizer gas flow L/h	1200	800	**Dry gas flow (L/min)**	8
Resolution	50,000	50-1000	**Nebulizer pressure (bar)**	1.8
Scan rate (sec)	0.1	0.08	**Collision Energy MS (eV)**	6
Automatic MS/MS colection mode	MS^E mode; Reinjection for single m/z feature.	MS^E mode, Reinjection for single m/z feature.	**Automatic MS/MS colection mode**	Not available
Collision Energy (eV)	1st function: 6eV; 2nd function 10-35eV	CID 10eV, 20eV, 30eV	**Collision Energy MS/MS (eV)**	10 to 40
Solvents	Solv. A: water containing 0.1% formic acid) Solv. B: acetonitrile containing 0.1% formic acid	Solv. A: (water containing 0.1% formic acid) Solv. B: acetonitrile/methanol 70/30 containing 0.1% formic acid	**Solvents**	Solv. A: 0.1% formic acid in Milli-Q water Solv. B: 0.1% formic acid in acetonitrile
	[82]	[84]		[85]

NMR SPECTRA QUALITY

Tips & Tricks

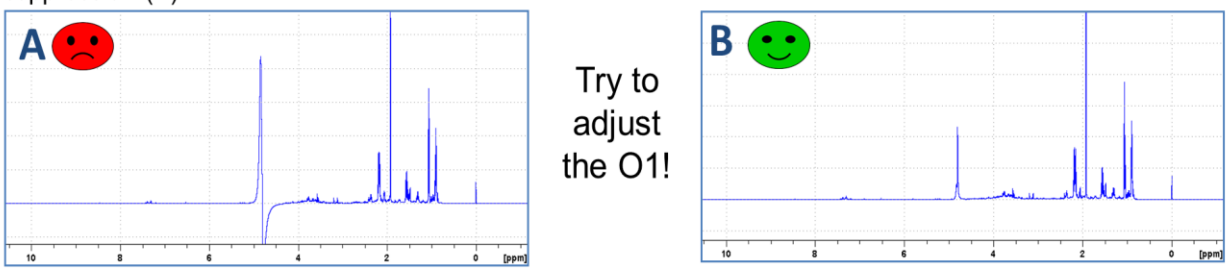
IMPACT OF SHIMMING PROCEDURE ON SPECTRA QUALITY

Shimming is a procedure to generate homogeneous magnetic field along the sample volume. If the field is not homogeneous the resulting spectra will suffer from distorted and large line shape (**A**) which in turn leads to poor resolution and sensitivity. For this reason, the field has to be optimized (**B**). The **peak width** (pw) measured on TSP or DSS signal should be < 1Hz!



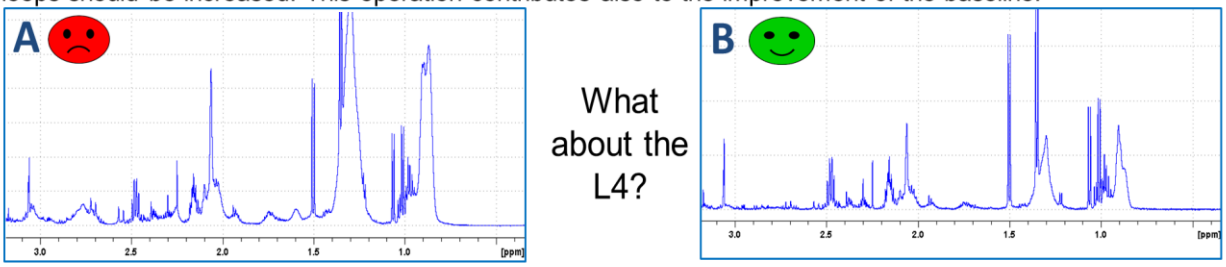
CARRIER FREQUENCY OFFSET FOR WATER (O1) CALIBRATION

As biological samples are in aqueous solution, typically, the carrier frequency ("O1") needs to be adjusted slightly to obtain the best water suppression (**B**). A bad calibration may significantly affect the result of a water suppression (**A**).



