Chapter 4
Metabolomic Strategies Involving Mass Spectrometry Combined with Liquid and Gas Chromatography

Aline Soriano Lopes, Elisa Castañeda Santa Cruz, Alessandra Sussulini, and Aline Klassen

Abstract Amongst all omics sciences, there is no doubt that metabolomics is undergoing the most important growth in the last decade. The advances in analytical techniques and data analysis tools are the main factors that make possible the development and establishment of metabolomics as a significant research field in systems biology. As metabolomic analysis demands high sensitivity for detecting metabolites present in low concentrations in biological samples, high-resolution power for identifying the metabolites and wide dynamic range to detect metabolites with variable concentrations in complex matrices, mass spectrometry is being the most extensively used analytical technique for fulfilling these requirements. Mass spectrometry alone can be used in a metabolomic analysis; however, some issues such as ion suppression may difficultate the quantification/identification of metabolites with lower concentrations or some metabolite classes that do not ionise as well as others. The best choice is coupling separation techniques, such as gas or liquid chromatography, to mass spectrometry, in order to improve the sensitivity and resolution power of the analysis, besides obtaining extra information (retention time) that facilitates the identification of the metabolites, especially when considering untargeted metabolomic strategies. In this chapter, the main aspects of mass spectrometry (MS), liquid chromatography (LC) and gas chromatography (GC) are discussed, and recent clinical applications of LC-MS and GC-MS are also presented.

Keywords Metabolomics • Mass spectrometry • Liquid chromatography • Gas chromatography • Mass analysers

A.S. Lopes • A. Klassen (✉)
Department of Exact and Earth Sciences, Federal University of São Paulo (UNIFESP), Diadema, SP, Brazil
e-mail: aline.klassen@unifesp.br; aline.klassen@gmail.com

E.C.S. Cruz • A. Sussulini
Laboratory of Bioanalytics and Integrated Omics (LaBIOmics), University of Campinas (UNICAMP), Campinas, SP, Brazil

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Abbreviations

AMDIS	Automated Mass Spectra Deconvolution and Identification System
APCI	Atmospheric pressure chemical ionisation
APPI	Atmospheric pressure photoionisation
BSTFA	N,O-bis-(trimethylsilyl)-trifluoroacetamide
CE	Capillary electrophoresis
CI	Chemical ionisation
DB-5MS	Equivalent to a (5 %-phenyl)-methylpolysiloxane
DC	Direct current
DIMS	Direct infusion mass spectrometry
EI	Electron ionisation
ERHILIC	Electrostatic repulsion hydrophilic interaction chromatography
ESI	Electrospray ionisation
FTICR	Fourier transform ion cyclotron resonance
FTMS	Fourier transform mass spectrometry
FWHM	Full-width half-maximum
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrometry
GCxGC	Comprehensive two-dimensional gas chromatography
HILIC	Hydrophilic interaction liquid chromatography
IT	Ion trap
LC	Liquid chromatography
LSER	Linear solvation energy relationship
MS	Mass spectrometry
MSTFA	N-methyl-N-(trimethylsilyl)-trifluoroacetamide
MTBSTFA	N-methyl-N-tertbutyldimethylsilyltrifluoroacetamide
NIST	National Institute of Standards and Technology
NPLC	Normal-phase liquid chromatography
OT	Orbitrap
Q	Quadrupole
QIT	Quadrupole ion trap
QqQ	Triple quadrupole
QTOF	Quadrupole time of flight
RF	Radio frequency
RPLC	Reverse-phase liquid chromatography
RTL	Runtime library
SIM	Single-ion monitoring
SRM	Selected reaction monitoring
THF	Tetrahydrofuran
TOF	Time of flight
UPLC	Ultra-performance liquid chromatography

4.1 Introduction

Metabolomic analysis in biological systems has become more and more important nowadays in different research areas, including the search for biomarkers that can support the understanding of the aetiology and biological/molecular bases of complex diseases, such as diabetes, coronary heart disease and cancer [1].

Mass spectrometry (MS) is the most suitable analytical technique in metabolomics for clinical studies, and the use of this technique has been growing in the last decades. In the clinical area, biological fluids, such as urine, blood and saliva, are commonly the object of study used for metabolomic analysis. Those biofluids are complex samples, with variable compound concentrations; consequently, the direct injection of the samples in the mass spectrometer compromises the detection of many metabolites due to ion suppression. Thus, separation techniques, such as gas chromatography (GC) and liquid chromatography (LC), coupled to the mass spectrometer, are required. In this way, all compounds, previously separated by one of the techniques mentioned before, will be introduced one after another into the mass spectrometer, which facilitates the metabolite identification by retention time and using authentic standards, in addition to the structural information (mass-to-charge ratio and relative abundance of the molecular ion/fragments) obtained by mass spectrometry.

In this chapter, we discuss the main aspects of mass spectrometry, including the most widely used mass analysers in targeted or untargeted metabolomics, and also GC- and LC-hyphenated techniques, considering the proper MS ionisation techniques in each case.

