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CICLO XXII

MASS SPECTROMETRY PNEUMATICALLY ASSISTED DESORPTION/IONIZATION IN FORENSIC TOXICOLOGY

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ABSTRACT

Mass spectrometry is one of the most relevant techniques in clinical and forensic toxicology. Its development and improvement are based on the invention and utilization of new ion sources, new ionization methods, new mass analyzers and new sample pre-treatment techniques. A recent innovation is the ability to record mass spectra on ordinary samples in their native environment, without sample preparation or pre-separation.

In this field, a new desorption ionization method called DESI (Desorption Electrospray Ionization) has been described; subsequently, method called DeSSI (Desorption Sonic Spray Ionization), at first sight similar to DESI, but in deep substantially different, has been developed.

This thesis consist in developing a new desorption/ionization interface to investigate the real mechanism involved in ions formation because we considered that propaedeutic for the extensive use of the method in the toxicological analytical field. We verified that the pneumatic contribution is preponderant to the obtained results. Hence, our new desorption/ionization interface uses only a spray of pure solvent with no high voltage on needle.

A key aspect of this project, applied to several complex matrix, is the number of controllable operating parameters that can be investigated and optimized to obtain an efficient surface analysis. The most important variables are taken in consideration were the source geometry (the spray angle and the ion uptake angle, as well as the various distances in aligning the spray, sample and mass spectrometer) and the characteristic of sprayer (contents of the solvent spray and gas flow rate).

All measurements have been performed in positive and negative ionization conditions, varying capillary voltage, nebulizing gas pressure, drying gas flow and end plate temperature. Acquisition was in multiple mass spectrometry mode (MS^n).

We have applied this new technical solution to compound identification, active principles and drugs identification in direct tablet analysis, active principles and drugs identification in vegetable species.

Future developments will be related to apply the direct analysis of analytes present on the original surfaces of interest in the toxicological field for in vivo sampling of living tissue surfaces, to identify drug and xenobiotic exposure, besides the chemical imaging of spatial distribution of analytes onto sample surfaces.

1. INTRODUCTION

Nowadays, mass spectrometry (MS) is one of the most frequently employed techniques in performing qualitative and quantitative analysis. Its specificity, selectivity and typical limits of detection are more than enough to deal with most analytical problems.

This is the result of significant effort, either from scientists working in the field or from the manufacturing industry, devoted to the development of new ionization methods, expanding the application fields of the technique, and new analysers capable of increasing the specificity mainly by collisional experiments (MS/MS or "tandem mass spectrometry") or by high mass accuracy measurements. [1]

Thus, the MS panorama is actually made up of many instrumental configurations, each of which have specific positive and negative aspects and different cost/benefit ratios. The development and improvement of MS can be seen, from one perspective, to be based on the invention and utilization of new ion sources, new ionization methods, new mass analyzers and new sample pre-treatment techniques.

MS is one of the most relevant technique available in forensic toxicology. Reliable qualitative and quantitative toxicological analysis is the basis of a competent toxicological judgment, consultation and expertise. A significant breakthrough in the analysis of drugs, poisons, and/or their metabolites in body samples was the hyphenation of chromatographic procedures with mass spectrometers, because in forensic toxicology high specificity and sensitivity is demanded.[2,3]

The particular task of analytical toxicology is to analyze complex biological matrices such as ante- or post-mortem blood, urine, tissues, or alternative matrices such as hair, sweat, oral fluid, or meconium. Besides, the compounds that have to be analyzed are often unknown. Therefore, the first step before quantification is identifying the compounds of interest. [4]

To correctly find and subsequently identify compounds, appropriate sample preparation prior to instrumental analysis is therefore a key step in such analytical methods. The trend towards automation and miniaturization has resulted in new and improved techniques of sample preparation, such as on- and offline solid-phase extraction and solid-phase microextraction, which are becoming increasingly popular. [5]

Currently, a new item of interest is the "direct" sample analysis with minimum sample pre-treatment. The dream of every chemist working in the analytical and toxicological fields is the possibility to analyze directly the substrate of interest without any (or with minor) sample treatment. This not only saves time and makes the chemist's life easier, but it potentially overcomes problems related to the recovery of the analytes, i.e. the efficiency of the extraction procedures of the analyte from the original matrix.

The efficient desorption, ionization, and further characterization via mass spectrometry of analytes performed directly from their natural matrixes via a fully "nonsample preparation" procedure, under atmospheric pressure and at room temperature, is certainly one of the most-welcomed advances of modern mass spectrometry. Identifying drugs, metabolites and xenobiotics on surfaces of living tissues would allow direct analysis of biological samples for forensic toxicology purposes.

This previously unimaginable feature of MS has been made possible recently with the introduction of a variety of new techniques such as desorption electrospray ionization (DESI),[6] direct analysis in real time (DART),[7] analysis of samples at atmospheric pressure,[8] desorption atmospheric pressure photoionization (DAPPI),[9] matrix-assisted laser desorption electrospray ionization (MALDI),[10] and desorption sonic spray ionization (DeSSI).[11] These new approaches have now been applied with success to several analytes and matrixes such as drug tablets, vegetable oils, perfumes and wine. [12]

All developed surface direct analysis methods use high voltage or acidic solvent to generate charged molecules from surface for subsequent MS analysis.

These solutions ensure ion formation but make the direct analysis of living surface (e.g. human finger skin) rather difficult.

A desorption/ionization interface, called Pneumatically Assisted Desorption/Ionization (PADI), has been developed in this study. PADI is a direct pneumatically assisted spray of pure solvent without the presence of any electric potential. This solution can be considered an innovative method for "in vivo" sampling of living tissue surfaces.

1.1 MS IN FORENSIC TOXICOLOGY

The mass spectrometric approaches are employed when linked to suitable chromatography (C) system. The synergism obtained allows C-MS to be used worldwide and is of considerable interest to researchers involved in basic chemistry, environmental and food controls, biochemistry, biology and medicine.[1]

In modern forensic toxicology and in the field of doping control, analytical procedures must not only be sensitive but also highly specific, because in most cases the analytes are not known in advance and many other xenobiotics or endogenous biomolecules may interfere with their detection. Gas chromatography coupled to mass spectrometry (GC/MS) ideally combines both requirements and has long been the gold standard in analytical toxicology.

Hyphenation of gas chromatography with mass spectrometry was in the 1960s. It took more than 30 years to hyphenate liquid chromatography with mass spectrometry (LC/MS) for routine application. In recent years liquid chromatography coupled to (tandem) mass spectrometry [LC–MS(/MS)]has become increasingly important, complementing and partly replacing GC/MS applications. [5]

Today, hyphenated mass spectrometric techniques like GC/MS and LC/MS are and will be indispensable tools in forensic toxicology and in doping control.

GC/MS in the Electron Ionization (EI) mode will play a major role particularly in comprehensive screening procedures, because very huge collections of reference spectra are available and the apparatus costs are moderate. LC/MS with the different mass analyzer types will become more and more a standard technique for automated target screening procedures and particularly for high-throughput quantification. Modern hybrid mass analyzers, such as QTRAPs with much higher sensitivity, will allow determination drugs in trace volumes of biosamples or modern very-low-dosed drugs. QTOF mass analyzers will find their place in forensic toxicology for detection of very rare

compounds via their empirical formula and in drug metabolism confirming the structural change during metabolism. [4]

1.2 MS ANALYTICAL APPLICATION

The choice of a method in analytical toxicology depends on the problems to be solved. The analytical strategy often includes a screening test and a confirmatory test before quantification. If a drug or category has to be monitored, immunoassays can be used for preliminary screening in order to differentiate between negative and presumptively positive samples. Positive results must be confirmed by a second independent method that is at least as sensitive as the screening test and that provides the highest level of confidence in the result.