THE L4 LOOP COUNTER

A common method for removing the contribution of the broad protein resonances is an NMR-edited pulse sequence such as CPMG. To reduce broad resonance from protein or other large molecules, the number of loops should be increased. This operation contributes also to the improvement of the baseline.



Box S6.6. Different factors affecting NMR spectra quality.

Table S6.5. Quality check parameters in NMR.

Signal to Noise Ratio (S/N)
Signal at 0.00 ppm (i.e. TSP)/Noise region between -0.50 and 0.80 ppm → S/N > 1000
Line-width at half height
For 2CLPYR5CA < 1.5 Hz (including 0 LB and fitting to Lorentzian)
Water suppression
If the residual signal of water is not sufficiently low, the O1 is reset and the spectrum is re-acquired

6.2.2. NMR Acquisition Parameters

The most important NMR acquisition parameters are found in **Table S6.6**. Examples of the sequences used for each type of biofluid are listed in **Table S6.7**.

Table S6.6 Acquisition parameters in NMR.

Key parameters
Number of scans The NMR experiment can be repeated many times on the same sample and each resulting FIDs can be accumulated, yielding an improved S/N ratio, linearly proportional to the square root of the number of scans. The time for each scan, generally about 5 s, is constituted by the sum of the pulse duration (a few microseconds), the acquisition, and the relaxation delay. The optimal number of scans depends on the following parameters
Field strength of the magnet The gain is a factor of 1.5 in S/N if a spectrometer of 600 MHz instead of 400 MHz is used). Cryoprobes provide a direct gain in the S/N ratio of a factor of approximately 5
Homogeneity of the magnetic field When non-optimal, broadened or distorted signals are obtained with loss in the S/N ratio and resolution
Concentration S/N is linearly proportional to the concentration at fixed volume
Relaxation delay For slowly relaxing nuclei, such as TMS, a long relaxation delay is required, i.e. at least 5× the T1
Acquisition Time If chosen too long, only noise is recorded and the S/N ratio decreases at constant experimental time
Additional parameters
Pulse width 3-10 μs – the shorter the pulse, the larger the excitation frequency windows
Number of points At least 32K complex points for high resolution
Spectral width At least 20 ppm centered on the solvent signal
Number of dummy scans Necessary to reach the spins steady-state before acquisition, minimizing the spectral distortions
Mixing time This time is optimized for a selective water suppression

Table S6.7. Examples of NMR acquisition parameters for three different biofluids.

Parameter	Urine	Serum	Serum	Feces
Pulse sequence	1D-NOESY-presat pulse sequence [-RD-90°-t-90°-tm-90°-ACQ]	CPMG spin-echo sequence [-RD-90x°-(t-180y°-t)L4-ACQ] (used to suppress the background signals from large molecules such as proteins)	1D-NOESY-presat pulse sequence [-RD-90°-t-90°-tm-90°-ACQ]	CPMG spin-echo sequence [-RD-90x°-(t-180y°-t)L4-ACQ] (used to suppress the background signals from large molecules such as proteins)
Number of time-domain data points	64k	32k	64k	32k
Spectral width	12 ppm	12 ppm	12 ppm	12 ppm
Number of scans per block	64	8	128	16
Number of blocks	1	32	1	16
Dummy scans	4	16	8	16
Acquisition time	2.72 s	2.28 s	4.0 s	2.27 s
Relaxation delay	4.0 s	4.0 s	2.0 s	5.0 s
Spin-echo delay (t)	200 µs	30 µs	0.5 ms	400 µs
Loop Counter (L4)		128 (used primarily for 2D experiments; does not usually need to be changed)		400 (used primarily for 2D experiments; does not usually need to be changed)
Mixing time	100 ms (the pre-saturation quality is not greatly influenced by this value)			
Total acquisition time	~4-5 min for 64 scans	~ 28 min 59 s for 256 scans	~13 min 47 s for 128 scans	~ 34m for 256 scans

6.2.3. Data Storage

FID (Free Induction Decay) data must be stored as is. Processed NMR spectra should be converted and stored in ASCII format

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