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Chapter

Perspective Chapter:
High-Performance Liquid Chromatography Coupled to Mass Spectrometry – The Advance in Chemical Analysis

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Abstract

How these techniques coupled, high-performance liquid chromatography and mass spectrometry (LC-MS), revolutionized the field of chemical analysis. For a long time, the field of instrumental chemical analysis was limited, as we could not reliably quantify low-concentration substances in complex matrices. This is certainly one of the main advantages of coupling these techniques. In this perspective chapter, the advantages and limitations of the LC-MS system will be presented, sample preparation for this type of analysis and examples of applications that have had analytical success. It will also be mentioned the analytical advance in relation to miniaturization for lab-in-syringe, lab-on-valve, lab-on-a-chip, organ-on-a-chip, among others.

Keywords: quantitative analysis, LC-MS, mass spectrometry, chemical analysis, miniaturization of LC

1. Introduction

High-performance liquid chromatography (HPLC) methods have been developed for the determination of chemicals at low concentration levels in complex samples. When we go deeper into the analytical instrumentation, we arrive at the application of ultra liquid chromatography (UHPLC) which has had a growing tendency to use this separation technique, guaranteeing faster assays compared to HPLC [1–3].

Among the main detectors used in such applications, stands out the mass spectrometers [4–6]. The application of mass spectrometry in qualitative and quantitative analysis has stood out in recent years, in the identification of analytes in complex mixtures, and especially in the structural characterization of unknown compounds [5, 7]. Thus, mass spectrometry provides accurate information about the compounds of interest. Currently, tandem mass spectrometry (MS/MS) has become indispensable in the characterization of analytes, mainly due to its high sensitivity [7–9].
High Performance Liquid Chromatography - Recent Advances and Applications

The literature mentions the use of triple quadrupoles, time of flight (TOF), ion trap, and orbitrap (electrostatic with ion trap) as the main analyzers in applications [4, 5, 7]. The most used ionization methods are electrospray (ESI), atmospheric pressure photoionization (APPI) or atmospheric pressure chemical ionization (APCI) [4, 5]. Figure 1 shows a representation of the parts of a mass spectrometer.

When we talk about the analysis of chemical compounds in complex matrices such as residues, contaminants, adulterants, metabolites, and degradation products, there are limitations that range from the costly preparation of some samples to the difficulty of using the same configuration of the LC-MS/MS for the identification and quantification of several analytes in the same sample [10, 11]. In this progress of analytical science, the more powerful the instruments are, the more complex their operation is, demanding a high level of expertise on the part of the operator.

We also need to mention, inspiring analytical advances that were made in relation to even more efficient strategies, through hyphenated systems, miniaturization for lab-in-syringe, lab-on-valve, lab-on-a-chip, organ-on-a-chip, 3D printed technologies, artificial intelligence, among others [12–16]. In this chapter, the power of hyphenated techniques such as LC-MS in the study and routine application of different analytes in different types of samples and fields of application will be presented.

2. The union of high-performance liquid chromatography (HPLC) with the mass spectrometer (MS)

The current trend in HPLC is to reduce analysis time and increase sample throughput without sacrificing the selectivity factor. The first possibility is to shorten the column length along with reducing the particle diameter. Another important parameter is the temperature, as the separation at a high temperature can reduce the analysis time [1, 2].

HPLC in reversed-phase mode (mainly using C18 columns), often referred to as RPLC, plays a dominant role for the determination of macromolecules, while UHPLC, in the same chromatographic mode, reduces runtime and improves sensitivity [1–3, 17].

Compared with GC, HPLC has some disadvantages in chromatographic performance such as resolution. The recent introduction of UHPLC employs porous particles with internal diameters smaller than 2 μm which, in combination with MS,
results in higher peak capacity, better resolution, and higher sensitivity compared to conventional HPLC columns [3, 17].

In parallel, mass spectrometry (MS) is widely recognized as a powerful analytical tool that provides quality quantitative data that may not be readily available by other instrumental techniques [4–6], for example, molecular weight, empirical formula (through precise mass measurement), isotopic ratios, detection of functional groups and other substituents, and structure elucidation, including in some cases, stereochemistry [7–9]. Among other advantages of the LC-MS coupling, we mention the extremely high sensitivity and low sample consumption [4, 8, 9].