For such highthroughput screening analysis, thin-layer chromatography,[13] GC with common detectors[14] or electrokinetic techniques[15] have been described. However, they are hardly used anywhere. Today, GC/MS is still the most frequently used technique in analytical toxicology, but single-stage or tandem LC/MS with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) have left the development stage and are becoming increasingly important in routine toxicological analysis, especially for quantification of the identified analytes.[16,17] LC/MS with atmospheric pressure photoionization (APPI) facilitates the measurement of nonpolar compounds that are inefficiently ionized by ESI or APCI, but is only rarely used in analytical toxicology.[18]

Whenever in toxicological analytical approach unknown compounds must be screened and reliably identified (prior to quantification), particularly in small amounts and/or in complex matrices, mass spectrometric techniques hyphenated with chromatographic systems are required. This task is an important part of the analytical strategy in forensic toxicology, in doping control or in environmental chemistry.[4] For target screening, e.g. confirming immunoassay results, the selected-ion monitoring (SIM) or the multiple-reaction monitoring (MRM) mode are generally used.[19-21] In order to increase the selectivity, at least three ions or two MRM transitions should be chosen

representing different parts of the molecule, preferably including the molecular ion. Identification criteria are the chromatographic retention time, the presence and abundance of the monitored ions and the ratios of the ion abundances. For the so-called general unknown screening procedures, the full-scan mode is the method of choice. Reconstructed mass chromatography with selected ions is used for screening and library search for the identification of peak underlying the spectra after subtraction of the background. The match factor in case of computer-aided library search should be as high as possible, but they have to be used with utmost caution, especially in the case of samples with a high matrix load. In such samples, overlaying mass spectra and therefore comparatively low match qualities are the rule rather than the exception. Therefore, the responsibility lies with the toxicologist to decide, depending on the case, how and when the minimum requirement for identity confirmation is reached. [8,22]

1.2.1 GC/MS

In the 1960s, hyphenation of gas chromatography with mass spectrometry had become the gold standard in all fields of analytical toxicology owing to its high identification and separation power combined with high sensitivity. [3]

GC/MS full-scan screening procedures in blood, in principle, allow the detection of a wide range of analytes, although their concentrations are generally lower than in urine. Target screening methods using the SIM mode have been published mainly for drugs of abuse, e.g. for application in the context of driving under the influence of drugs (DUID).

The full-scan GC/MS screening is based on reconstructed mass chromatography using macros for selection of suspected drugs followed by identification of the unknown spectra by library search.

Use of extensive mass spectral reference libraries from the fields of toxicology and general chemistry often allow the detection of even unexpected compounds amenable to GC and EI. They allow the identification of unknown compounds even in the absence of reference substances, if certain prerequisites are fulfilled. Suitable mass spectral reference libraries should be built under standardized conditions and not only be collected from various sources. In case of GC/MS, the spectra of derivatizable compounds should be recorded after common derivatization, because otherwise such compounds cannot be sensitively detected. Spectra of artefacts formed from the analyte during sample preparation or GC must be included, as well as those of matrix compounds and of typical impurities/contaminants such as softeners. For use in forensic toxicology and doping control, the reference library should not only include spectra of reference substances of drugs, poisons, pesticides and pollutants but also of their metabolites.[23] Finally, adequate procedures should be developed for isolation (derivatization) and chromatographic separation to enable a sensitive and reliable detection of these compounds in biosamples.

Thus, GC/MS screening fulfils most demands of high-throughput screening in urine at least in the meaning of analytical toxicology, providing screening for thousands of compounds within one procedure[4,24] and allowing an easy identification of unknown analytes (of course previously studied).

The EI is surely the ionization method most widely employed. [25] But EI method suffers of two main limitations: it is based on the gas phase interactions between the neutral molecules of analyte and an electron beam of mean energy 70 eV. This interaction leads to the deposition of internal energy in the molecules of analyte, reflecting in the production of odd electron molecular ions ($[M]^{+\bullet}$) and of fragment ions, highly diagnostic from the structural point of view.

Then the first limitation of EI is related to the sample vaporization, usually obtained by heating the sample under the vacuum conditions (10^{-5} – 10^{-6} Torr) present in the ion source. Unfortunately, for many classes of compounds the

intermolecular bonds (usually through H bridges) are stronger than the intramolecular ones and the result of the heating is the pyrolysis of the analyte: the EI spectrum so obtained is not that of analyte, but that of its pyrolysis products. This happens in general with all the highly polar compounds.

The second limitation of EI is related to the internal energy deposition: for many classes of compounds it is too high, leading to the extensive fragmentation of the molecule and to the absence of molecular ion, generally considered the primary information of a mass spectrometric measurement.

To overcome the second limitation above described, in the 60s of last century a new ionization method was proposed, based not on a physical interaction but on gas-phase reactions of the analyte with acid or basic ions present in excess inside an ion source, operating at a pressure in the order of 10^{-1} – 10^{-2} Torr; this method is usually called Chemical Ionization (CI). [26]

Generally, protonation reactions of the analyte are those more widely employed. The occurrence of such reactions is related to the proton affinity (PA) of M and that of the reactant gas, and the internal energy of the obtained species are related to the difference between these proton affinities. From the operative point of view CI is simply obtained by introducing the neutral reactant species inside an EI ion source in a 'close' configuration, by which quite high reactant pressure can be obtained.

CI, as well as EI, requires the presence of samples in vapour phase and consequently it cannot be applied for non-volatile analytes. [27]

1.2.2 LC/MS

After the successfully application of GC/MS, it took more than 30 years to hyphenate liquid chromatography with mass spectrometry for routine application. In the early 1990s, several working groups started with LC-MS in

analytical toxicology. [28, 29] Some of them started with a transfer of existing LC-UV or GC-MS procedures so that the scientific and/or practical progress was rather limited, particularly when considering the limitations of this new technique such as the rather poor spectral information (in single-stage apparatus), the poor reproducibility of the ionization, and the susceptibility to matrix effects (ion suppression or enhancements). In recent years, the apparatus have been improved and the analysts have learned to more or less overcome the disadvantages and challenges with LC/MS analysis. For example, relevant matrix effects can often be avoided by suitable specimen clean-up, chromatographic changes, reagent modifications, and effective internal standardization[3]. After 15 years, single-stage or tandem LC-MS with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) have now definitively left the development stage and are becoming increasingly important in routine toxicological analysis, especially for quantification of the identified analytes [17, 30]. They have even opened the door to new fields of toxicological interpretation and expertise such as, e.g., sensitive detection of chemical agents in hair in the case of drug-facilitated crimes [31] or determination of a chronic alcohol consumption by determination of ethanol conjugates in plasma, urine, or hair [32].

Today, LC/MS has been shown to be an ideal supplement to GC/MS, especially for quantification of more polar, thermolabile, or low-dosed drugs[3] ; liquid chromatography coupled with a single-stage or tandem mass spectrometer is becoming more and more a routine apparatus, especially in blood and plasma analysis, because it guarantees the stability of analytes in the biosample before work-up and during the whole analytical procedure.[17,19,21] However, when establishing LC/MS screening procedures in routine work, several limitations should be kept in mind. The spectral information of EI mass spectra can be reached only by product ion spectra, while the information of single-stage ESI and/or APCI spectra is rather poor. In the latter case, in-source fragmentation caused by increasing the orifice or fragmentor voltage allows the

formation of structure related fragments. Modern apparatus allow switching very fast between the different voltages so that different traces with different orifice/fragmentor voltages can be recorded during LC separation. Thus, different compounds with different fragmentation properties can be analyzed in one run without the loss of spectral information. [22, 27, 33]

The question whether ESI or APCI cannot be answered in general. APCI is more appropriate for unionized analytes. The sensitivity depends on the analyte structure and the apparatus type used and should always be tested for. Dams et al. found that ESI and APCI showed matrix effects, with ESI being much more susceptible than APCI. Finally, Beyer et al. could show that the accuracy and precision data of a plasma quantification method for toxic alkaloids were very similar when using LC-APCI-MS or LC-ESI-MS/MS. [3]

1.2.2.1 ELECTROSPRAY IONIZATION (ESI)

Electrospray is based on the droplet production in presence of strong electrical fields. The first experiments on electrospray can be considered that performed by Jean-Antoine Nollet, who observed in 1750 that the water flowing from a small hole of an electrified metal container aerosolizes when placed near the electrical ground. At that time physics, chemistry, physiology and medicine were very often seen as a unique science and some experiments were performed at physiological level. Abbé Nollet observed that "a person, electrified by connection to a high voltage generator (hopefully well insulated from the ground! –authors' note-) would not bleed normally if he was to cut himself; blood sprays from the wound".

