5
LC–MS Interfaces

Pierangela Palma, Elisabetta Pierini, and Achille Cappiello

5.1
Introduction

Liquid chromatography (LC) coupled to mass spectrometry (MS) is today a well-established analytical technique (LC–MS) that, in the last few decades, has opened the door to many challenging applications. MS is undoubtedly the most powerful detector that can exploit the separation capability of an LC column, and although coupling these two techniques was a demanding operation that required many efforts, now it can depend on reliable and rugged instrumentation that allows the determination of a large number of thermally labile molecules with quite different chemical properties.

From a historical point of view, the thermospray interface developed by Vestal and coworkers can be considered one of the first attempts of LC–MS techniques. It was based on a spray formation followed by solvent elimination using differential pumping stages [1]. More recently, the development of "soft" ionization techniques and the availability of efficient LC columns that operate at lower flow rates opened the way to a skyrocketing success of LC–MS. As a matter of fact, if we look carefully at the literature and the instruments available on the market, we can observe that most of the new benefits of LC–MS come from developments in each of the two coupled techniques, LC and MS, rather than from the interface itself. If we take into consideration the improvements in the chromatographic separations offered by ultra high-pressure liquid chromatography (UHPLC), solid-core packing materials, and nano-flow, chip technologies coupled to faster, more accurate, and sensitive MS analyzers, such as orbitrap, linear trap, and time-of-flight, we have an idea of the arsenal of today's LC–MS.

As opposed to GC–MS that relies on electron ionization (EI), LC–MS mostly utilizes atmospheric pressure ionization (API) techniques, a totally different approach that leads to radically different MS results. EI is a high-energy, gas-phase ionization technique that generates odd electron ions ($M^{+\bullet}$) and provides a reproducible fragmentation of molecules that can be recorded in electronic

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libraries for unparalleled identification purposes. Soft ionization techniques provide ions with low-energy transfer that typically results in a single-molecular ion $(M+H)^+$ or $(M-H)^-$, and several adduct ions. In-source fragmentation can be achieved through collision-induced dissociation (CID), but the quality and the reproducibility of the resulting mass spectra are not sufficient to create suitable electronic libraries. High-resolution or tandem (MS/MS) instruments are needed to compensate for the lack of mass spectral information.

The major issues in coupling LC with MS include both mobile phase and sample restrictions. The mobile phase can have a variable composition and carries the sample to the MS influencing, in many cases, the ionization of analytes. The physicochemical properties of the analytes, on the other hand, may be very different, imposing a huge demand in terms of system requirements. All these difficulties have stimulated the development of different approaches to satisfy the demand arising from many application fields that can benefit from LC–MS [2].

In this chapter, attention will be focused on the ionization process. The most commonly used interfaces and ion sources, as well as several new approaches, will be discussed [2–4]. Developments and improvements in the widely used API sources technology, including atmospheric pressure photoionization (APPI) and atmospheric pressure laser ionization (APLI), will be discussed. In addition, non-API sources, such as direct EI and supersonic molecular beams (SMB) LC–MS, will be taken into account, due to the growing attention gained in the analysis of low molecular weight compounds, including nonpolar molecules that are difficult to ionize with the two mostly used API sources, namely, electrospray interface (ESI) and atmospheric pressure chemical ionization (APCI).

5.2 API Sources

API ionization techniques are the driving force in LC–MS and are at the basis of the enormous success of LC–MS. To date, no other approach can compete with API interfaces for the number of instruments, applications, and presentation at conferences. They are all "soft" ionization techniques, which are widely used for the analysis of a large number of compounds in a wide range of molecular weights and polarities.

Among them, ESI ionization is the most widespread source, present in the majority of LC–MS systems, in many cases associated with APCI for the analysis of organic molecules in a wide range of polarities [4,5]. To a lesser extent, APPI and APLI sources are employed. The operational principle of API interfaces is the nebulization of a liquid phase into an atmospheric pressure ion source region, followed by the separation of ions from neutral molecules. Nebulization can be achieved by an electric field (ESI), which can be assisted pneumatically (ion spray), ultrasonically, or by heat (such as in APCI). Whatever ions are generated in the gas phase, they are sampled through an orifice that acts as a fixed restriction between the pressure region and the first sampling stage before

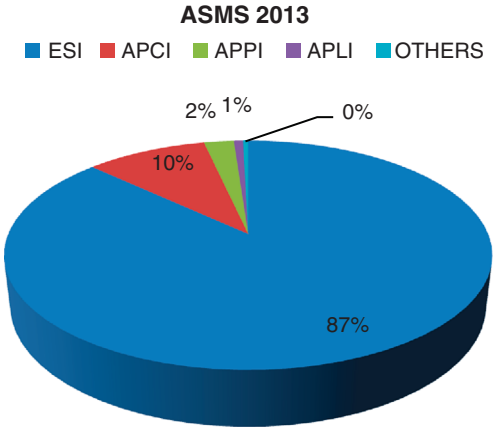

Figure 5.1 Orientative distribution of the different LC–MS interfaces in the abstract presented at the annual conference of the ASMS in 2013 (nonofficial data).

reaching the analyzer. Modern API instruments have become very much user-friendly, and this has contributed to their widespread diffusion.

API platforms have reached a solid level of maturity in terms of reliability and robustness so much so that most of the research is addressed to new applications (proteomics, cancer research, glycomics, metabolomics, and many others) rather than to technological improvements. In particular, the advent of ESI has revolutionized biological and biomedical research, opening the door to a great number of new research lines. In the abstracts presented at the annual conference of the American Society for Mass Spectrometry (ASMS) in 2013, approximately 87% of the presentations contained the word "ESI" or "electrospray" in their title, nearly 13% contained either the word "APCI" or "APPI" or "APLI," whereas only less than 1% of the presentations had the use of non-API sources (Figure 5.1, nonofficial data).

5.2.1
Electrospray Interface (ESI)

ESI was developed in the 1980s by John Fenn, who was awarded the Noble Prize for his studies on "soft desorption ionization methods for mass spectrometric analyses of biological macromolecules." ESI rapidly became the LC–MS interface everybody was waiting for, opening the door to a wide variety of applications for high- to medium-polarity compounds in an extended range of molecular weights, due to its high sensitivity and versatility. ESI technology has a dominant role in the LC–MS market; in fact, nearly all the LC–MS instruments are equipped with an ESI interface, which can be coupled to an APCI interface for the analysis of less polar compounds. As it can be seen in Figure 5.2, modern instrument configurations, due to improvements in both LC and MS, allow very high detection specificity and sensitivity [6].