4.2 Mass Spectrometry

Mass spectrometry (MS) is often the technique of choice in clinical metabolomics for identifying/quantifying different classes of metabolites. Briefly, MS consists in producing gas-phase ions that are further detected and characterised by their mass and charge [2].

A sample inlet, an ion source, a mass analyser and a detector compose a mass spectrometer. The sample inlet has the function of introducing the sample into the mass spectrometer, the ion source generates gas-phase ions via an ionisation technique, the mass analyser separates the ions according to their mass-to-charge ratio (m/z), and the detector generates an electric current from the incident ions that is proportional to their abundances [3].

As considered by this chapter, the sample inlet is a separation technique (either gas or liquid chromatography), and proper ionisation techniques will be discussed for each chromatography-MS coupling in the following sections. Of course, the sample can be also directly injected into a mass spectrometer, being this technique called direct infusion mass spectrometry (DIMS). However, the major drawbacks are ion suppression

effects, which cause an enormous loss of metabolite information, and the requirement of a high-resolution mass analyser, which increases the cost of the analysis.

Mass analysers can be used either alone or combined. This combination can be made between the same type of mass analyser or between different mass analysers (hybrid instruments) and is called tandem mass spectrometry (MS/MS). In MS/MS, the ions that arrive at the first mass analyser (precursor ions) are isolated, subsequently fragmented, and finally those fragment ions are separated according to their *m/z* in a second mass analyser and detected. For some types of mass analysers, the number of mass analysis steps can be increased, i.e. the fragment ions can be re-fragmented and further detected. In this case, the experiment is termed multiple-stage mass spectrometry (MS^n, where n refers to the number of mass analysis steps). Tandem mass spectrometry and multiple-stage mass spectrometry improve the identification of a molecule, because not only the molecular ions are detected but also the fragments generated from precursor ions.

The main performance characteristics of a mass analyser are [2–4]:

(a) Mass accuracy (accuracy of the measured *m/z* provided by the mass analyser, directly related to the mass resolving power and stability of the mass analyser)
(b) Mass resolving power (ability of a mass spectrometer to provide a specified value of mass resolution, i.e. generate distinct signals for two ions with a small *m/z* difference)
(c) Mass range (limits of *m/z* over which a mass spectrometer can detect ions or is operated to record a mass spectrum)
(d) Transmission efficiency (ratio of the number of ions reaching the detector and the number of ions leaving the mass analyser, related to the sensitivity of the mass spectrometer, i.e. the minimal concentration of a compound leading to a peak intensity greater than a specified signal-to-noise ratio)
(e) Scan speed (rate at which the analyser measures over a certain mass range)
(f) Scan cycle time (the time required to obtain a mass spectrum, also called duty cycle)

Mass analysers that can be used for LC-MS or GC-MS are the same. The particularity that guides the mass analyser choice is the type of metabolomic analysis to be performed, either targeted or untargeted.

4.2.1 Mass Analysers Used for Targeted Metabolomics

As previously described in Chap. 1, the goal of targeted metabolomics is to perform a quantitative analysis of specific metabolites (or a defined set of metabolites). The main features for a mass analyser to be used in targeted metabolomics are transmission efficiency, scan cycle time and scan speed. In this case, single quadrupole (Q), triple quadrupole (QqQ), quadrupole ion trap (QIT) and Orbitrap (OT) are the most employed mass analysers.

Single quadrupole (Q) is the simplest mass analyser, which operates either in the single-ion monitoring (SIM) or scan mode. For targeted metabolomics, the SIM

mode is preferred because it provides a significantly better sensitivity. In the SIM mode, the quadrupole parameters (RF and DC voltages) are adjusted to filter and select only one specific *m/z* [5].

Triple quadrupole (QqQ) instruments have the advantage over single quadrupole ones for being able to perform selected reaction monitoring (SRM) experiments. In the first mass analysis stage, a specific precursor ion is selected by the first quadrupole. Then, fragmentation occurs in the collision cell (second quadrupole or other multipole, operating at RF only) by collisions with an inert gas. Finally, specific fragment ions are monitored in the third quadrupole to increase both the sensitivity and the selectivity compared to the single quadrupole operating in the SIM mode [3, 5].

Quadrupole ion trap (QIT) has similar principles as the single quadrupole mass analyser, which uses an electric field applied in the electrodes for ion separation by mass-to-charge ratio. Once the ions, with certain *m/z*, enter into the electrode area, the applied field promotes the orbit of these ions. As the radio frequency is increased, the ions with higher *m/z* become more stabilised, whereas the ions with lower *m/z* become less stabilised and are not detected due to the collisions with the walls of the mass analyser [6]. This analyser is not suitable to be used in combination with UPLC due to its low sensitivity, once the injection volume is reduced in this chromatography type.