LC-MS applications generally employ reversed-phase separations; however, when hydrophilic interaction chromatography (HILIC) and ion pair chromatography (IPC) are applied, compounds of greater polarity can be accommodated, due to the limitations of RPLC [9, 18]. HILIC has an important unique benefit over normal phase LC (NPLC) in that aqueous samples have a much higher compatibility with the mobile phase, simplifying sample introduction. However, the load capacity of the HILIC is generally much less than that of the RPLC. HILIC also tends to generate wider peaks than RPLC, resulting in a lower selectivity factor and greater reliance on the mass spectrometer for peak resolution [4, 8, 9, 18].

With the development of mass spectrometry, a whole new field has opened up within analytical chemistry, providing new opportunities for the analysis of diverse substances. The data acquisition modes in the LC-MS/MS instruments made the chemical profile of a sample accessible, within the limitations of the instrument [8–10].

The main advantages of LC-MS are as follows: the ability to identify unknown compounds from complex matrices (i.e., food, environmental, biological samples); solve co-elution problems, since there are two identification parameters (retention time and m/z fragmentation), making it possible to select and separate the desired transitions even if the compounds are co-eluted in the chromatogram [2, 4, 6, 10].

In coupling LC with sequential mass spectrometry (LC-MS/MS), three atmospheric pressure ionization (API) techniques cover virtually the entire range of compound polarities, namely electro spray ionization (ESI), chemical ionization at atmospheric pressure (APCI), and atmospheric pressure photoionization (APPI) [5–7, 19]. Figure 2 shows the ionizations sources. However, a single form of ionization cannot cover all types of molecules: polar, neutral, ionic, and non-polar. Thus, the ionization mode can be chosen according to the acidic, neutral, or basic character of the analytes [19].

In some cases, the correct choice of ionization technique and polarity mode (positive or negative) is not so obvious. ESI ionization in positive mode, for example, is the most common mode in LC-MS/MS because it can effectively ionize a wide range of low and medium polarity molecules [4, 7, 19]. Negative ionization provides better results for certain analyte classes (e.g., organic acids or carbohydrates) [19]. APCI, on the other hand, is preferred for more non-polar analytes, for example [4–6, 19]. Thus, tests in all available ionization modes are very important as a first step in method optimization.

The characteristic ions are the same for all API techniques: the base peak of the positive ion mass spectrum in full scan mode is generally the [M+H]⁺ ion often accompanied by less abundant alkali metal adducts [M+Na]⁺ and [M+K]⁺ [5, 19, 20]. On the other hand, the negative ion mode normally shows the peak of the deprotonated molecule [M−H]⁻ as the base peak [20]. APPI more often shows the formation of M⁺ radical molecular ions, unlike the other API techniques. Rarely, adducts with HPLC mobile phase molecules are found as [M+H+solvent]⁺ in HPLC-MS/MS coupling [5, 20].
Recently, there has also been considerable interest in laser desorption ionization (LDI) techniques such as matrix-assisted laser desorption Ionization (MALDI) and surface-enhanced laser desorption ionization (SELDI). MALDI is the second most important ionization technique in complex applications, but its role in metabolite identification is not as comprehensive [20–22]. Several other ionization modes are constantly being proposed, including direct analysis in real time (DART), extractive electrospray ionization (EESI), laser ablation electrospray ionization (LAESI), and paper spray ionization, among others [21, 22].

The function of a mass analyzer is the separation of ions according to their different mass-to-charge ratios (m/z). The quality of mass separation is characterized by the degree to which close m/z values can be separated by the analyzer. Mass analyzers are classified into low- and high-resolution groups [6–9]. In general, low-resolution mass analyzers (e.g., quadrupoles and ion traps) have resolution in the many thousand range, while high-resolution analyzers should provide a minimum resolution of
15,000, for time-of-flight (TOF)-based analyzers, or more than 50,000, for Fourier transform mass analyzers (FTMS) [21, 22].

The advantage of ion traps is the possibility of studying fragmentation mechanisms [20, 22]. Some papers showed that the selectivity of the peaks and the number of tracer ions detected using the fast separations of UHPLC and TOF/MS were similar to those generated by the conventional methods of HPLC/MS with a reduction of 10 minutes in the analysis time [10, 22]. Fourier transform ion cyclotron resonance mass spectrometry (FTICR) provides the highest resolving power available currently. However, due to its complexity and high cost, FTICR is rarely used in conjunction with LC [10, 20–22].