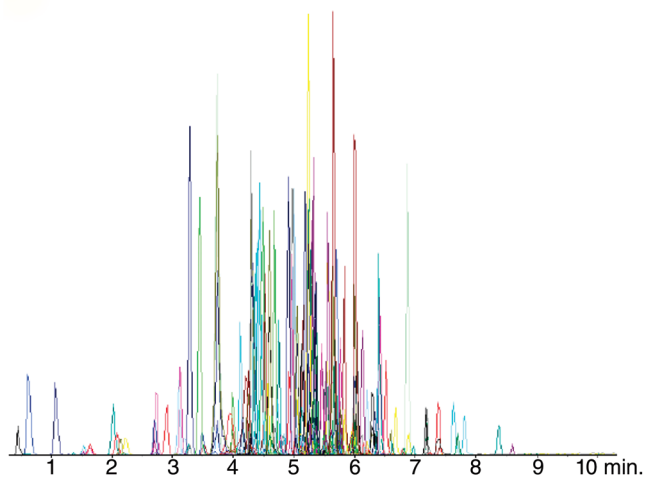

Figure 5.2 High-throughput UHPLC–ESI–MS/MS analysis of 125 pesticide residues. Column: Pinnacle DB Aqueous C_{18}; 50 mm × 2.1 mm ID; particle size: 1.9 µm; pore size: 140 Å; temperature: 35 °C; mobile phase A: 10 mM NH_4OAc in water; B: 10 mM NH_4OAc in methanol. Gradient: from 10 to 90% B in 10 min. Flow rate: 0.60 ml/min. (Reproduced with permission).

5.2.1.1 Principles of Operation and Ion Formation

As illustrated in Figure 5.3, ESI ions and molecules, already present in bulk, are carried by a mobile phase inside the interface and converted into ions in the gas phase at atmospheric pressure by the vaporization of the charged droplets of the solution [7–9]. Heat may be applied to compensate for the heat of vaporization of the solvent. When the mobile phase passes through metallic capillary tubing, a strong electrical field, of the order of 10^6 V/m, causes the formation of charged droplets. Under the effect of the voltage applied, the ions tend to move toward the surface of the liquid, and at a proper voltage, the solution forms a typical Taylor cone, from which a spray plume of charged droplets breaks out. A coaxial low gas flow is also used to keep the droplets in a limited space. As the droplets' size decreases, the ratio of surface charge to surface area increases. When the charge repulsion overcomes the surface tension, the Rayleigh stability limit is reached and, at that point, a cascade fission process begins [10,11]. A heated curtain gas or a heated capillary can support the evaporation of the solvent. Several theories have been proposed to describe the ion formation in ESI [12–14] that depends on many different mechanisms that may occur in the bulk solution, or during the formation of the charged droplets, or in the gas phase by protonation or deprotonation or salt adduct formation, or by an electrochemical redox reaction. Molecules with more than one protonation or deprotonation site can form multiple charged ions. It is a peculiarity of ESI to form ions such as $[M + nH]^{n+}$ and $[M - nH]^{n-}$. In this case, depending on the molecular mass, a

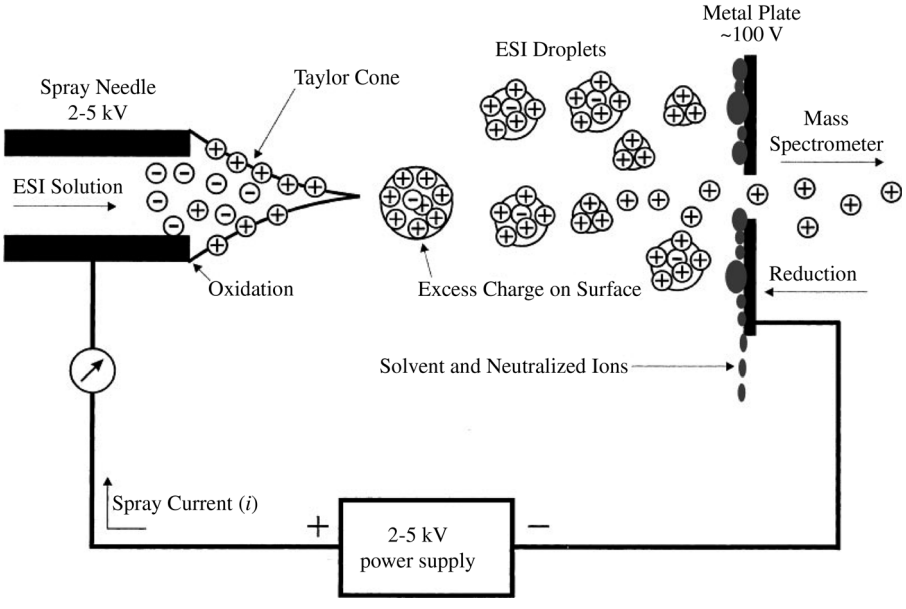

Figure 5.3 Schematic of the ESI nebulization process (reproduced with permission).

number of molecular ions are present in the spectrum at different m/z values. With appropriate software, it is possible to resolve all these signals in a deconvoluted mass spectrum to calculate the molecular mass of the analyte [5,7]. The possibility of forming multiple charged ions allows the detection of high molecular weight molecules with mass analyzers having a limited m/z scan range. The behavior of high molecular weight compounds, in particular protein and peptides, as well as many others, to form multiple charged ions has made this ionization method essential in biomedical investigations.

Adducts with various ions (Na^+, K^+, NH_4^+, Cl^-, and acetate) can be formed at different stages of the ESI process [15]. The adduct formation can be stimulated to promote the ionization of weakly basic or polar analytes, adding proper salts bearing the desired cations. Other adducts, such as noncovalently bound ones, can involve enzyme and substrate, protein and ligand, protein and protein, and antigen and antibody, can be studied using ESI–MS with the advantage of requiring a low amount of sample and a short time to provide structural information [14,17].

ESI is a soft ionization technique, which means that the ion fragmentation is limited; therefore, the mass spectrum provides information primarily on the molecular ion. As a consequence, tandem mass spectrometry (MS/MS) is mandatory to obtain the identification and structural elucidation of the analytes. ESI–MS^n is a basic technique in the identification and sequencing of high molecular weight molecules, such as proteins, by using bottom-up and/or top-down strategies.

5.2.1.2 Factors Influencing ESI Response

The response in ESI–MS strongly depends on the physico-chemical properties of the analytes; however, the signal can also be influenced by the presence of coeluted compounds or by changes in the mobile phase composition [11,18–21].

This phenomenon, peculiar to API, is commonly described as matrix effects (ME), and it is responsible for either the suppression or the enhancement of the signal. The many factors that contribute to ME in API have been described elsewhere, and they can occur either in the solution or in the gas phase, or both [22]. The ESI interface is more prone to ME among API interfaces because ionization reactions occur mainly in the liquid phase, wherein the concentration of interfering compounds is higher. ME greatly affects not only the ionization but also the vaporization process.