The most recently developed mass analyser, with high acquisition speed, is the Orbitrap (OT). It operates at acquisition rates of 12 Hz. The analyser operation is based on harmonic ion oscillations in electrostatic field. The ions around a central electrode are trapped, and the *m/z* values are measured from the frequency of ion oscillations. As the ions are tangentially introduced into a logarithmic electric DC field between these two electrodes, they start to oscillate radially around the wire and are eventually ejected at the ends of the trap. This device provides high mass resolution (>100,000 FWHM), high mass accuracy (2–5 ppm) and acceptable dynamic range (10^3). However, the scan speed is inversely related to mass resolution, i.e. one scan per second can be acquired when selecting 100,000 mass resolution; as a result, the reproduction of the correct chromatographic peak shape is affected. Whereas, when faster scanning is selected (10 scans s^{-1}), mass resolution is decreased (10,000 FWHM) [7]. Recently, a modification of the Orbitrap Elite™ instrument has provided a resolution above 1,000,000 at a transient length of 3 s [8, 9].

Thus, for targeted metabolomics, high sensitivity can be achieved with OT mass analyser operating at high acquisition speed. In addition, the high-resolution power helps to associate the fragment ions to precursor ions in complex mixtures.

4.2.2 Mass Analysers Used for Untargeted Metabolomics

Untargeted metabolomics consists in an exploratory analysis that aims to identify the entire set (or at least the majority) of metabolites contained in a biological sample, as previously described in Chap. 1. The main features for a mass analyser to be used in untargeted metabolomics are mass resolution power, mass range and mass accuracy. In this case, time of flight (TOF), quadrupole time of flight (QTOF),

Fourier transform ion cyclotron resonance (FTICR) and Orbitrap (OT) are the most employed mass analysers.

Time of flight and quadrupole time of flight are the most used mass analysers for untargeted metabolomics due to the data acquisition over a wide mass range with high mass accuracy and resolving power. Their performance involves the time measurement that ions take to travel from the beginning to the end of a field-free flight tube. Ions are accelerated in an electric field reaching a terminal linear velocity, which depends on their *m/z* ratio. About 10,000 consecutive scanning events per second at sampling rate of 50 Hz can be achieved with a mass error of 5 ppm, whereas a resolution of 40,000 at *m/z* 956 and maximum acquisition speed of 30 Hz can be achieved by the integration of ion mobility separation to TOF. QTOF mass analyser is distinguished of TOF by the possibility to integrate MS/MS at the same resolution of the precursor ion [9].

FTICR is a high-resolution mass analyser that employs cyclotron frequency in a fixed magnetic field for the determination of the ions *m/z*. The disadvantage of FTICR instruments is their relatively slow acquisition rates. At a scan rate of 1 Hz with mass resolution of 100,000 at *m/z* 4000, the number of points over the chromatographic peak, especially if additional MS/MS scans are required, is low when FTMS is combined with modern fast chromatography systems. This limits the application of FTICR in liquid chromatography mass spectrometry (LC-MS)- and capillary electrophoresis mass spectrometry (CE-MS)-based metabolomics [10].

About OT mass analysers, as its performance was already explained in Sect. 4.2.1, it is important to mention that an OT mass analyser allows untargeted metabolomic experiments due to the high resolution at high speed acquisition and mass accuracy (<5 ppm), which is much higher in comparison to a TOF mass analyser. According to the literature, OT has become a mainstream instrument for metabolomics, for providing more complete results by LC-OT in comparison to LC-FTICR and LC-TOF MS [9].

4.3 Liquid Chromatography Coupled to Mass Spectrometry in Clinical Metabolomics

Amongst all separation techniques that can be coupled to mass spectrometry in metabolomics, liquid chromatography (LC) is the most employed, mainly due to its versatility, i.e. the possibility of separating different classes of compounds, from very polar up to very non-polar compounds. This versatility is possibly owed to the many chromatographic columns with a variety of stationary phases available [11].

The separation in the chromatographic system depends, basically, on properties such as hydrophobicity, molecular size and polarity of the compounds. The separation of compounds occurs into a chromatographic column composed by a stationary phase with polar or non-polar properties. In chromatography using polar stationary phase columns, the solvent used to elute the compounds from the stationary phase

(mobile phase) presents higher polarity than the stationary phase, which is called normal-phase liquid chromatography (NPLC). However, in chromatography using non-polar stationary phase columns, the mobile phase presents lower polarity than the stationary phase, which is called reversed-phase liquid chromatography (RPLC). Then, non-polar compounds, such as lipids, elute first in NPLC, whereas polar compounds, such as amino acids, elute first in RPLC [12].

Clinical samples contain very polar compounds (amino acids) and also compounds with high hydrophobicity (phospholipids). Thus, the stationary phase can be chosen based on the compound classes of interest, if the aim of the study is targeted metabolomics. However, if the interest is to reach the most information as possible (untargeted metabolomics), more than one type of column is necessary [13]. Table 4.1 summarises LC-MS applications in clinical metabolomics, considering different column types.