About one century later Lord Kelvin studied the charging between water dripping from two different liquid nozzles, which leads to electrospray phenomena at the nozzles themselves. In the last century a series of systematic

studies on electrospray were carried out by Zeleny and Taylor allowing a detailed description of the phenomenon. In the middle of the century electrospray started to be used on the industrial scale, in the application of paints and coatings to metal surfaces. The fine spray results in very smooth even films, with the paint actually attracted to the metal. Miniaturized versions of electrospray are even finding their way into the next generation of micro-satellites: the electrostatic plume makes an efficient, although very low power, ion propulsion engine.

Electrospray became of analytical interest in 1968, when Dole and co-workers produced gas-phase, high molecular weight polystyrene ions by electrospraying a benzene/acetone solution of the polymer. Quite strangely these results do not lead to further applications until 1984, when the studies of Yamashita and Fenn brought electrospray in the analytical world and from which electrospray applications have shown a fantastic growth.

This technique can be considered the ionization method which all the scientific community was waiting for an effective and valid approach for the direct study of analytes present in solution, without the need of analyte vaporization and, consequently, for an easy coupling of MS with LC methods.

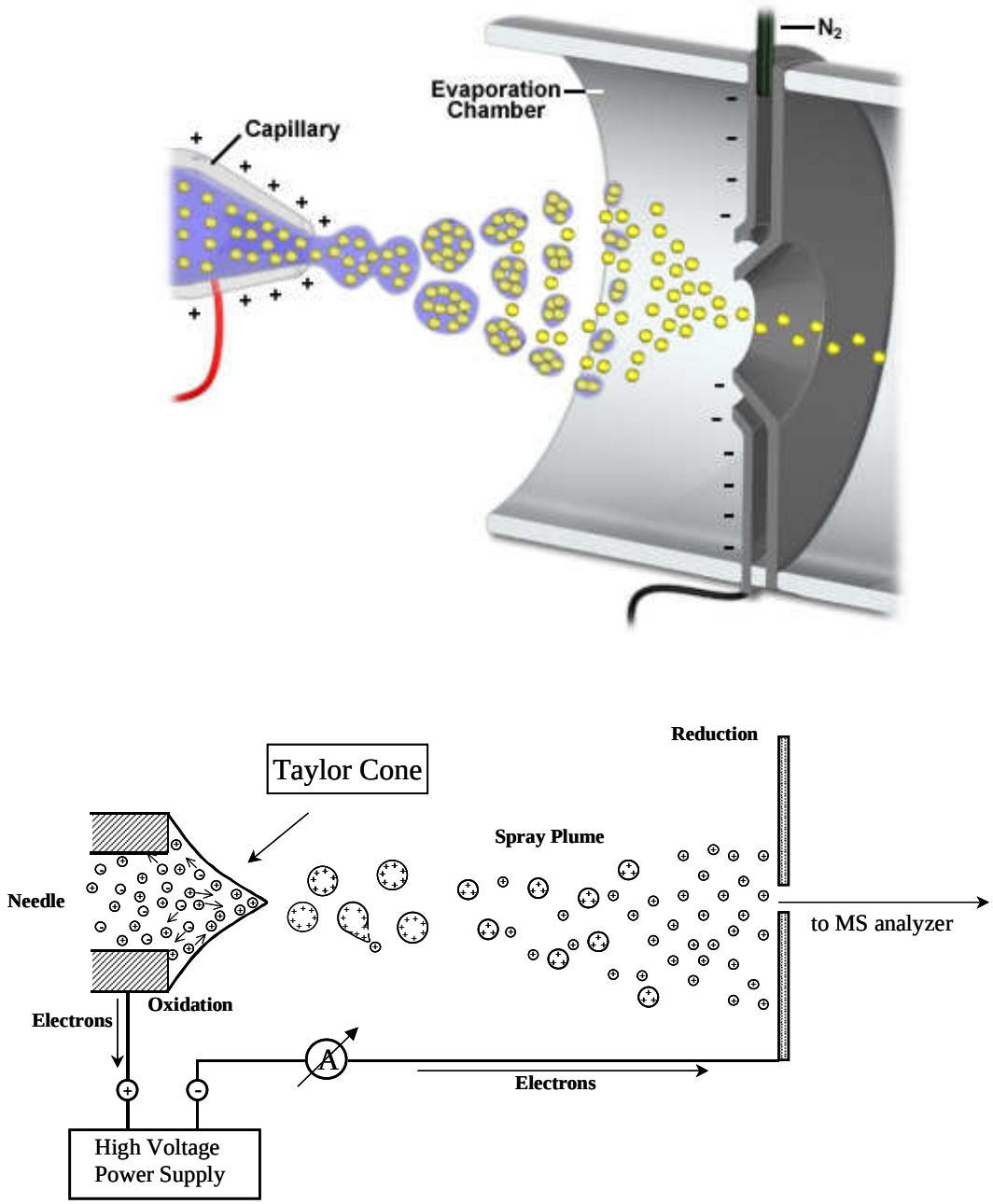


Figure 1. Schematic of an electrospray source showing the production of charged droplets from the Taylor Cone

The instrumental set-up for electrospray ionisation experiments is schematized in figure 1. The solution is injected in a stainless steel capillary. Between this capillary and a counter electrode, placed few-tenth of millimetres far from it, a voltage in the order of some kV is applied. In general, as the liquid

begins to exit from the needle, it charges up and assumes a conical shape, referred to as the Taylor cone, in honor of Taylor who firstly described the phenomenon in 1964. The liquid assumes this shape because when charged up, a conic shape can hold more charge than a sphere. The formation of this cone shaped structure can be justified by the presence of charged species inside the solution which experiment the effect of the electrostatic field existing between the capillary and the counter electrode. What is the origin of this charged species, in absence of ionic solute? It is to emphasize that even in absence of ionic analytes, protic solvents produce ionic species, due to their dissociation. Thus, for example, taking into account that K_w at 20°C is $10^{-14.16}$, the H_3O^+ concentration at 20°C is in the order of $8.3\ 10^{-8}$ M. Analogously $K_a(CH_3OH)=10^{-15.5}$. Consequently the solvents usually employed for electrospray experiments already produces ions in solution, which can be considered responsible of the cone formation. Of course the presence of dopant analytes (e.g. acids), as well as traces of inorganic salts, strongly enhance this phenomenon .

If the applied electrical field is enough high, the formation of charged droplets from the cone apex is observed which, due to their charge, further migrate through the atmosphere to the counter electrode. Experimental data have shown that the droplet formation is strongly influenced by:

- Solvent chemical-physical characteristics (viscosity, surface tension, pKa);
- Concentration and chemical nature of ionic analytes;
- Concentration and chemical nature of inorganic salts;
- Voltage applied between capillary and counterelectrode.

In the case of positive ion analysis the capillary is usually placed at a positive voltage while the counterelectrode is placed to a negative voltage (this is the case shown in figure 1). The vice versa is used in the case of negative ion

analysis. In both cases a high number of positive (or negative) charges are present on the droplet surface.

The formation of the Taylor cone and the subsequent charged droplet generation can be enhanced by the use of coaxial nitrogen gas stream. This is the instrumental setup usually employed in the commercially available electrospray sources: then the formation of charged droplets is due to either electrical and pneumatic forces.

The electrical current due to the droplets motion can be easily measured by the amperometer (A) shown in figure 1. This measurement allows to estimate, from the quantitative point of view, the total number of elementary charges leaving the capillary and which, theoretically, may correspond to gas phase ions.