The consequence of ME is an unreliable quantitation of analytes, which can lead to erroneous results. While making the method setup, it is wise to prevent ME, for example, by using sample cleanup procedures to get rid of the matrix components. Enhancing the chromatography is also a good way to separate coeluted compounds from those of interest. Another approach to compensating for ME is dilution or the use of costly isotope-labeled internal standards (ISs), whenever available [23–26].

5.2.1.3 Modes of Operation

Depending on the nature of analytes, the ESI analysis can be performed either in a positive or in a negative ion mode. Although modern instruments allow switching modes during a single run, this operation can end up in spray instability; therefore, if possible, it is always preferable to run separate analyses of the same sample.

The typical mobile phase in ESI is primarily composed of polar solvents, such as water, methanol, and, to a lesser extent, acetonitrile with or without the addition of volatile buffers. Pure water as a mobile phase might decrease sensitivity because of its low vapor pressure. When adding a modifier, for example, for pH adjustment or ion pairing, it is mandatory to avoid the introduction of nonvolatile salts or compounds that can induce signal alteration [24].

The ESI technology can work at nano-flow and high flow rates, with the purpose of optimizing ionization efficiency, minimizing ME, and improving sensitivity, in both cases. In Table 5.1, ion sources working at different flow rates are

Table 5.1 Different ESI sources.

Source	Flow rate (μl/min)	Aerosol generation
Nano-ESI	0.01–0.1	Electrostatic
Micro-ESI	0.1–4	Electrostatic
ESI	1–10	Electrostatic
Ionspray	10–500	Pneumatic/electrostatic
Turboionspray	500–1000	Pneumatic/electrostatic

summarized. The capability of ESI to operate in a very wide range of liquid intake permits easy hyphenation with LC and capillary electrophoresis (CE).

Low flow rates (1–1000 nl/min) have the advantage to achieve higher sensitivity because the ionization efficiency is optimal. Nano-ESI is particularly useful in biochemistry and protein analysis where very small amounts of sample are available. Smaller inner diameter tips give a more efficient generation of gas phase ions and allow placement of the tip closer to the sampling orifice resulting in optimal sampling of ions [5–27]. However, nowadays the sensitivity has approached its theoretical limit with nano-ESI systems. As a consequence, future efforts are mainly aimed at enhancing the high-flow ESI technique, which has an ionization efficiency lower than the theoretical limit considering that most commercial LC instruments operate at high flow rates (1–1000 μl/min). At high flow rates, it is possible to inject large volumes, compensating for the minor absolute sensitivity, thus lowering the detection limits. At high flow rates, nebulization is often assisted pneumatically (ion spray), by heat (turboionspray), or ultrasonically with the aim of enhancing the efficiency and tolerance to mobile phase composition, reducing ME.

In the routine analysis of biological samples, source contamination from salts and nonvolatile compounds can occur. In this case, off-axis ESI geometries (with respect to the heated capillary) perform better than on-axis geometries.

Some splitting devices have been developed with the aim of reducing the MS input flow rates. Concentric nanosplitter can be considered an interesting example, which adopts a second tube, with a smaller inner diameter, coaxial to that exiting from the HPLC column developed by Vouros and coworkers. Part of the LC effluent enters into this smaller tube, whereas the rest of the mobile phase is split into a waste or directed to a second detector or to a fraction collector. This splitter device reduces the flow rates entering into the analyzer from 200–400 μl/min to 200 nl/min, avoiding turbulences and peak broadening that occur with splitting devices with T or Y configurations [28,29]. As a result, a significant increase in sensitivity is achieved, regardless of the removal of more than 99% of the sample.

5.2.2
Atmospheric Pressure Chemical Ionization

Chemical ionization in MS was first used in the 1960s by Munson and Field in a typical electron ionization source [30]. A controlled amount of reagent gas introduced into the ion source is ionized in the classical EI mode. The gas ions transfer their charge to the analytes' molecules via an acid–base reaction, generating an intense molecular ion and a limited fragmentation. This approach is helpful in the determination of compounds that tend to give a very weak molecular ion in EI. The first attempt of an atmospheric pressure chemical ionization source used a ^{63}Ni foil or, alternatively, a corona discharge needle as a primary source of electrons [31,32]. However, in those days, a suitable LC–MS instrumentation capable of accommodating an APCI source was not fully developed yet. It was

not before the end of the 1980 that this valuable approach could become commercially available, and today the APCI interface is essential in the analysis of low-polarity compounds for its sensitivity and robustness [5,33–35].

5.2.2.1 Principles of Operation and Ion Formation

APCI is a soft ionization technique and an LC–MS interface that operates in gas phase. APCI and ESI interfaces are hosted normally in the same instrumentation. A liquid effluent coming from the LC at flow rates between 0.2 and 2 ml/min is nebulized into a heated (350–600 °C) vaporization tube. The high temperature compensates for the latent heat of vaporization and does not spoil the analytes that remain at temperatures slightly higher than the ambient. A buffer gas (air, nitrogen) is also used at this point to favor the ionization. A schematic of an APCI source is reported in Figure 5.4. The gas–vapor mixture enters the atmospheric pressure region in which a corona discharge needle, kept at a high voltage (5–6 kV), is positioned. A beam of electrons is accelerated in this electric field accomplishing the task of ionizing the buffer gas, in most cases nitrogen, generating the so-called reagent or primary ions In positive ion mode mainly $N_2^{+\bullet}$, $O_2^{+\bullet}$, $H_2O^{+\bullet}$, and $NO^{+\bullet}$ ions are generated, whereas in negative ion mode $O_2^{-\bullet}$, $O^{-\bullet}$, $NO_2^{-\bullet}$, $NO_3^{-\bullet}$, $O_3^{-\bullet}$, and $CO_3^{-\bullet}$ ions are formed as shown in Figure 5.5 [36,37]. A series of reactions take place, involving the charge transfer from the primary ions to solvent molecules for the production of "reactant ions." These reactant ions transfer the charge to the analyte molecules that, depending on their proton affinity (PA), form either protonated or deprotonated molecular ions. The ionization process leads to $[M+H]^+$ ions' formation in the positive ion mode and $[M-H]^-$ ions' formation in the negative ion mode. The formation of adduct ions is less prevalent in APCI than in ESI; however, $[M\text{-}HCOO]^-$ or $[M\text{-}CH_3COO]^-$ ions may be observed.