Table 4.1 Applications of LC-MS in clinical metabolomics, considering the most used column types

Column	LC-MS system	Metabolites	Biological matrix	Reference(s)
C18	UPLC-QTOF MS	Amino acids, sugars, peptides, lipids, nitrogenous bases, organic acids, nucleotides, phospholipids	Human liver cancer cell line, mammary cancer cell line, breast cancer cell line	[14–17]
	UPLC-TOF MS, HPLC-QTOF MS, UPLC-QqQ MS	Amino acids, α-hidroxy esters, sterol lipids, sugars, organic acids	Diabetic mouse kidney and liver tissue	[18]
		Lipids, sugars, organic acids, amino acids, sphingolipids	Diabetic mouse plasma, rat plasma, human myocardial ischemia plasma, rat haemolytic and aplastic anaemia plasma	[18–22]
		Amino acids, lipids, organic acids	Mouse hair	[18]
		Organic acids	Rat faeces	[19]
	HPLC-FTICR MS, HPLC-QTOF MS, UPLC-QqQ MS, UPLC-QTOF MS	Nucleosides, amino acids, organic acids, nitrogenous bases, sphingolipids	Human bladder cancer urine, human urine, human type 2 diabetic urine, rat haemolytic and aplastic anaemia urine	[20, 22–24]
	UPLC-IT-FTICR MS, HPLC-TOF MS, HPLC-QTOF MS	Steroids, lipids, amino acids, dipeptides, glycerolipids, nitrogenous bases, organic acids	Human hepatitis disease serum, rat myocardial infarction serum, human oesophageal cancer serum	[25–27]

(continued)

Table 4.1 (continued)

Column	LC-MS system	Metabolites	Biological matrix	Reference(s)
C8	UPLC-QTOF MS	Lipids, amino acids, organic acids	Diabetic rat liver tissue	[28]
	HPLC-QTOF MS	Amino acids, organic acids, lipids	Human oesophageal cancer serum	[27]
	HPLC-TOF MS, HPLC-QTOF MS	Lipids, steroid lipids, glycophospholipids, sugars, amino acids, sphingolipids	Human plasma, rat diabetic plasma	[29, 30]
	HPLC-QTOF MS	Organic acids, sterol lipids	Human urine	[20]
HILIC	UPLC-QTOF MS, HPLC-QqQ MS	Sugars, amino acids, nucleosides, organic acids, nitrogenous bases, peptides	Human liver cancer cell line, mouse mammary tumour model, human pancreatic cancer cells, human colon cancer cells	[14, 31–33]
	HPLC-IT MS, UPLC-TOF MS, UPLC-QqQ MS	Organic acids, amino acids, nucleosides, amino sugars, sugars, nitrogenous bases	Rat urine, human urine	[34–37]
	UPLC-QqQ MS, HPLC-TOF MS, UPLC-QTOF MS, UPLC-OT MS, HPLC-QqQ MS	Amino acids, organic acids, phospholipids	Rat plasma, human plasma, human cardiovascular disease plasma	[21, 29, 38]
		Amino acids, lipids, glycerolipids, nitrogenous bases	Rat brain and liver tissue	[39, 40]

In order to obtain information of non-polar and weakly polar compounds, C18 and C8 are the most used columns, mainly due to their robustness, ease to handle, fast conditioning, versatility, the ability to cover a wide range of chemical classes and good performance for clinical untargeted metabolomics [41, 42]. However, for hydrophilic, ionic and polar compounds, which are poorly retained in C18 or C8 columns, or insufficiently charged to be retained by ion-exchange chromatography, hydrophilic interaction liquid chromatography (HILIC) is recommended. HILIC is similar to NPLC; the difference comes from the mobile phase, which is composed of polar and/or aprotic organic solvent miscible in water.

HILIC combines highly hydrophilic stationary phase, such as unmodified, chemically modified silica (amino, cyano, amide, diol, zwitterionic and/or polar polymer), with mobile phase, such as any polar organic solvents (acetonitrile, methanol,

isopropanol, etc.) and aprotic solvents (tetrahydrofuran, THF and dioxane) containing small amount of water (<5 %).

In comparison to RPLC, in HILIC the strong solvent is water; thus, the gradient starts with high percentage of organic solvent, and the separation begins after increasing the percentage of aqueous phase, ending with high percentage of this phase.

In metabolomics, polar compounds are preferentially determined by HILIC, mainly due to the mobile-phase compatibility to mass spectrometry, in comparison to NPLC, high-detection sensitivity and high amount of organic solvent in the mobile-phase composition, which allows desolvation in the mass spectrometer ionisation process. In addition, a low back pressure due to minor viscosity of organic solvent used in the organic phase and the possibility to inject the sample dissolved in organic solvents are advantageous [43]. However, a disadvantage in comparison to RPLC is low injection volume capability, which reduces the sensitivity and generates wider peaks, resulting in low-peak resolution. Applications of RPLC and HILIC in metabolomics field have been reported in the literature [44, 45].