Sophisticated HPLC or UHPLC systems associated with mass spectrometry (MS) exposed the difficulties associated with the analysis of new analytes in complex samples and analytes at very low concentrations (from nanogram to picogram) [6–9, 20, 21]. The basic incompatibility between LC and MS stems mainly from two factors.

First, the mass spectrometer requires ions in the gas phase at very low pressures compared to the pressures that are applied in HPLC. Another reason is that HPLC employs solutions often containing non-volatile solutes and buffers at atmospheric pressure, at flow rates of 1 mL/min. This flux typically corresponds to about 100
times the amount that can be accommodated by the vacuum system of the mass spectrometer [5, 7, 10].

The second point is that in LC, sample volatility is not an issue. Therefore, for the coupling between the techniques to become useful, the mass spectrometer must use an ionization technique that produces ions in the gaseous phase without necessarily requiring thermal vaporization of the sample [10]. During the last few years, several ionization techniques have been developed for MS providing optimal ionization of non-volatile molecules, such as those already mentioned [4–10, 20–22].

Given the limitations, one could question the role of the LC-MS technique (and its derivations such as HPLC, UHPLC, HILIC, MS/MS, etc.). Is it an attractive instrumentation that generates a multitude of data that no one can reproduce? Or is it a useful technology that will serve as a basis for routine and quality testing? Will it be (or perhaps already is) established as the main chemical analysis tool?

It is clear that LC-MS/MS is evolving very quickly (also based on remarkable technological advances and large investments by manufacturers). The fact is that mass spectrometry is taking the place of other technologies and it looks like it will eventually replace them in the different types of laboratories and fields of application of analytical chemistry. Figure 3 shows the schematic of LC-MS procedure.

3. Sample preparation and other analytical issues in LC-MS analysis

Despite the development of automated methods, much of sample preparation still depends on manual human effort, so that this step of the analytical process is the most error-prone, and the most time and resource-consuming in modern chemical analysis [10, 24].

Sample preparation is considered by many scientists to be the work of unskilled personnel, and, as a result, there is a tendency to apply rather outdated sample preparation methods or protocols and liquid-liquid extraction schemes that use large volumes of solvents [10, 24]. As a result, the adoption of new techniques becomes urgently needed.

The more extensive the sample preparation in a complex matrix, the more targeted and less comprehensive the analysis. But, of course, minimal sample preparation is always indicated, for representativeness of the compounds of interest, reducing matrix interference or co-elution, among other important requirements for LC-MS analyses [7, 24, 25].

For analysis of solid samples, for example, the majority only need to be solubilized to obtain a liquid solution to be submitted to the analytical system [10, 24]. When we talk about other types of samples, extraction procedures are employed, such as liquid-liquid extraction, solid phase extraction, solid phase microextraction, supercritical fluid extraction, and specific extraction using polymers [7, 10, 24, 25].

Overall, this depends a lot on the characteristics of that sample. For urine, normally centrifugation and dilution are sufficient [20, 22]. However, for blood-derived samples (e.g., plasma and serum), proteins must be precipitated using organic solvents to avoid compromising the integrity of the LC-MS system [11, 19, 20].

The application of the mass spectrometer does not mean that we will be able to analyze all the relevant compounds in a sample, since not all of them are ionizable [8, 24]. A portion of these compounds are always disadvantaged and discriminated against, even for comprehensive ionization strategies. The signal strength of the compounds varies depending on the matrix, sample type, and system performance [22, 24].
Derivatization is a chemical modification of the analyte(s) in the sample leading to the stabilization of the molecule(s) in the chromatographic analysis, reducing separation problems during the analytical run [5, 22]. When HPLC-MS analyses are used, great care must be taken and very well understood about the molecules of interest. Derivatization can lead to characteristic fragmentation applicable, for example, to distinguish positional isomers, when we talk about LC-MS [22].

Another common tool in LC-MS studies is the use of standards labeled with stable isotopes of the compounds of interest [22, 26]. However, not all classes of these compounds are commercially available in this form. Combining a labeled and unlabeled analyte leads to twin peaks with a known mass difference, which is useful for studying fragmentation pathways in tandem mass spectra (MS/MS) [5, 10, 26].