During the APCI process, parallel reactions can occur, some of which can cause detrimental effects on the overall process. External components, such as impurities or additives of the mobile phase, can provoke ion suppression or may generate interfering adduct ions. These matrix effects are observed to a lesser

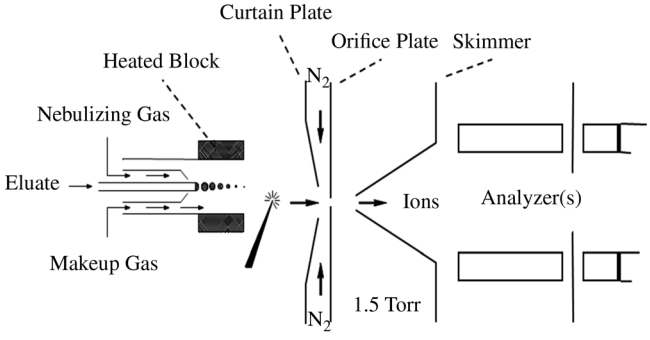

Figure 5.4 Schematic of the APCI interface (reproduced with permission).

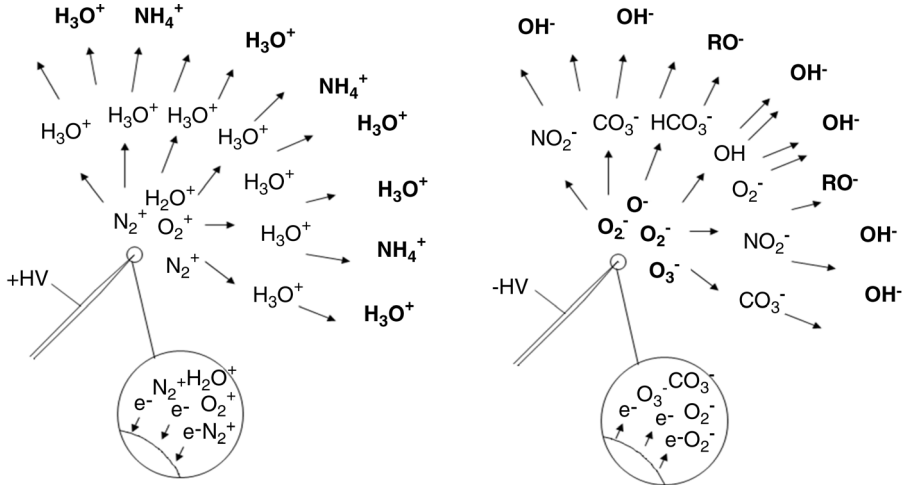

Figure 5.5 Ion formation in (a) positive and (b) negative ion mode in APCI (reproduced with permission).

extent in APCI than in ESI. Air components can enter the plasma as well, producing unwanted species that have an effect on the signal response.

The APCI interface is successfully used in the analysis of volatile and semivolatile molecules of low polarity that are neither strong acids nor strong bases, with a molecular weight up to 1500 u. Polar and nonpolar solvents can be used as mobile phase, and the pH does not have a significant influence on the ionization of the analytes. The ionization process produces a few fragments that do not provide structural information; therefore, multiple-stage MS is required for compound characterization. In contrast to ESI, APCI does not produce multiple charged ions. MS/MS can also be helpful to enhance the sensitivity and the selectivity, especially when dealing with trace determinations in complex matrices.

The APCI interface is used in a large number of applications due to its robustness and high sensitivity. In particular, it is widely used in pharmaceutical chemistry, pharmacology, biotechnology, biochemistry, food, and environmental analyses.

5.2.3
Atmospheric Pressure Photoionization

The principle of photoionization (PI) was already used a few decades ago in gas chromatography (GC) and LC, although only recently it has found a widespread use as an ionization method for MS. Atmospheric pressure photoionization (APPI) is a relatively new LC–MS interface, presented in 2000 for the analysis of nonpolar molecules that do not ionize with APCI. Like the other API interfaces, it is a soft ionization technique [38–45]. Ions are generated from the interaction of a molecule with a photon in an ion source with a design very similar to that of

an APCI source. Combined APCI/APPI sources are available on the market and allow one to operate in either one or both modes. In APPI, a vacuum UV (VUV) lamp is used for the ionization of the molecules in the gas phase.

5.2.3.1 Principle of Operation

In Figure 5.6, a schematic of the interface is shown in comparison with an APCI source [45]. A kripton VUV lamp emits photons at 123.9 nm and 116.5 nm with energies of 10.03 and 10.64 eV, respectively. This energy is not sufficient to ionize the major components of air and most solvents. The radiation beam can be placed orthogonally or in-line with respect to the MS ion path, depending on the manufacturer [40–42,45].

The molecules (M) absorb the UV photons leading to electronically excited species; if the photons' energy ($E = h\nu$) exceeds the ionization energy (IE) of the analyte, it is ionized forming a radical molecular ion (primary ionization).

$$M + h\nu \rightarrow M^* \tag{5.1}$$

$$M^* \rightarrow M^{\bullet +} + e^- \tag{5.2}$$

However, solvent molecules, present at higher percentage, can also undergo the same primary process depleting the emitted photons [43]. Therefore, direct ionization is not so efficient to be competitive with other API sources. Moreover, at atmospheric pressure, the ion's free pathway being very short, it is very likely

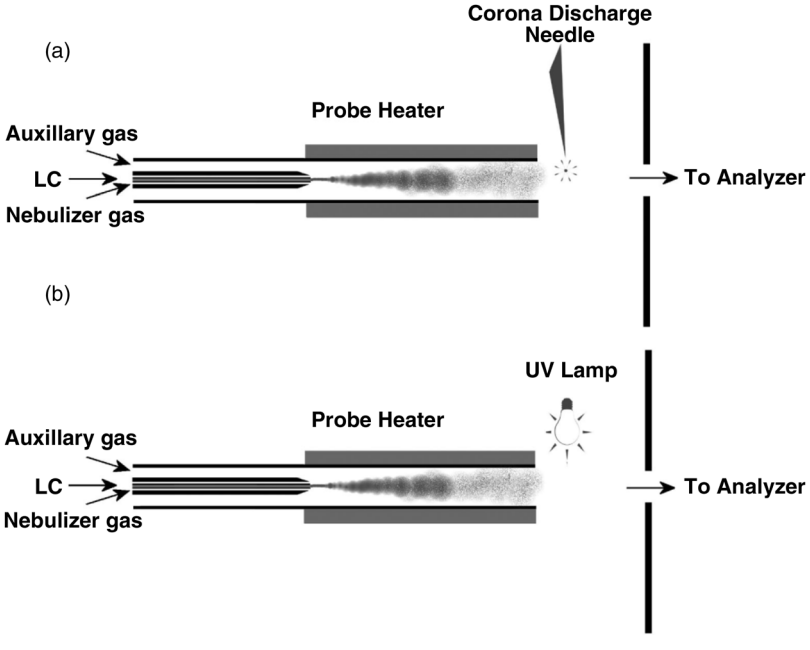

Figure 5.6 (a,b) Schematic of APPI source in comparison with an APCI one (reproduced with permission).

that radical molecular ions undergo collisional reactions. As a consequence, secondary reactions can occur. The main mechanisms involved in a secondary ionization are charge exchange, electron capture (EC), and proton transfer.