The charged droplets, generated by solution spraying, decrease their radius due to solvent evaporation but their total charge amount remains constant. The energy required for the solvent evaporation is in a first step due to the environment thermal energy. In a second step this process is enhanced through further heating obtained by the use of a heated capillary or by collisions with heated gas molecules. The maintenance of the total charge during this evaporation phase can be explained by the fact that the ion emission from the solution to the gas phase is an endothermic process.

The decrease of the droplet radius with respect to time leads to an increase of the surface charge density. When the radius reaches the Rayleigh stability limit the electrostatic repulsion is identical to the attraction due to the surface tension. For lower radius the charged droplet is unstable and decomposes through a process generally defined "Columbic Fission". This fission is not regular (in other words the two parts originated by it do not have necessarily analogous dimensions).

Until now two different mechanisms have been proposed to give a rationale for the formation of ions from small charged droplets. The first of them has been recently discussed by Cole and Kebarle & Peschke in 2000. It describes the

process as a series of scissions which lead at the end to the production of small droplets bringing one or more charges but only one analyte molecule. When the last, few solvent molecules evaporate, the charges are localized on the analyte substructure, giving rise to the most stable gas phase ion. This model is usually called "the Charged Residue Mechanism" (CRM) (see lower part of figure 2).

Thomson and Iribarne in 1979 have proposed a different mechanism, in which a direct emission of ions from the droplet is considered. It occurs only after that the droplets have reached a critical radius. This process is called "Ionic Evaporation" (IEM) and is dominant with respect to Columbic Fission from particles with radius r <10nm (see upper part of figure 2).

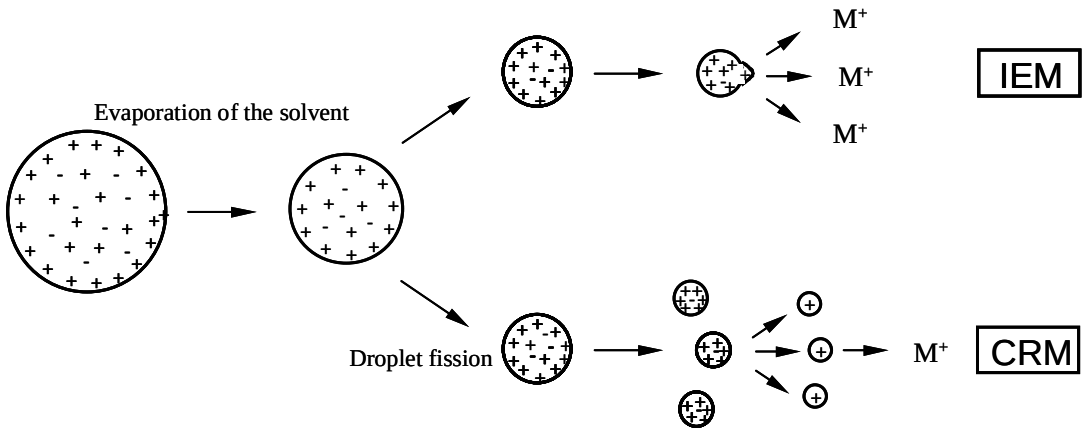


Figure 2. Graphical representation of ions formation models: Ionic Evaporation and Charged Residue Mechanism

Both CRM and IEM are able to explain many of the behaviours observed in ESI experiments. However a clear distinction between the two mechanisms lies in the way by which an analyte molecule is separated from the other molecules (either of analyte or solvent present in droplets). In the case of IEM this separation takes place when a single analyte molecule, bringing a part of the charge in excess of the droplet, is desorbed in the gas phase, so reducing the columbic repulsion of the droplets. In CRM mechanism this separation occurs

through successive scissions, reducing the droplet dimensions until when only one single molecule of analyte is present in them. In general the CRM model is retained valid in the process of gas phase ion formation for high molecular weight molecules.

Then, the ESI process in complex: the ion formation depends on many different mechanisms occurring either in solution or during the charged droplets production and ion generation from the droplets themselves. Thus, the concentration of the analyte present in the original solution does not correspond to that present in the droplets generating the gas phase ions, especially if the original solution is far from neutrality. In the case of polar compounds the surface charge density present in the droplet can activate some decomposition reaction of the analyte.

The ESI source can lead to the production of positive or negative ions, depending on the potentials applied on the sprayer and the related counterelectrode. Some producers follow the original ESI source design, placing the sprayer at some kV (positive for positive ion analysis, negative for negative ion production) and the counterelectrode (i.e. the entrance to the mass analyzer) grounded or at few Volts, as shown in the left hand side of figure 3 for positive ion analysis. Some other producers use a different potential profile, placing the sprayer at ground potential and the counterelectrode at + or – kV for production of negative or positive ion respectively (right hand side of figure 3).

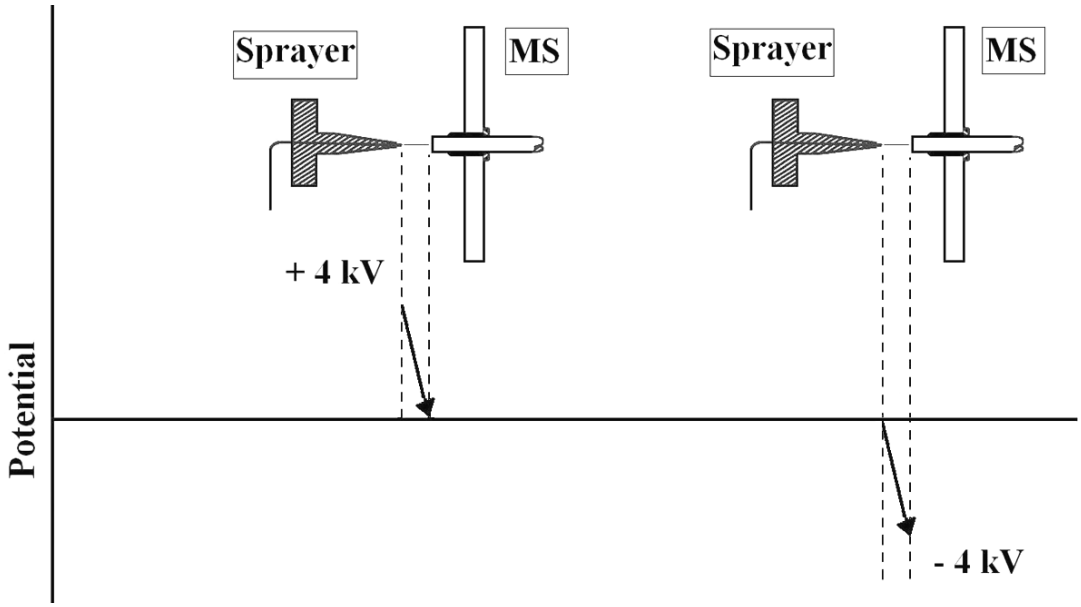


Figure 3. Potential profiles usually employed in ESI/MS for positive ion analysis

The privileged formation of molecular species makes the ESI method highly interesting for the analysis of complex mixtures, without the need of previous chromatographic separation. By direct infusion of the mixture dissolved in a suitable solvent it is possible to obtain a map of the molecular species present in the mixture itself. Furthermore operating in positive ion mode it is possible to evidence the compounds with the highest proton affinity values (i.e. the most basic ones) while in negative ion mode the formation of ions from the most acidic species will be privileged .

Electrospray is surely the ionization method most widely employed for the LC/MS coupling. The possibility to perform ionization at atmospheric pressure (also obtained in the case of atmospheric pressure chemical ionization and atmospheric pressure photoionization, APCI and APPI respectively) allows the direct analysis of analyte solutions. However some problems arise from the intrinsically different operative conditions of the two analytical methods, first of all the high vacuum conditions that must be present at the mass analyzer level. Furthermore mass spectrometers exhibit generally a low tolerance for non-

volatile mobile-phase components, usually employed in LC conditions to achieve high chromatographic resolution.