About HILIC, the retention mechanisms are based by partitioning, hydrogen bonds and electrostatic interactions [46]. Partitioning phenomenon is considered as a liquid-liquid separation system because polar compounds are partitioned between two liquid phases, acetonitrile-rich phase and water-rich layer immobilised on the hydrophilic stationary phase, which is formed by strong water attraction by the polar groups present in the stationary phase. The lower the water concentration in mobile phase, the higher the water layer. For mobile phases with water at concentration lower than 20 %, an excess of water adsorbed at stationary phase is created; as a result, a multilayer is formed. At high concentrations of organic phase, only water layer is adsorbed (closer) to the stationary phase, because only molecules of water can interact with the residual silanols of the stationary phase. Therefore, the polar compounds are solubilised in the water layer; thus, the more hydrophilic compounds are, the more solubilised in the water layer, the more retained in the stationary phase. On the other hand, the elution of polar compounds happens at high concentrations of water due to the water layer reduction. A condition for the water layer formation is the presence of small amount of water (lower than 5 %) in the organic phase [42].

In addition to the partitioning mechanism, hydrogen bonds (H-bond) of the analyte with stationary phase are considered. This mechanism was related by LSER (linear solvation energy relationship) and proved that compounds that contain functional groups with hydrogen donor or hydrogen acceptor can interact with stationary phase by H-bond [47]. This separation mechanism is pronounced if low quantity of water is used in mobile phase.

Finally, electrostatic interactions of basic and acid compounds can be considered. The ionised residual silanols, present in the stationary phase, which were not eliminated by hydrogen bond or steric effects, can interact with basic compounds, such as occurs in ion-exchange mechanism, whereas acid compounds (e.g. acid phosphopeptides) can be retained due to hydrophilic interaction or electrostatic repulsion hydrophilic interaction chromatography (ERHLIC), generated when enough organic solvent is employed in a mobile phase [48].

The extension of each mechanism depends mainly on the kind of stationary and mobile phase. As explained above, the percentage of water in the organic phase affects directly the water layer formation; as a result, the partitioning mechanism is affected. However, the salt present in aqueous phase decreases the electrostatic attraction or repulsion. On the other hand, analyte retention occurs due to the water layer increasing, which is affected by presence of salt in mobile phase. In addition, the pH of the mobile phase influences the extension of retention because the pKa of the analytes are affected; thus, the charge of the analytes can be altered [42].

As the stationary phase is classified in neutral, charged and zwitterionic, the separation mechanism extension can be affected distinctly. Neutral stationary phase contains polar functional groups, which are uncharged at pH 3 up to 8; thus, in this case, hydrophilic interaction is the main separation mechanism [42]. However, if charged stationary phase is employed, which contains polar groups, and is dependent on the pH of the mobile phase, the extension of the separation mechanism is based on ion-exchange mechanism and hydrophilic partitioning [49].

Finally, the three separation mechanisms (partition, electrostatic and hydrogen bond) can occur in a zwitterionic stationary phase, mainly due to the presence of zwitterionic ligands (sulfobetaines) that have negative and positive charges. Many applications of HILIC have been presented in the literature and specific to the clinical field, as well as the use of RPLC and HILIC simultaneously [13, 50–53].

Currently, RPLC and HILIC columns with lower internal diameter (e.g, 1 mm) and lower length have been proposed to improve metabolite detection. Therefore, an introduction of instruments that are able to operate at very high pressure – ultra-performance liquid chromatography (UPLC) – coupled to mass spectrometry has been proposed to improve metabolite detection. This technology allows an increased resolution, better sensitivity and ion suppression reduction. As a result, more analytes are detected in a sample in comparison with conventional HPLC. In addition, lower solvent consumption is observed, due to the low flow rate (150–250 μL min^{-1}), which is possible because of the internal column diameter reduction (e.g. 1 mm). This kind of columns allows the same linear velocities obtained in conventional columns (i.d. 2.1 mm). The use of UPLC technology for biofluid analysis is increasing [54, 55].

In order to enhance the column lifetime, as to RPLC as to HILIC, sample preparation is necessary for clinical samples. The introduction of the samples without sample preparation can clog the chromatographic column and in the mass spectrometer can cause ion suppression and ion source deterioration because of the presence of salts in relatively high concentrations. The sample preparation method depends on the aim of the analysis. The main challenge in sample treatment for LC-MS in clinical applications is to isolate a compound and/or compound classes (targeted metabolomics) from the biofluids or the variety of concentrations of different classes (untargeted metabolomics). Different sample cleanup techniques for clinical applications, for targeted metabolomics, have been reviewed [56]. However, for untargeted analysis, as the interest is to obtain the most information as possible, only the protein precipitation procedure is recommended. More details about sample preparation for clinical metabolomics can be found in Chap. 2.