Other reactions are also possible; when the IE > $h\nu$, M^* may undergo a deexcitation process, such as photodissociation, photon emission, or collisional quenching with a nonexcited molecule. All these reactions are undesirable.

Essentially, it was demonstrated that the ionization efficiency can greatly be improved (two to three orders of magnitude) when a photoionizable compound (dopant) is present in the LC effluent that undergoes primary ionization and promotes the analyte ionization exploiting secondary ionization pathways [44]. The dopant acts as an intermediate between photons and analytes. The dopant forms radical molecular ions with high recombination energy and/or a low PA. The use of the dopant (D) enhancing gas-phase reactions allows to obtain a higher yield of certain species and is necessary to achieve higher sensitivities. The ionization mechanism depends on the PA values of the molecules involved (dopant, solvent, and analyte) and on their capacity to capture an electron in the gas phase, called electron affinity (EA) [40–42]. Charge exchange is the most important mechanism for apolar and low-polarity compounds. Proton transfer is common for acidic or basic compounds.

$$D^{\bullet+} + M \rightarrow D + M^{\bullet+} \quad \text{if } EA_D > EA_M \quad \text{(charge exchange)} \tag{5.3}$$

$$D^{\bullet+} + M \rightarrow [D - H]^{\bullet}[M + H]^{+} \quad \text{if } PA_M > PA_{[D\text{-}H]^{\bullet}} \quad \text{(proton transfer)} \tag{5.4}$$

In the negative ion mode, the ionization process is initiated by the photoionization of the dopant according to reaction (5.2). If electron affinitive species are present in the source, such as oxygen, for example, they can capture the low-energy electrons, as in reaction (5.5).

$$O_2 + e^{-} \rightarrow {O_2}^{-\bullet} \quad \text{electron capture} \tag{5.5}$$

The superoxide ${O_2}^{-\bullet}$ ion can promote charge exchange (reaction (5.6)), proton transfer (reaction (5.7)), or substitution reactions (reaction (5.8)) with analytes or solvent molecules.

$$M + {O_2}^{-\bullet} \rightarrow M^{-\bullet} + O_2 \quad \text{if } EA(M) > EA(O_2) = 0.451 \text{ eV} \tag{5.6}$$

$$M + {O_2}^{-\bullet} \rightarrow [M - H]^{-} + HO_2^{\bullet} \tag{5.7}$$

$$M + {O_2}^{-\bullet} \rightarrow [M - H + O]^{-} + OH^{\bullet} \tag{5.8}$$

In any case, the reactions must be addressed to obtain a high yield of a single species choosing the proper solvent and dopant. Commonly used dopants are benzene (it leads mainly to protonated analytes), acetone, toluene, anisole, tetrahydrofuran, chlorobenzene, bromobenzene, and hexafluorobenzene. However, it is necessary to have a good knowledge of the chemistry involved in the gas-phase reactions to control the ionization process and to obtain the expected results. The sensitivity strongly depends upon the method conditions and the operator

ability. An expert operator can achieve detection limits in the low nmol/l range [41,43–50].

For reversed phase (RP) analysis, the combination water/methanol yields a higher sensitivity than water/acetonitrile, due to the higher PA of acetonitrile [47].

Typical normal-phase (NP) mobile phases can be added to nonpolar solvents with low PA. Toluene, as well as acetone, is often used as a dopant [48,49]. The addition of weak acids may have a negative influence on the ionization process in negative ion mode. Flow rate has the main role in the ionization efficiency by proton transfer. Generally, high flow rates are reported to reduce ionization efficiency [50].

Recently, Robb and Blades proposed an improved PI source, an atmospheric pressure electron capture dissociation (APECD) source, for the analysis of polar, nonvolatile, and thermally labile compounds, such as peptide and protein at subpicomolar concentrations, obtaining performances comparable to ESI [51]. This source greatly extends the range of compounds analyzable with APPI, which can become an a alternative technique to ESI. The apparatus consists of a commercial APPI source equipped with an electropneumatic heated nebulizer obtained by electrifying the sprayer, using an external high-voltage power supply. The results are interesting; in fact, the response increased by two-order magnitude [40,51]. A future development of the technique could be the study of a nano-spray model, which might give better performances.

5.2.4
Atmospheric Pressure Laser Ionization

APLI has recently been introduced in the realm of API sources as an alternative or complementary ionization for APCI and APPI with a high specificity in the analysis of small, nonpolar molecules, in particular aromatic compounds. This method employs laser beams to promote the ionization of the analytes [52–54]. It is a soft ionization method, and modulating the laser power density can induce fragmentation.

5.2.4.1 Principle of Operation and Ion Formation

In Figure 5.7, a schematic of the APLI interface is illustrated [55]. Ionization is promoted at atmospheric pressure by a pulsed fixed frequency laser beam placed in front of the MS sampling orifice. The position of the laser beam does not seem to be crucial. The corona discharge needle is disconnected, and a field gradient of 50–200 V/cm is used to improve ion transmission.

Resonantly enhanced multiphoton ionization (REMPI) at atmospheric pressure is the process that governs the ion formation through very well-described reactions [56–60].

The adsorption of the radiation leads to the formation of the excited molecule and to loss of an electron, with the formation of a radical molecular ion, $M^{+\bullet}$. This reaction is unlikely to have any efficiency to be useful in analytical

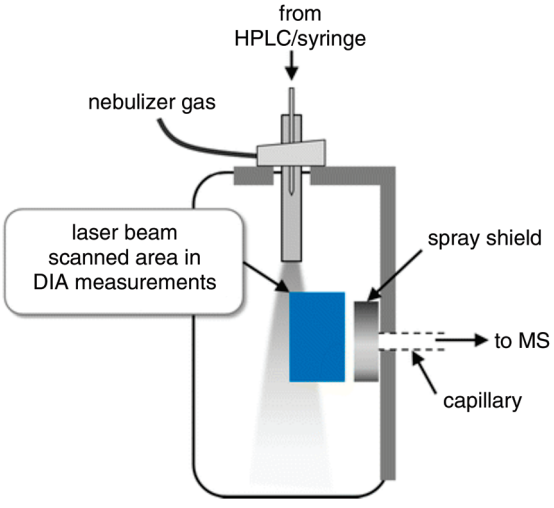

Figure 5.7 Schematic of APLI source (reproduced with permission).

applications. Due to laser pulses, further absorption of photons can lead to the fragmentation of the precursor ion. Photodissociation can occur; however, it does not provide ions in sufficient numbers. When an additional light source is applied, an intersystem crossing (ISC) phenomenon can deactivate resonantly excited states and promote ionization.

Mobile phase and solvent molecules do not interfere in the ionization process because they do not absorb at the UV wavelengths used.