Summarizing, the difficulties in LC-MS coupling can be related to:

- Sample restriction: the differences among different classes of samples in terms of molecular weight, polarity and stability (either from the chemical or from the chemical-physical point of view) require an accurate set-up of the ESI source conditions;
- Solvent restriction: the LC mobile phase is generally a solvent mixture of variable composition. This variability necessarily reflect on the formation in ESI conditions of droplets of different dimension and different life, i.e. in ionization conditions different in some extent. Also in this case an indeep evaluation of the ESI source parameters must be performed to achieve results as close as possible.
- Chromatographic eluate flow, that must be compatible with the sprayer operative flow.

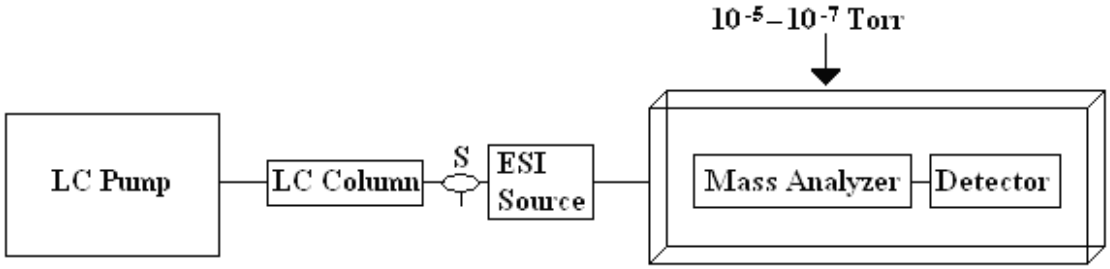


Figure 4. Scheme of the LC/ESI/MS system

The scheme of a LC/ESI/MS system is shown in figure 4. Depending of the LC solvent flow, the splitter S can be employed to reduce the flow itself to values suitable for the ESI sprayer. Of course the split ratio reflects on a decrease of sensitivity (a portion of the sample is dropped away). Analytical columns with internal diameters (i.d.) in the range 2.1-4.6 mm require the use of

the splitter, while columns with i.d. ≤1mm can be directly connected with the ESI source.

It is worth of noting that, aside the splitting problem, the i.d. reduction of LC columns leads to a sensible increase in sensitivity of the LC/ESI/MS system. In fact, as shown schematically figure 5, the i.d. reduction leads to a higher analyte concentration, due to the volume reduction: then, passing from a 4.6 mm i.d. column to a 1.5 mm one, a decrease of volume of one order of magnitude is obtained, reflecting in a ten times increase of analyte concentration and the consequent increase of the MS signal.

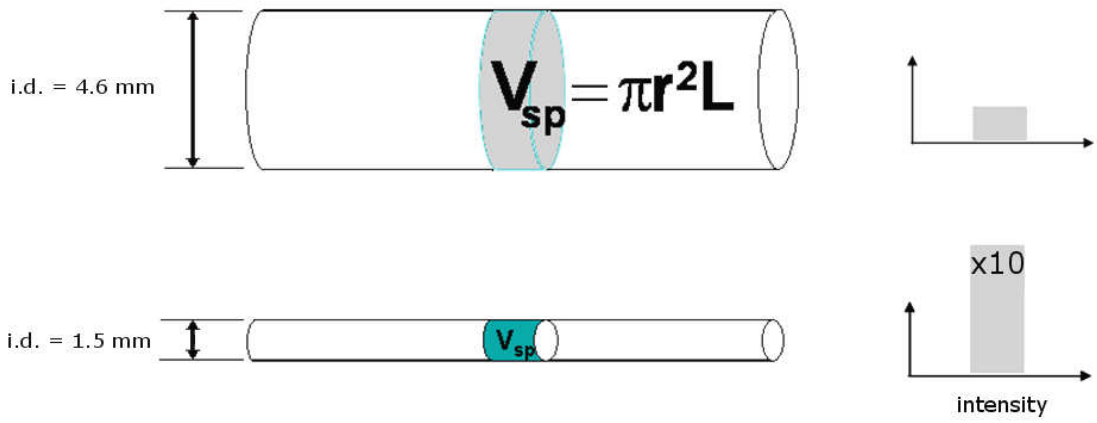


Figure 5. Comparison of the behaviour of two LC columns of different internal diameter, operating with the same linear velocity. In the case of low i.d., a higher analyte concentration is present, reflecting in a higher signal intensity

This aspect has led to the production of micro- and nano- electrospray sources, in which the chromatographic eluate flow are in the range $1\text{-}10^{-2}$ µL/min. A typical instrument set up for nano-ESI experiments is shown in figure 6. In this case the supplementary gas flow for the spray generation is no more present and the spray formation is only due to the action of the electrical field. The sprayer capillary, with internal diameter in the range 5-20 µm, is

coated with a conductive film (e.g. gold film) in order to be placed at the correct electrical potential.

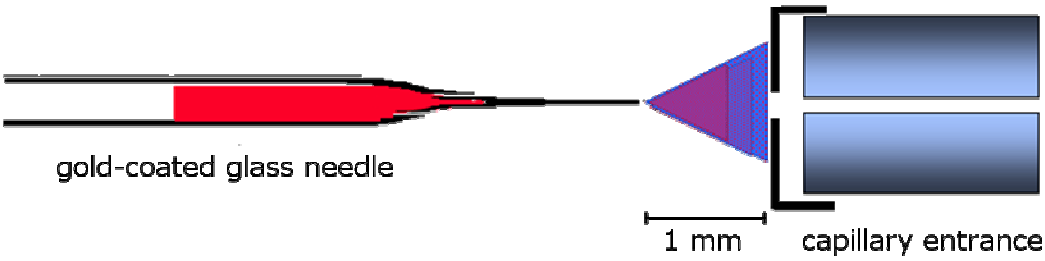


Figure 6. Typical instrumental configuration for nano ESI experiments

Just to give an idea of the nano-ESI performances, when the electrical field is applied, a spray is generated with a flow rate in the order of 25-100 nL/min. This means that 1 µL of sample can be sprayed for about 40 min! And this reflects in a system with high sensitivity and requiring a very low sample quantity. [34]

1.2.2.2 ATMOSPHERIC PRESSURE CHEMICAL IONIZATION (APCI)

APCI was developed starting from the consideration that the yield of a gas phase reaction in CI does not depend only by the partial pressure of the two reactants, but also by the total pressure of the reaction environment. For this reason the passage from the operative pressure of 0.1-1 Torr, present inside a classical CI source, to atmospheric pressure would, in principle, lead to a relevant increase in ion production and, consequently, to a relevant sensitivity

increase. Furthermore the presence of air at atmospheric pressure can play a positive role in promoting ionization processes.

At the beginning of the researches devoted to the development of the APCI method, the problem was the choice of the ionizing device. The most suitable and effective one was, and still is, a corona discharge. The important role of this ionization method mainly lies in its possible applications to the analysis of compounds of interest dissolved in suitable solvents: the solution is injected in a heated capillary (typical temperatures in the range 350-400°C), which behaves as a vaporizer. The solution is vaporized and reaches, outside from the capillary, the atmospheric pressure region were the corona discharge takes place. Usually the vaporization is assisted by a nitrogen flow coaxial to the capillary (figure 7 and figure 8). The ionization mechanisms are typically the same present in CI experiments.