4.3.1 MS Ionisation Techniques Used in LC-MS

Mass spectrometry is considered a powerful technique to detect metabolites and has high sensitivity to quantify them, as mentioned in Sect. 4.2. Metabolites present in biofluids must be ionised before MS detection. Hence, an interface between LC and MS is necessary, and this is usually the ion source. Three ionisation techniques are commonly used in metabolomics: electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI) and atmospheric pressure photoionisation (APPI). Amongst them, ESI is the most widely used ionisation technique for untargeted metabolomics, mainly because ESI is considered a soft ionisation technique, e.g. generates ions with little or no fragmentation, which can help in the identification of unknown metabolites, and also for being able to ionise compounds at an extensive polarity range. This approach has been extensively implemented in clinical metabolomics [57, 58]. In addition, ESI requires no sample derivatisation, ionises a large mass range of compounds and is suitable for non-volatile and polar compound analyses with high sensitivity.

Electrospray is a process that creates or transfers intact ions from solution to gas phase at atmospheric pressure. A spray is induced after a high-voltage application on a capillary. Thus, charged droplets are formed and emerge from a Taylor cone (jet of charged particles formed by an electric field after voltage application) to the mass analyser after solvent evaporation, which release ions with multiple charges (z) to the gas phase, even though metabolomics has interest in $z = 1$.

Other ionisation techniques are employed for LC-MS systems in metabolomics, such as APCI and APPI. Similar to ESI, APCI and APPI are considered soft ionisation techniques. Both can be used in positive and negative modes and are employed for non-polar and thermally stable compounds (e.g. lipids), respectively.

Each ionisation technique is able to detect compounds with different polarities and mass ranges; therefore, complementary information is obtained if more than one ionisation technique is employed. In addition, to provide complementary metabolomic information, analyses in the positive and negative ionisation modes are required. Comparing ESI analysis alone, an increase of 20 % of detected metabolites in human blood was observed when APCI analysis was employed [59]. Some MS instruments can provide analysis in positive and negative modes and detect compounds with different polarities at the same mass spectrometry analysis [60].

4.4 Gas Chromatography Coupled to Mass Spectrometry in Clinical Metabolomics

Gas chromatography (GC) is a well-established analytical technique used routinely in metabolomics platforms when coupled with some types of mass spectrometers. This instrumentation is based on separation of volatile (or made more volatile by chemical derivatisation) and thermally stable metabolites. The chemical classes that

can be considered naturally volatile are [61]: ketones, aldehydes, alcohols, esters, furan and pyrrole derivatives, heterocyclic compounds, sulphides, some lipids, isocyanates, isothiocyanates and hydrocarbons with 1–12 carbons. The classes that can be made volatile by derivatisation are sugars, sugar phosphates, amino acids, lipids, peptides, long-chain alcohols, amines, amides, alkaloids, sugar alcohols and organic acids [61].

When the sample is injected in the GC instrument (usually a volume of 0.5–2 μL), the metabolites are volatilised immediately, and an inert gas (helium or nitrogen) carries the sample from a heated injection system (200–250 °C) to a coated capillary column. Capillary column coating is composed by a solid or liquid phase (called as stationary phase), where an inert gas flows through, carrying the metabolites. The capillary column is maintained within an oven, which has a fine temperature control. As the temperature increases, the compounds that have low boiling points elute from the column sooner than those that have higher boiling points. Columns with varying chemical composition of stationary phases have been utilised in clinical metabolomic analysis; however, DB-5MS (chemically bonded with 5 % diphenyl cross-linked 95 % dimethylpolysiloxane) columns or columns with equivalent stationary phase (HP-5MS and RTX-5MS) are more commonly used [62, 63]. The separation of metabolites occurs by a chemical interaction between the stationary phase (polarity) and the temperature (volatility). A result of this complex mechanism of separation when coupled to a mass spectrometer is high-resolution power and sensitivity. However, the main limitation of the use of gas chromatography is the derivatisation step, necessary for some metabolite classes. For many applications of GC, the metabolites are not naturally volatile, and it is necessary to add this sample preparation step, which is time consuming, low throughput and can be error prone, introducing variability and artefacts [64].

The derivatisation step in metabolomic studies is usually made by a two-step process, which encompasses oximation and silylation/chloroformate reagent. The oximation protects ketone functional groups from keto-enol tautomerism and decarboxylation and inhibits the ring formation of reducing sugars [65]. Derivatisation based on silylation is the most popular; nevertheless, reagents based on chloroformate are also used for clinical metabolites [66]. Derivatisation based on silylation includes the following agents: N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA), N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) and N-methyl-N-tertbutyldimethylsilyltrifluoroacetamide (MTBSTFA) that can react with nearly all polar functional groups, including –COOH, −OH, −NH and –SH, increasing the compound volatility by replacing the active hydrogen with an alkylsilyl group [65, 67]. Before the silylation step, it is necessary to check for complete dryness of the sample to avoid hydrolysis that is typical of these reagents, whose efficiency indeed depends upon the preservation of the anhydrous environment [66]. Xiong et al. [68] used the oximation-silylation (with BSTFA reagent) method to derivative metabolites in urine samples, focusing to discriminate patients with phenylketonuria. The authors detected, simultaneously, amino acids, organic acids, carbohydrates, amides and fatty acids. Begley et al. [69] also used the oximation-silylation method, but with MSTFA reagent for untargeted metabolomics of human serum samples.