With APLI, instead of APPI, even without dopants, an efficient formation of radical cations is observed in most cases. In addition, the sensitivity for nonpolar to very low-polarity aromatic or highly conjugated compounds is up to three orders of magnitude higher compared to APPI [52]. At present, APLI outperforms all other API techniques for the analysis of polycyclic aromatic hydrocarbons (PAH) in terms of sensitivity and detection limits. However, its use is limited to some specific applications, mainly concerning PAH.

5.3 Non-API Sources

All API sources are soft ionization-based interfaces and therefore are not very informative, because they produce poor fragmentation or none at all. To get more structural information and to increase the identification capability, two strategies are generally adopted.

The first consists in causing the fragmentation by collisional-induced dissociation (CID) with argon or helium molecules with adjustable kinetic energy after the ionization process. To this aim, two analyzers (MS/MS) and a collision cell are

required, increasing the analysis cost. Furthermore, CID is not very reproducible and the mass spectra cannot be collected into reliable libraries. The second highly expensive strategy is to use high-resolution analyzers such as Orbitrap or TOF for exact mass measurements. In this case, a number of possible molecular formulae, depending on mass accuracy, are obtained for the analyte identification.

In addition, at atmospheric pressure, the ion's mean free pathway is just 65 nm. As a consequence, the probability of collisions with molecules present in the gas phase, solvent, for example, is extremely high and many reactions can occur at the same time. An expert operator must govern these reactions accurately to get the desired ionization yield. In ESI, the matrix could also affect vaporization and ion evaporation leading to worse performances in quantitative analysis [7,8,54].

The setting up of the analytical method with an API source and the interpretation of multicharged spectra, when present (in ESI or ESI–APPI interface), could be difficult and time-consuming. For all these reasons, a few groups are working to realize a suitable non-API interface aimed at overcoming all the listed drawbacks, with challenging results. Predecessors of these interfaces could be considered the particle beam and the thermospray interfaces, based on different physical properties, used to remove the solvent from the LC effluent before entering the MS [1,61].

5.3.1 Direct-EI

Electron ionization is a well-known ionization technique, widely used in hyphenated GC–MS systems. It offers plenty of fragmentation for an unparalleled identification capacity. In the past, many authors made considerable efforts to generate EI spectra from LC effluent. However, with the advent of ESI, researchers' interest was completely drawn to exploit the potential of the new interface, which opened the way to new applications in many fields, particularly of biological interest. However, soft ionization techniques are less informative than EI and do not completely fill the gap toward the detection of small nonpolar molecules.

For these reasons, further attempts at LC–EI–MS coupling were done by Cappiello *et al.* who realized a microparticle beam interface. This interface was still an external interface between a high-vacuum source and the atmospheric pressure liquid phase, necessary to evaporate and eliminate the solvent before entering the ion source, without hampering the analyte access. The interface could cause contamination, analyte losses, poor linearity, and reproducibility, especially at a high concentration of water in the mobile phase. Thus, the particle beam interface resulted in being less attractive than the GC–EI–MS interface and not competitive with the new API sources for LC. In recent years, due to the advent of nano-LC technology, a new, more challenging interface was realized by Cappiello *et al.*, which introduced all the column effluents directly into the EI source, avoiding sample loss, contaminations, and irreproducibility. Because of its straightforward design, the interface was called direct Direct-EI.

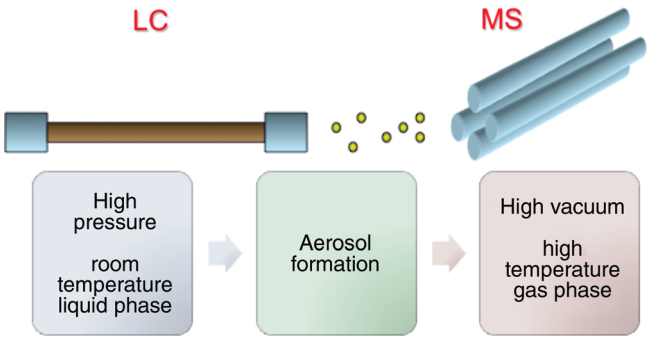

Figure 5.8 Schematic of the Direct-EI interface and mechanism of ion formation.

Nano-LC columns work at submicroliter/min flow rates. The LC effluent enters directly into the EI source through the new interface, and solvent evaporation occurs inside the source in which the solvent excess is eliminated by diffusive or turbomolecular pumps, which are similar to those used in a commercial GC–MS system. In fact, a submicroliter/min flow rate produces a submilliter/min vapor rate, which is within the pumping capacity of most MS systems. The lowest flow rate able to generate a fine aerosol is 100 nl/min. The system works at flow rates ranging from 100 to 900 nl/min. At higher flow rates (more than 1 µl/min), chemical ionization activated by solvent vapors can occur, and the protonated molecule abundance increases. It is worth noting that the interface is concentration sensitive so that higher flow rates imply lower signal intensity. Therefore, flow rates higher than 900 nl/min imply not only chemical ionization but also a reduced response.

Commercial GC–MS can easily be adapted to work as a nano-LC–MS system. The new interfacing apparatus, schematized in Figure 5.8, is entirely held into the source. This internal interface insulates the LC effluent, which must enter the source as a liquid phase to prevent premature in-tube solvent evaporation and clogging. Analytes enter the MS source through a capillary tubing (25-µm internal diameter (ID)) that protrudes a few millimeters inside the source. The ion source temperature must range from 250 to 350 °C. EI differs from other ionization techniques in that it produces a hard ionization, it operates under high-vacuum and high-temperature conditions, and it is based on a physical ionization process. In all API sources, ionization is due, to some extent, to a chemical reaction so that coeluted species, such as a matrix or a solvent, can strongly interfere with analyte ionization changing the reaction yield. EI ionization is, on the opposite, a physical process; the interaction with 70-eV energy electrons released from a tungsten filament produces a radical molecular ion that undergoes abundant fragmentation inside the source (caused by a surplus of vibrational energy that exceeds bondage energy), without interfering with the coeluted substances. In fact, under high-vacuum conditions, ion–molecule collisions (and reactions) are not likely to occur. As a consequence, signal intensity is

related only to the concentration of each analyte and ionization is unaffected by chemical reactions with the matrix (or to a very low extent). The coeluted compounds are ionized independent of one another.

Fragmentation is highly reproducible and generates high-quality and informative spectra, which can be compared with those present in reliable and commercially available libraries (National Institute of Standard and Technology (NIST), Wiley). NIST and Spectral Deconvolution and Identification System (AMDIS) also developed algorithms, which extract the single-analyte mass spectrum in the case of unresolved chromatographic peaks [62,63].

Solvent ions are present in the low mass range of the spectrum recorded; however, they can be eliminated by background subtraction, an operation commonly used in the GC–MS analysis also to get a better quality spectrum [64,65].