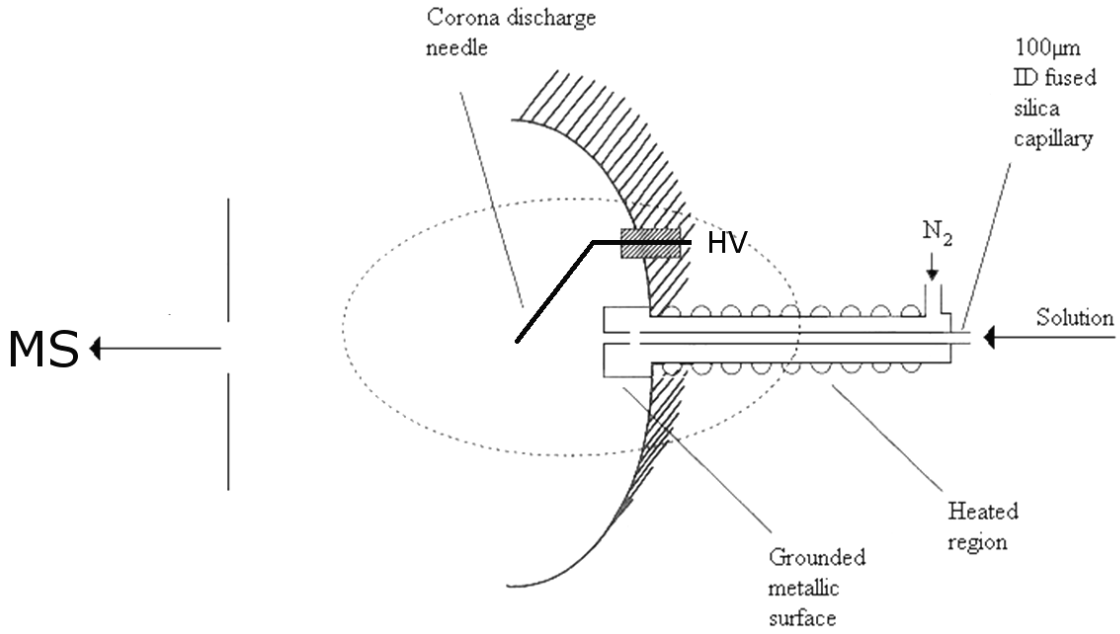


Figure 7. Scheme of the APCI ion source

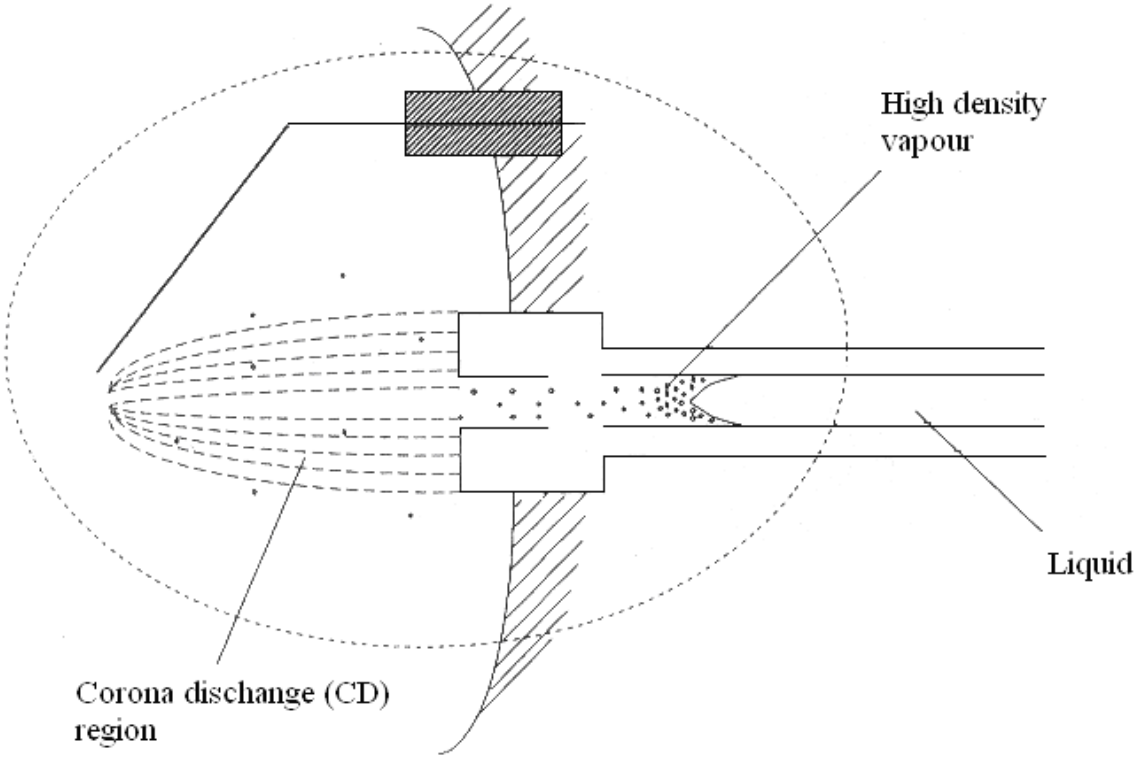


Figure 8. Corona discharge region of an APCI source

The needle generates a discharge current of ca. 2-3μA, which ionizes air producing primary ions (mainly $N_2^{+\bullet}$, $O_2^{+\bullet}$, $H_2O^{+\bullet}$ and $NO^{+\bullet}$ in positive mode, $O_2^{-\bullet}$, $O^{-\bullet}$, $NO_2^{-\bullet}$, $NO_3^{-\bullet}$, $O_3^{-\bullet}$ and CO_3^- in negative mode). The primary ions react very rapidly (within 10^{-6}sec) transferring their charge to solvent molecules, in a reaction controlled by the recombination energy of the primary ions themselves, to produce the effective CI reactant ions. These are characterized by a longer lifetime (about 0.5×10^{-3}sec) and react with analyte molecules to produce analyte quasi-molecular ions by charge or proton transfer reactions, according to the proton affinity of the analyte itself. The whole ionization cascade is represented in the figure 9. In these conditions the formation of protonated ($[M+H]^+$) or deprotonated ($[M-H]^-$) molecules is generally observed operating in positive or negative ion mode respectively.

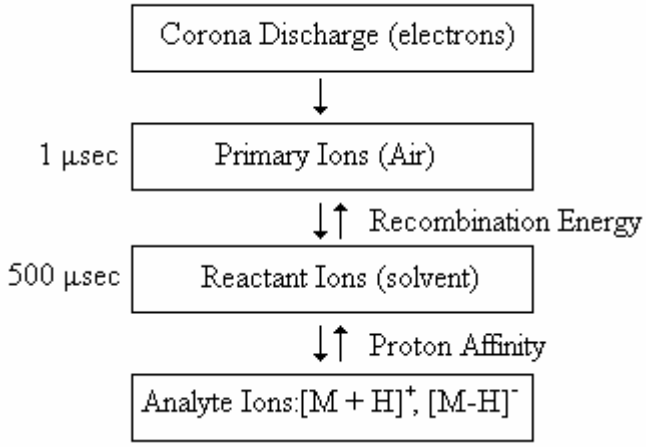


Figure 9. Sequence and time scale of the reactions occurring in an APCI ion source

Besides APCI there is the Atmospheric pressure photoionization (APPI). Photoionization exhibits some theoretical advantages with respect to electron ionization, but also some severe limitations: the main to the extensive use of photoionization in mass spectrometry was that at the light frequencies suitable to produce ionization of most organic compounds (IE ranging up to 13 eV) it is not possible to use optical windows in the path of the light beam. All the window materials are essentially opaque at this photon energy. Consequently, the light source, usually involving a gas-discharge, must be mounted inside the ion source housing operating in high vacuum conditions. A further aspect that in the past limited the common use of photoionization was surely the low sensitivity of the method. When operating in high vacuum conditions, typical of classical ion sources, the formation of ions is some orders of magnitude lower than is observed with the same sample density in EI conditions, and this can be related to the photon cross section.

However, it should be emphasized that photoionization has been used since 1976 as a detection method in gas chromatography, proving that, when the sample density is high enough, good sensitivity can be achieved, together with the specificity related to the wavelength employed. [34]

1.2.3 MASS ANALYZERS AND HIGH SPECIFICITY

A fundamental device of Mass Spectrometer is mass analyzer, devoted to the separation of ions with respect to their mass-to-charge (m/z) ratio. It is based on the knowledge of the physical laws that govern the interaction of charged particles with electrical and magnetic fields.