Derivatisation based on chloroformate has the advantage of being conducted in aqueous media. Zheng et al. [70] quantified, simultaneously, short-chain fatty acids and branched-chain amino acids using propyl chloroformate reagent in complex biological samples, including faeces, plasma and urine, from animal and human subjects. Other derivatisation reagents can also be used for specific metabolites on targeted studies [71, 72].

After the separation by GC, the metabolites enter into the mass spectrometer for identification and/or quantification. For this purpose, the molecule (metabolite) needs to be ionised, in order to be further separated according to the *m/z* ratio, which is detected and converted into electronic signal. The production of charged metabolites separated by GC occurs by two forms: electron ionisation (EI) or chemical ionisation (CI). EI is performed in a high-vacuum ion source (10^{-7} to 10^{-5} mbar, 200–250 °C) where the gas-phase molecules are bombarded by a fixed electron voltage, typically −70 eV [73]. This electron bombarding gives the sample molecules excess of energy, and many fragment ions are formed. Fragmentation pattern is characteristic to a particular molecule and therefore can be useful in determining the structure of the analyte, which is easily compared to available databases [62]. However, some compounds fragment completely and do not provide the molecular ion; thus, CI can be utilised as an alternative ionisation technique for these specific metabolites. In CI, a gas (methane or ammonia) reacts with the metabolite resulting in a charged molecule. CI is a relatively softer ionisation technique, producing spectra with reduced fragmentation when compared to EI [62]; in conjunction with exact mass, it can aid in the confirmation or identification of metabolites and hence potential biomarker candidates [74]. EI is the most commonly used ionisation technique in GC-MS-based metabolomic studies, generating reproducible mass spectra with minimal instrument-to-instrument variations. CI can produce molecular ions for some volatile compounds that do not give molecular ions in EI. The main use of CI is to confirm the molecular mass of some compounds [74, 75].

After the fragmentation in the ion source, the fragments are separated in mass analysers and detected. The generated mass spectrum is characteristic for each molecule and can be compared with mass libraries available in databases together with the retention time (or retention index) reported from chromatograms. Data treatment involves computational tools used to validate the metabolite identification and can usually be made using software packages provided by the instrument's manufacturer or using free access softwares. Nowadays, there are many software packages available [76, 77]. In general, after analysis, data treatment includes data preprocessing, data processing, statistical analysis and validation.

Data preprocessing includes the following important steps: deconvolution, library-based identification and alignment [65]. Deconvolution is a very important step for an untargeted metabolomic study, extracting only valuable signals from a complex mixture of signals in the chromatogram, and, in addition, performs treatment of noise, correction for baseline drift and extraction of co-eluting components [65]. Amongst the softwares, the AMDIS (Automated Mass Spectra Deconvolution and Identification System) presents free access and is commonly used for this task by many research groups. Subsequently, metabolite identification by GC-MS is usually

made by library databases. The two most used libraries for GC-MS metabolite identification are Fiehn RTL library and NIST (National Institute of Standards and Technology) mass spectra database. Fiehn library has the advantage of including retention index and retention time information that can be compared with experiments performed following the same analytical method [78]. This additional information increases the reliability in the analysis and decreases false positive results. The availability of spectral libraries to metabolite identification is the main advantage of the GC-MS over LC-MS methods for clinical metabolomic studies. Therefore, few preprocessing software packages and available databases are used for both low- and high-resolution data. The study performed by Peralbo-Molina et al. [79] represents the difficulty of identifying compounds using databases: NIST database (used by the authors) does not contain high-resolution MS information as provided by the TOF analyser; thus, more steps in the data processing were necessary to validate the identification of each compound. Finally, alignment is needed for correcting retention time differences between chromatographic runs and matching data from different samples. This preprocessing is based on retention time and mass spectra similarity: compounds from different samples are compared together by computing a spectral score from their respective spectra [65, 77]. Some software packages are available to perform alignment alone or as consequential process of peak finding and deconvolution. Bioinformatics tools for GC-MS data preprocessing were compared recently on the literature [80].

After data preprocessing, it is necessary to explore the data and to remove any mystifying information, meanly for untargeted metabolomics [81]. This step is known as data processing, which includes discard of contaminants (derivatisation reagents, compounds from column bleeding, etc.), normalisation, scaling and transformation [65]. Finally, the statistical analysis (univariate or multivariate data analysis) is applied to the conclusion of the research goal.

The mass analysers that can be coupled to gas chromatography are single quadrupole (Q), triple quadrupole (QqQ), time of flight (TOF) and ion trap (IT). Recent GC-MS applications using these analysers can be seen in Table 4.2. The coupling between Orbitrap (OT) and GC was recently developed [106]; nevertheless, until the present moment, it was only applied for plant extract [107], and there are expectations that it will be applied to clinical metabolomics.