Indeed, the new interface can work like a GC–MS interface enlarging the range of compounds that can be analyzed to thermolabile, polar, and nonpolar molecules with low molecular weight (approximately up to 500 u), as shown in Figure 5.9, in which 11 compounds (9–19) are not suitable for GC–MS analysis, whereas the first group of substances is not amenable to ESI detection [66].

Therefore, with a low-cost LC–MS instrumentation, without the use of a high-resolution MS, a highly informative or highly selective analysis can be performed for a very wide range of small-molecule applications. The direct EI interface is compatible with volatile buffers, acids, and modifiers and shows an increased tolerance to nonvolatile buffers. This characteristic widens the choice of suitable chromatographic methods, improving *de facto* the LC–MS application potential.

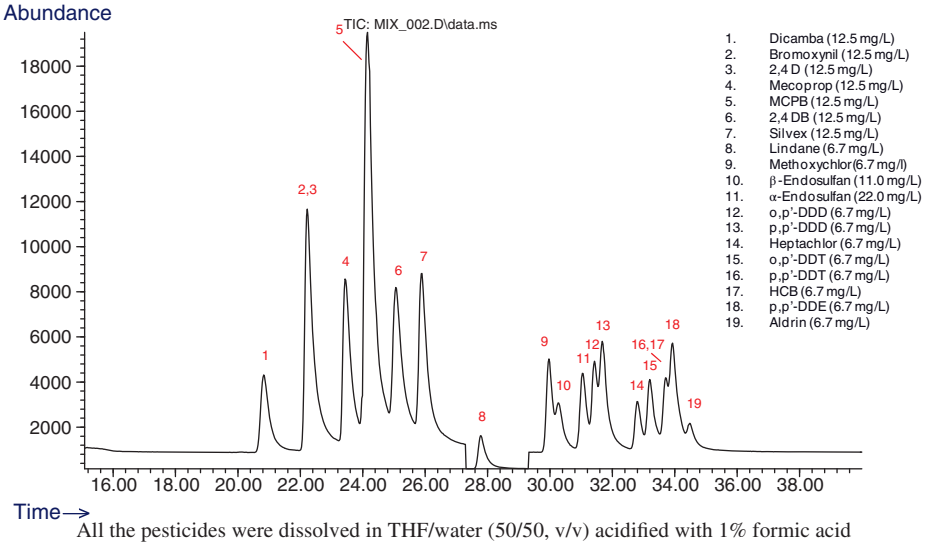

Figure 5.9 HPLC–EI–MS separation of a mixture of pesticides. Compounds from 9 to 19 are not suitable for GC–MS analysis.

Moreover, the technique is easy to use and easy to automatize with respect to API techniques and does not require an expert operator.

Direct-EI allows an accurate quantitative detection giving up to four to five orders of magnitude of linear response. An improvement in LODs, hitherto in the order of picograms in SIM mode, is expected by interfacing the direct EI to a triple quadrupole operating in selected reaction monitoring (SRM) mode.

5.3.2
EI of Cold Molecules in Supersonic Molecular Beam (SMB)

The SMB approach proposed by Amirav and coworkers can be considered an improvement upon the particle beam technology, focused on achieving an EI spectrum with an enhanced molecular ion so that the identification of the analytes is improved. The radical molecular ion intensity is enhanced preserving the typical EI library searchable fragmentation, which is the main advantage of EI.

The schematic of the apparatus is reported in Figure 5.10.

The vaporization is realized in two steps. In the first step, the liquid effluent is heated by a thermospray system, as in an APCI interface. In the second step, the sample expands from the supersonic nozzle. The LC effluent is vaporized at about 1–2 bar inside a glass tube, connected to a supersonic nozzle by a short fused silica capillary transfer line. The capillary impedes the flow, leading to an effective high-pressure sample vaporization combined with 0.1 bar low pressure behind a 300-μm supersonic nozzle to suppress cluster formation during the supersonic expansion, yet obtaining an efficient vibrational cooling. Sample vaporization is obtained by the pneumatically assisted spray formation followed by a fast thermal vaporization of the analytes before their expansion from the

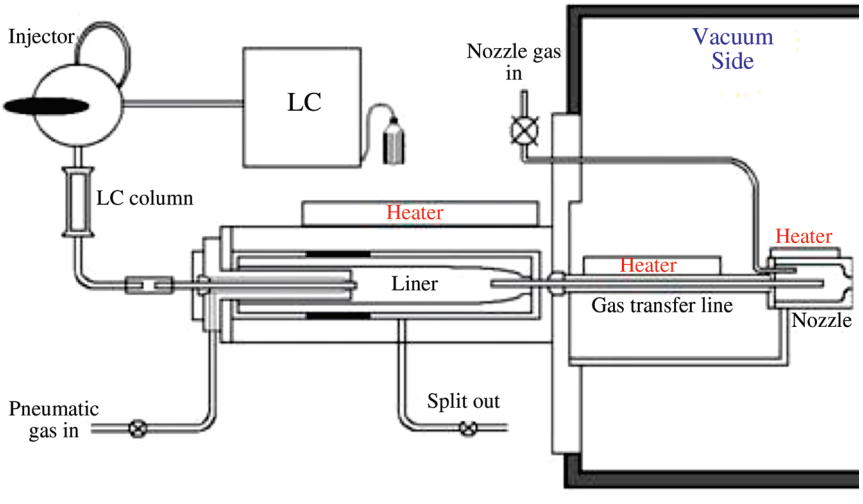

Figure 5.10 Schematic of supersonic EI– LC–MS apparatus (reproduced with permission).

supersonic nozzle. This new approach of EI–LC–MS, called capillary separated vaporization chamber and nozzle (CSVCN) system, gives performances comparable to APCI in terms of robustness and usability. Because the sample flow rate is more than 100-fold greater in the capillary transfer line than in the vaporization chamber, thermal degradation can be considered negligible in the transfer line, and it could eventually occur in the chamber. However, this thermal degradation does not occur because the molecules are supercooled, avoiding any further dissociation, as soon as they expand from the supersonic nozzle. The CSTNV vaporization chamber is heated at an external temperature of about 500–800 °C, as well as in APCI, although with respect to APCI, it is fully thermally assisted and not thermally/gas assisted. No nitrogen gas generator is required (in contrast to APCI and ESI) because the vaporized solvent acts as a carrier gas. The SMB of vibrationally cold indissociated molecules is collimated by a skimmer into a second vacuum chamber, equipped with two diffusion pumps. The analytes are ionized by an EI source and ions are deflected at 90 °C through an ion mirror and sent to the analyzer. Unlike APCI, all the analytes can be ionized regardless of their polarity and no gas-phase ion–molecule reactions occur, so that no ME is observed. The spectra are the same way obtainable from an EI ionization source; thus, they can be compared with NIST spectra, although they have an enhanced molecular ion. In Figure 5.11, a supersonic EI spectrum compared with the EI spectrum of the same compound in the NIST library is shown. Amirav also developed a software that converts experimental MS data into a chemical formula, confirming or rejecting automatically the NIST library identification [67].