Two main characteristics are relevant for a mass analyzer; the ion transmission and the mass resolution. The former can be defined as the capability of a mass analyzer to bring to the detector all the ions that have entered into it. Of course the ion transmission will reflect on the sensitivity (or, better, to the detection limit) of the instrument. The mass resolution is usually defined as the analyzer capability to separate two neighbouring ions. The resolution necessary to separate two ions of mass M and (M + ΔM) is defined as:

$$R = \frac{M}{\Delta M}$$

From the theoretical point of view, the resolution parameters can be described as shown in figure 10.

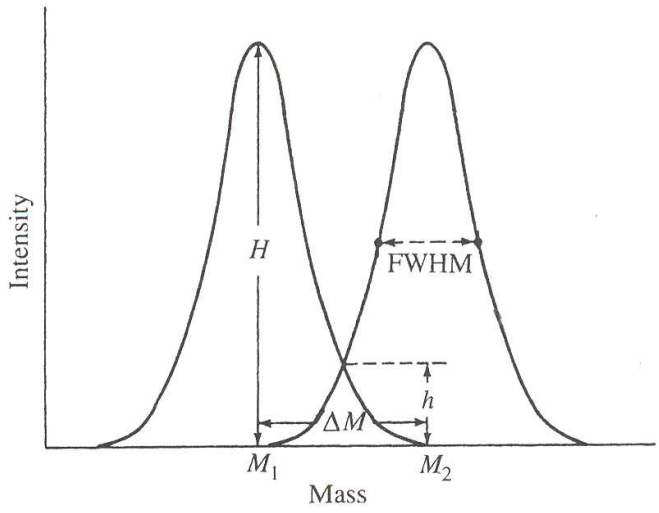


Figure 10. Mass resolution parameters

The most of mass analyzer have been projected and developed during the last century, but in the few years of the new one some interesting, highly promising, new approaches have been proposed: accurate mass measurement and multistage mass spectral analysis.

1.2.3.1 ACCURATE MASS MEASUREMENT

The high discriminative power of accurate mass measurement offers great potential for identification of compounds without (at first instance) comparison with standard and / or knowledge of the pattern of fragmentation. The approach is very attractive for toxicological generic screening including unpredictable compounds or analytes for which no standards are available (eg metabolites). The complexity and variety of biological matrices of forensic interest (blood, urine, hair, cadaverous tissues) require anyway a preliminary stages of separation with chromatographic techniques.

The identification of the compounds will initially be based on a comparison of accurate mass and isotopic ratio measured on the peaks of interest with those measured on standard compounds, but will also run a constant comparison between measured data and calculated theoretically using the formula compounds.

In case of multiple candidates' identification (isobaric compounds) it is necessary proceed to discrimination by using other physical-chemical parameters calculated according to the chemical structure of analytes (eg. chromatographic elution). In this case, to avoid the approximation of the results of these calculations, it is useful an internal standard for comparison.

To perform accurate mass measurements, the gold choice in forensic toxicology is the Orbitrap system [35].

The Orbitrap mass analyzer was invented by Makarov and it can be considered the evolution of an early ion storage device, developed by Kingdom in 1923. It utilizes a purely electrostatic field for trapping the ions: no magnetic or electro-dynamic fields are present. The Kingdom trap can be schematized as shown in figure 11: it consists in a wire, a coaxial cylindrical electrode and two end cap electrodes, each component electrically isolated from the other ones. A voltage is applied between wire and cylinder. In these conditions, if ions are injected into the device with a velocity perpendicular to the wire, those with appropriate perpendicular velocities will follows stable orbits around the central wire. A weak voltage applied on the two end cap electrodes allows to confine ions in the centre of the trap.

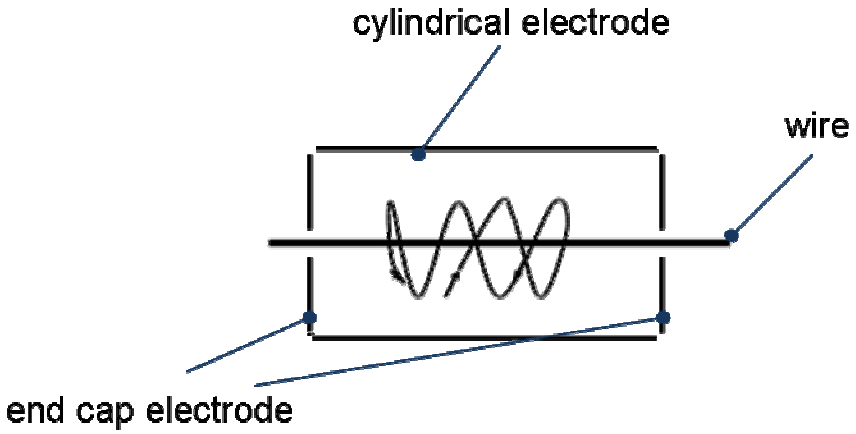


Figure 11. Scheme of the Kingdom ion trap

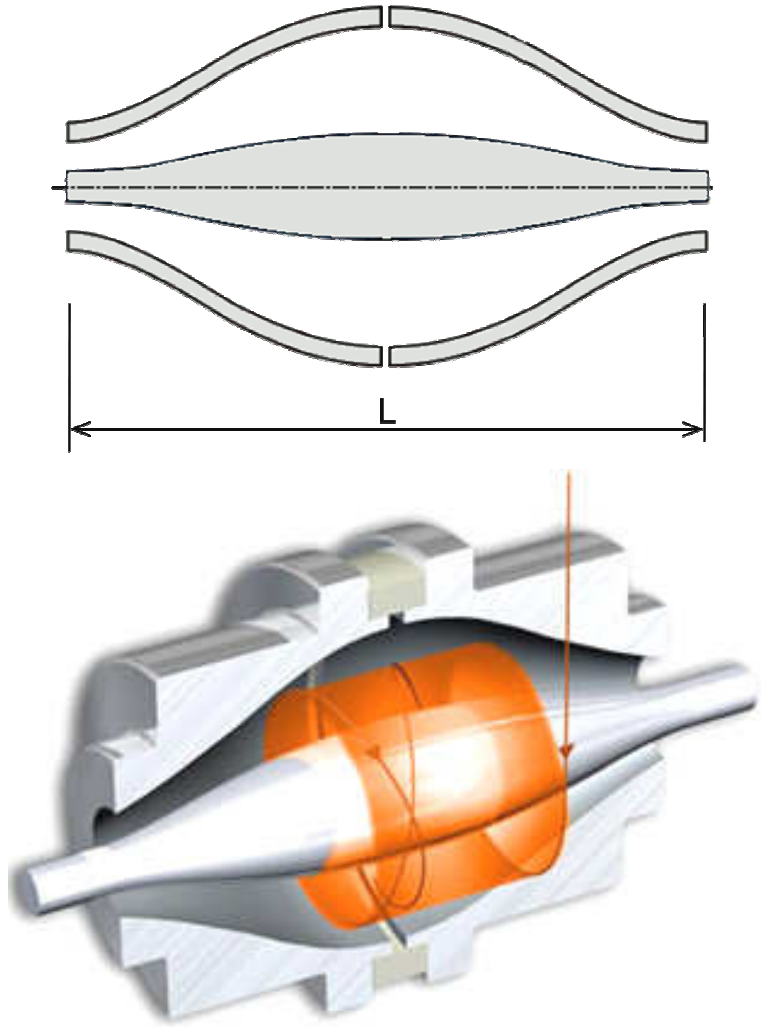


Figure 12. Shape of the electrodes of an Orbitrap. The length L is in the order of few centimetres

Makarov invented a new type of mass spectrometer by modifying the Kingdom trap with specially shaped outer and inner electrodes (see figure 12). Also in this case a purely electrostatic field is obtained by a d.c. voltage applied to the inner electrode. Ions injected in the device undergo a periodical motion that can be considered the result of three different periodical motions:

- rotation around the inner electrode;
- radial oscillation;
- axial oscillations.