Many LC methods without MS detection often rely on the use of non-volatile buffers such as phosphate buffers and other inorganic additives. These non-volatile compounds cannot be recommended for HPLC-MS coupling due to possible contamination of the ion source and also due to ionic suppression (matrix effect) [12, 16, 22].

Inorganic acids in the mobile phase must be replaced by organic acids (formic or acetic acid) with typical concentrations below 0.1%, but sometimes higher [5, 8, 11]. For an alkaline environment, ammonium hydroxide in a similar concentration range can be used with alkaline pH extended stability columns for LC [10, 11]. Typical volatile salts in HPLC-MS/MS are ammonium acetate or formate, with a common concentration range of 2–10 mmol/L [5, 8, 10, 11].

4. Applications of the LC-MS/MS technique

That the LC-MS/MS technique has high resolving power, accuracy, and detection of analytes in complex samples, or at low concentrations, has already been discussed. In this part of the chapter, will be presented some outstanding applications in the area and its advances.

The first application cited for analysis by mass spectrometry is in the study of xenobiotic chemical compounds (those that can be harmful to human and animal health as a whole). These compounds may be present in drugs, industrial chemicals, pollutants, pesticides, pyrolytic products from cooked foods, secondary plant metabolites (polyphenols, flavonoids, glycosides, terpenes, steroids, alkaloids, and antibiotics) and toxins from bacteria, fungi, plants, and animals [1, 8, 9, 11, 17, 20, 22, 24–26].

As it is known, due to the complexity and diversity of metabolite properties, a single analytical technique cannot be applied to detect all thousands of metabolites in a biological sample [11, 17, 22, 24, 26]. However, the detection of these xenobiotic compounds by masses must be preceded by separation techniques such as HPLC, in the HPLC-MS/MS junction [1, 8, 9, 11, 17, 22, 24]. Thus, it is possible to perform the structural discrimination of positional isomers and stereoisomers based on different retention times [17, 20, 22, 24].

The metabolomics community realized that the application of hyphenated techniques is an appropriate strategy to analyze most of the detected metabolites that are in different ranges of polarity and molecular weight [22, 24–26].

The GC-MS technique is widely used for metabolomic studies and provides efficient and reproducible analyses [1, 8, 9, 11, 17, 20]. However, non-volatile compounds that do not derivatize and thermolabile compounds will not be observed in the
GC-MS analysis, which attracts even more interest for the addition of HPLC-MS/MS as an analytical tool, mainly for the analysis of human blood plasma [9, 11, 22, 24].

UHPLC-MS/MS-based metabolomics has also been applied to profile urinary metabolites for processes related to toxicity and pathogenesis, with tests in rats [11, 17, 22, 26]. It was realized that the introduction of UHPLC coupled to high-resolution mass spectrometers provides high analytical accuracy for the discovery and identification of target metabolites or biomarkers. However, the implementation of the technique still requires substantial experience and know-how, being limited to a very small number of routine clinical laboratories.

To further extend the coverage of the metabolome, APCI ionization has shown the potential to reveal some additional metabolites, which exhibit high ionization potential in this mode compared to ESI [1, 8, 9, 11, 17, 20]. Most global metabolite profiling studies are currently performed on TOF-MS machines due to the sensitivity, rapid data acquisition, and high mass accuracy they offer [8, 9, 11, 17, 20, 22].

The ability of the mass spectrometer to separate molecules based on their mass-to-charge ratio (m/z) has led many researchers to conclude that results can be obtained more quickly using direct introduction [i.e., direct infusion/direct insertion] and that the separation of molecules on the basis of their m/z is sufficient, especially when using very high-resolution mass spectrometers [1, 8, 20, 24–27]. As an example, the application of UHPLC-MS/MS resulted in the quantitative determination of >140 polar metabolites in a single injection of wine or fruit extracts [27].

When we look at environmental samples, with regard to the presence of pollutants in aquatic and terrestrial ecosystems, some compounds can spread through the environment contaminating living organisms, which may affect the health of these ecosystems [8, 9, 24, 25, 27]. In short, the HPLC-MS/MS technique has shown the best results in most common cases such as monitoring of drugs, cosmetics, and related preservatives [8, 9, 27].

These are just some of the cited applications for such a rich and important technique in the analysis of chemical compounds in different matrices and areas of study.