The response is rather uniform due to constant ionization efficiency so that an estimation of the concentration of unknown samples can be done without knowing the single compound identity. Higher EI sensitivities are obtained compared to particle beam MS. LODs are in the low picogram range in SIM mode, comparable to those obtained with the Direct-EI approach. Furthermore, like Direct-EI, the MS system is low cost and can be used with both in GC–MS and in LC–MS.

5.3.3
Combined Single-Photon Low-Pressure Photoionization and EI Ionization

An interesting attempt was recently made by Zimmerman and Cappiello who realized an interface that uses photoionization under vacuum conditions in order to avoid the main drawbacks of working at atmospheric pressure: ion–molecule reactions and matrix effect.

In Figure 5.12, the ion source asset is shown. The LC effluent enters the ion source through a Direct-EI interface. Inside the source, an electron beam (12 keV) is directed through a 300-nm-thin SiN_x foil into a reservoir filled with argon (2000 hPa pressure). As a result, these atoms are excited and form excimers that, upon their decay, emit VUV radiation at 126 nm focused by an optical system and used for single-photon ionization (SPI).

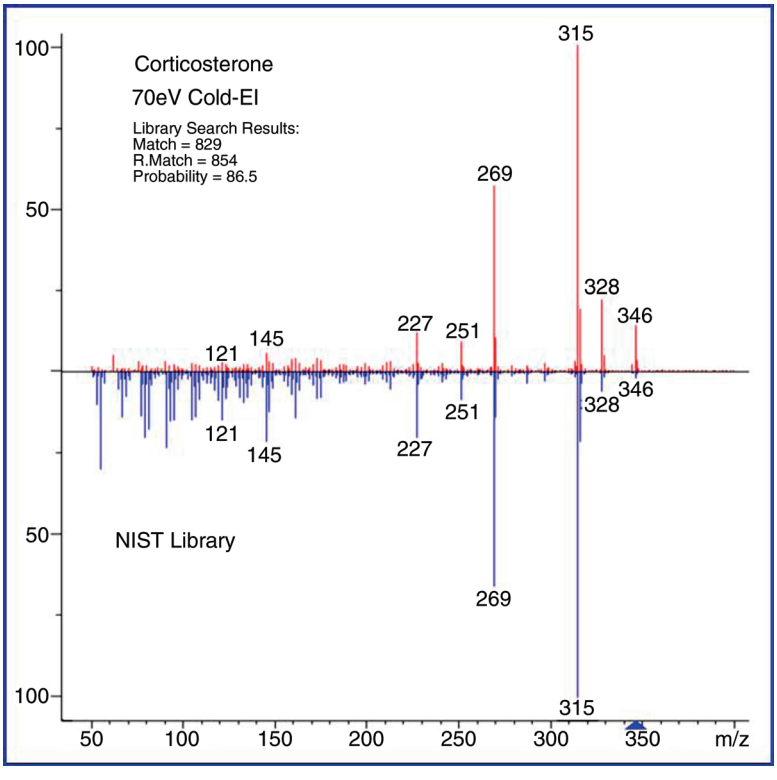

Figure 5.11 A comparison of cold EI mass spectrum of corticosterone obtained with the supersonic LC–EI–MS system and the NIST spectrum, with NIST library matching factors and probability of identification (reproduced with permission).

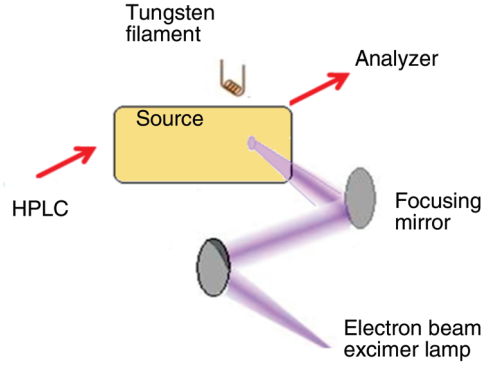

Figure 5.12 Schematic of the single photon low-pressure photoionization–electron ionization interface.

The quadrupole analyzer is set orthogonally to the capillary carrying the LC effluent [68]. With this combined interface, both reproducible, library-matchable EI–MS spectra and soft PI spectra can be obtained. As a consequence, the advantages of the two ionization techniques are summed. In fact, good sensitivities can be obtained working in PI and, at the same time, collateral reactions and ME are avoided because ionization is realized under high-vacuum conditions. Furthermore, the technique has the potential of EI in getting structural information on analytes.

5.3.4
LC/DESI–MS Interface

An interesting approach has been proposed recently by Cai *et al.* [69]. An LC effluent passes through a PEEK capillary tube with a microdrilled orifice on its wall, as shown in the schematic reported in Figure 5.13. A small droplet of liquid emerges out of the orifice, whereas the LC effluent runs toward the outlet. The droplet is ionized by desorption electrospray ionization (DESI), whereas the great amount of analytes remains into the peak capillary tube and can be collected for preparative purposes or analyzed by other methods. This new technique allows to avoid dead volume formation, which could determine chromatographic peak band broadening. Reactive DESI can also be applied for online derivatization. The interface works efficiently also with higher mobile phase flow rates, such as the flow rates employed working with monolithic columns.

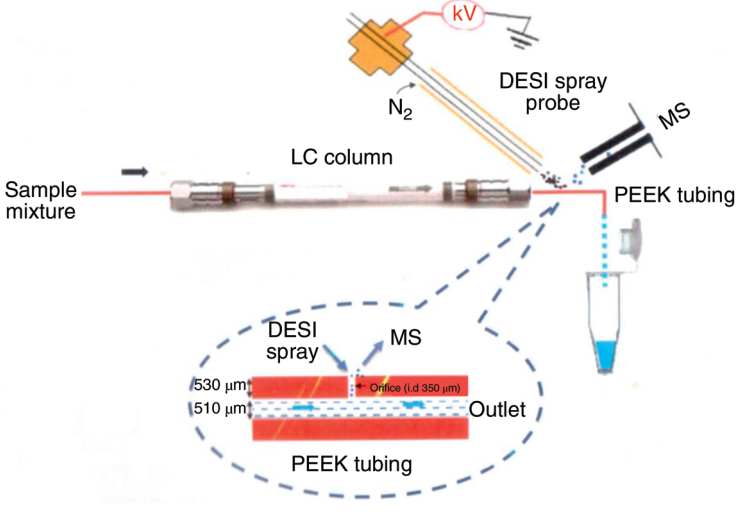

Figure 5.13 Schematic of the new LC/DESI–MS interface (reproduced with permission).

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