Although the new developments of fast and high-resolution power mass analysers coupled to GC, the tendency of this chromatography separation technique in metabolomics includes an increase in the use of comprehensive two-dimensional gas chromatography (GCxGC) using columns with different selectivities, thus enhancing the resolution power. GCxGC combining two columns with orthogonal separation characteristics yields a multiplicative increase in peak capacity [108]. A thermal- or pressure-based modulator is located between the columns to periodically focus the effluent from the first column and transfer it to the second column in small concentrated segments [109]. GCxGC has been applied to targeted and untargeted metabolomics in the clinical area and is preferentially coupled to TOF-MS [110, 111].

Table 4.2 Applications of GC-MS in clinical metabolomics

Column	GC-MS system	Metabolites	Biological matrix	Reference
DB-5MS	GC-QTOF MS	Organic acids, esters, alcohols, lipids	Exhaled breath condensate	[79]
DB-5	GC-TOF MS	Amino acids, esters, steroids, sugar, organic acids, sugar alcohols	Serum	[82]
VF-1 ms and HP-1[a]	GC-Q MS and GC-IT MS	Steroids	Urine	[83]
AT-5MS	GC-IT MS	Steroids	Plasma	[84]
DB-5MS	GC-QTOF MS	Alcohols, organic acids, esters, sugar alcohols, sugars, amino acids, piperidines, non-metal oxoanionic compounds, lipids	Human sweat	[85]
CP-SIL 8 CB	GC-Q MS	Organic acids, amino acids, sugars, nitrogenous bases, amides	Serum	[86]
Rtx5Sil-MS	GC-TOF MS	Amines, sugars, organic acids, amino acids, steroids, lipids, amino acids, non-metal oxoanionic compounds	Cerebrospinal fluid	[87]
DB-50[a]	GC-TOF MS	Untargeted analysis (no identification performed)	Plasma	[88]
Rtx5Sil-MS	GC-TOF MS	Sugars, amines, sugar alcohols, lipids, organic acids	Blood (plasma/serum)	[89]
Rxi-1 ms	GC-TOF MS	Amines, alcohols, nucleosides, organic acids, nitrogenous bases, sugars	Saliva	[90]
RTX-5Sil MS[a]	GC-TOF MS	Amino acids, organic acids, sugars, lipids, amino acids, nucleosides, amines	Breast tissue	[91]
ZB-5MS	GC-QqQ MS	Amino acids, organic acids, sugar alcohols, sugars, amines, alcohols	Urine	[92]
TR-5MS	GC-QqQ MS	Organic acids	Urine	[93]
DB-5 + DB-17	GCxGC-TOF MS	Organic acids, amino acids, lipids	Serum	[94]
BPX-5 + BPX-50	GCxGC-TOF MS	Organic acids	Urine	[95]
DB-5 + DB-1701	GCxGC-TOF MS	Sugars, lipids, non-metal oxoanionic compounds	Plasma	[96]
DB-5MS and HP-5	GC-Q MS and GC-TOF MS	Amino acids, organic acids, vitamins, sugars, steroids	Plasma	[97]

(continued)

Table 4.2 (continued)

Column	GC-MS system	Metabolites	Biological matrix	Reference
DB-5MS	GC-Q MS	Amino acids, organic acids, amines	Urine	[98]
HP-5MS	GC-Q MS	Amino acids, sugars, amines, nucleosides, inorganic acids, organic acids, alcohols, amides, heterocyclic compounds, steroids	Mucosal tissue	[99]
DB-50[a]	GC-TOF MS	Nucleosides, organic acids, sugar alcohols, alcohols, sugars	Serum	[100]
DB-5MS	GC-TOF MS	Lipids, organic acids derivatives, amino acids, amines, sugars, organic acids, sugars, sugar alcohols	Serum	[101]
VF-5 ms	GC-IT MS	Lipids, amino acids, sugars, sugar alcohols, organic acids derivatives, heterocyclic compounds	Plasma	[102]
DB-5[a]	GC-Q MS	Pyrimidine bases	Urine	[103]
DB-1MS[a]	GC-Q MS	Amino acids, organic acids	Urine	[104]
DB-5MS	GC-IT MS	Amino acids, organic acids, esters, amides	Urine	[105]

[a]Cited in a reference therein

4.5 Conclusions and Future Prospects

The use of chromatographic techniques coupled to mass spectrometry is a perfect combination for clinical metabolomics field. When combining efficient separation techniques with high sensitivity and/or high-resolution mass spectrometers, the quantification/identification of metabolites contained in complex biological samples can be successfully achieved for targeted and untargeted clinical metabolomics.

LC-MS and GC-MS are the most employed analytical platforms in clinical metabolomics, and the tendency is that they will remain with this status, since many improvements have been developed in the last decade, both in chromatography (new stationary phases for columns, two-dimensional separations, etc.) and in mass spectrometry (mass analysers with increased resolution power, nanospray ion sources, etc.).

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