5. The future of analytics power

Currently, as applications based on miniaturized instrumental analyses cover several areas of research (toxicology, medicine, food safety and quality, environmental surveillance, among others), work aimed at the development of new technologies that make separations by nano-LC commonplace is also encouraged [12, 13, 16].

In summary, these automated systems work as follows: first, the sample is loaded onto the miniaturized extraction column in order to clean the sample, extract, and pre-concentrate the compounds of interest. After that, a valve is turned to the elution position, and the compounds are transferred to the miniaturized analytical column, promoting chromatographic separation and detection [12–15].

New approaches such as microchips are emerging as highly miniaturized column types, suitable for field or real-time analyses. This increase in the search for new stationary phases and analytical column technologies is helping miniaturized LC to become popular and to cover several fields of application of analytical chemistry [15, 16].

The main objectives of miniaturization are to increase portability, reliability, analysis speed, and jointly reduce costs, as well as simplify the chromatographic step making it more understandable for inexperienced operators [12–15]. The fact of
being able to gather most of the LC components in a micro-sized planar structure, combined with the reduced number of connections in the fluidic system, can mitigate problems of chromatographic peak broadening [11–14].

As is widely known, both capillary-LC and nano-LC have notable advantages when compared to standard HPLC, including increased sensitivity (decreasing LOD and LOQ values), as well as a decrease in the need for stationary phase, in solvent consumption and waste generation. Furthermore, recent investigations have focused on the development of miniaturized sample preparation methods to reduce the required sample volume [3, 12–16]. Figure 4 shows a miniaturized system of nano-LC.

Automation using an online coupling approach, integrating sample preparation and chromatographic separation, has been shown to avoid multi-step procedures and time-consuming analyses. For these reasons, combining the advantages of LC and sample preparation on a miniaturized scale emerges as an attractive approach to perform direct extraction, separation, detection, and quantification of target analytes [13, 14].

Despite the known benefits of chip-based LC, there are also several challenges to be overcome, such as simplifying procedures for making chips in the laboratory; process reproducibility; accuracy and precision in the injection as well as in the pumping system; in addition to coupling to sample detection or pre-treatment steps [3, 11–14].

It should be noted that such a reduction in the analytical work scale requires a high sensitivity detector suitable for working at low flow rates and lower concentration levels. And in this regard, the mass spectrometer (MS) is in practice the detector responsible for meeting the current sensitivity requirements, thus becoming an ideal detector for miniaturized LC [3, 11–16].

Currently, several different nano-ESI systems are commercially available, designed to improve ion emissions by creating constant flows from the sample through the miniaturized LC to the ion source [12, 13, 15, 16]. However, even with this successful coupling between the two techniques, the analysis of complex samples still requires more precise and selective results, which has driven the application of tandem mass spectrometry (MS/MS) at this juncture.

This “tandem” configuration results in higher levels of specificity [3, 11, 12]. Furthermore, reducing the flow rate to the order of microliters or nanoliters per minute results in the formation of sub-micrometer droplets. With this, there is an improvement in the ionization of the analytes and in the signal-to-noise response [15, 16].

Figure 4. Schematic of how a nano-LC system. Note that the system is made up of the main parts that a conventional HPLC has, such as sampler, column, and flow system (pump).
Despite the known advantages of mass spectrometry coupled to miniaturized LC, the complexity of their physical communication still represents an obstacle to the advancement of these systems. This obstacle occurs mainly because the LC works with a higher pressure in the liquid phase, while the MS requires a vacuum condition for proper operation [3, 11, 12, 15].

6. Conclusions

With everything that has been presented in this chapter, we can consider that even if we cannot obtain a configuration between LC, with regard to ion sources and mass analyzers, fully comprehensive for the different fields of application of chemical analysis, the hyphenated technique LC-MS/MS still represents the most promising way forward. On the one hand, selectivity of the analyte and reproducibility of separation by chromatography (HPLC, UHPLC, HILIC, etc.) participate, and on the other hand, we can generate embedded chemical information, confirming the identity of the analytes based on their molecular mass and specific ion fragments, by through tandem mass spectrometry. On miniaturization, the development and improvement of miniaturized or portable LC-MS systems can represent a significant achievement for the “in the field” analysis of forensic, environmental, food, medical, military and public interest exposures.

Conflict of interest

The authors declare no conflict of interest.
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