These three components (axis) exhibit well defined frequencies:

- Frequency of rotation ωφ
- Frequency of radial oscillations ωr
- Frequency of axial oscillation ωz

In particular the ion motion in the z (axial) direction may be described as an harmonic oscillation in a relationship whit the m/z value of the trapped ion. The ion detection is obtained by image current detection on the two outside electrodes, and by Fourier Tranform algorithm the complex signal due to the copresence of ions of different m/z values (and hence exhibiting different ωz values) is separated in the single m/z components. The typical mass resolution obtained by this analyzer is up to 105.

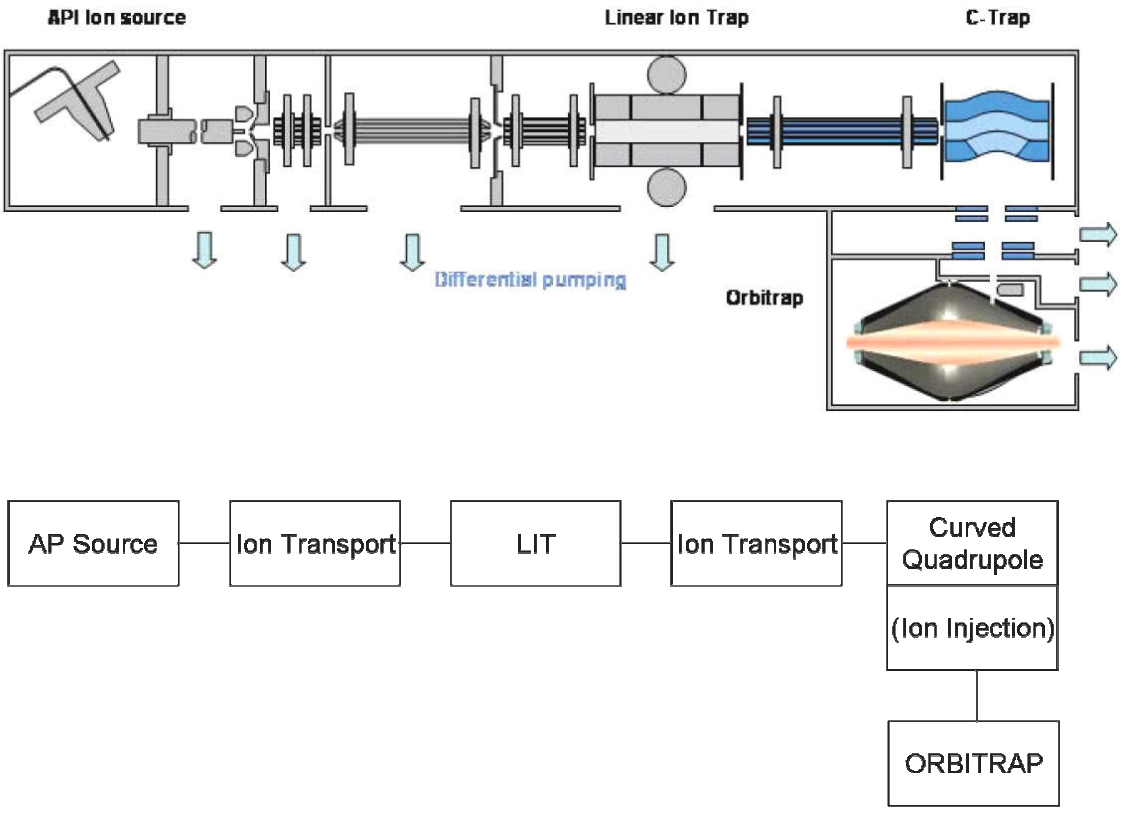


Figure 13. Scheme of a commercial Orbitrap instrument

The nowadays commercially available instrument including the orbitrap technology is schematized in figure 13. The orbitrap represent the last step of a long journey done by the ions generated by an AP ion source (ESI, APCI, APPI). The ion are firstly transported in a linear ion trap by which MS/MS experiments can be effectively performed with high sensitivity. The ions (either precursor or collisionally generated fragments) are then transported to a "curved quadrupole" (c-trap), a highly effective device for ion storing, focusing and ejection. By its action a well focalized ion beam is injected, through a further series of lenses, along the appropriate direction inside the orbitrap and analyzed in the high resolution conditions required by the analytical problem: as long is the trapping time, as high is the achieved resolution.

Aside the FT-MS-based instruments (eg. Orbitrap), able to obtain specificity through either MS/MS or accurate mass measurements, a further system is commercially available, based on the use of quadrupole analyzers coupled with time of flight (Q-TOF) [36, 37]

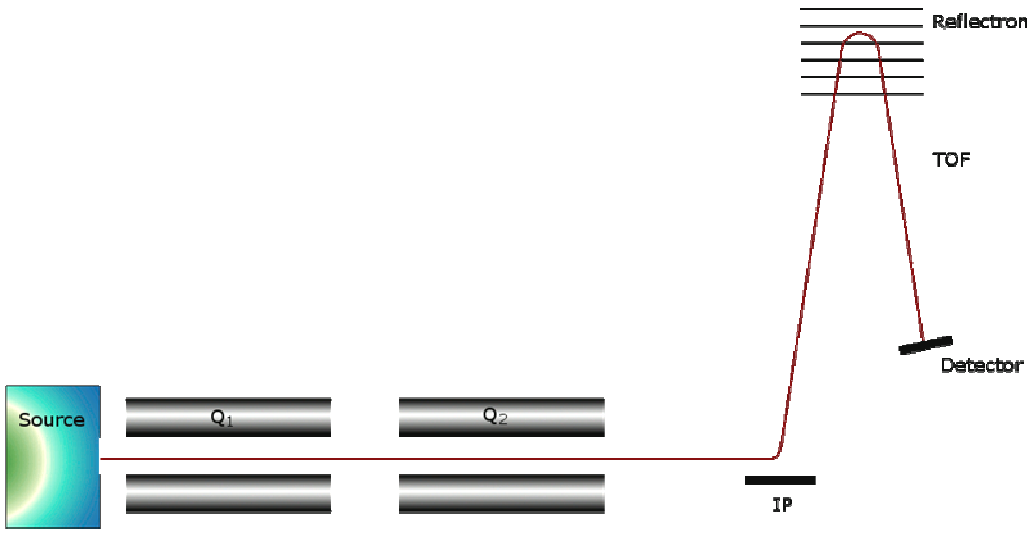


Figure 14. Scheme of a Q-TOF system

Its basic structure is reported in figure 14. It can operate in TOF mode or in Product ion scan mode. In the former case both Q1 and Q2 operate in rf only mode; in other words they transmit all the ions from the ion source to the ion pusher (IP). Once reached IP, the ions are pulsed, by the application of a suitable electrical field (typical voltage applied in the order of 104 V) for 100ns every 100µs, in the TOF analyzer, in a direction orthogonal to the original pathway. By this experimental set up the mass spectrum of all the ions generated into the ion source can be obtained, with resolution in the order of 15000-20000 and accuracy in the ppm range.

In the product ion scan mode Q1 is used to select the ionic species of interest, by applying the suitable U, V voltages on the rods. In Q2, used as collision cell and operating in rf only mode, the selected ions collide with the target gas and the product ions are analyzed by TOF, so obtaining their accurate mass value.

The advantages of Q-TOF can be so summarized:

i) high efficiency in MS/MS experiments
ii) high mass accuracy for both normal mass spectra or product ion spectra
iii) a price surely lower of the FT/MS-based instrument.

1.2.3.2 MULTISTAGE MASS SPECTRA ANALYSIS (MS^n)

In recent years, the power of mass spectral analysis in forensic applications has been greatly augmented by multistage mass spectral analysis. Use of MS/MS analysis is important in forensic toxicology applications.

MS/MS analysis can be thought of as occurring in space or in time. The process commonly used in many instruments is based on linking several quadrupoles together. These are typically referred to as triple quads, owing to the presence of three quadrupole analyzers in series (QQQ). In its simpler form it consists in an arrangement, along the same axis, of three different quadrupole

mass filter (see figure 15). The instruments generate ions in the same manner as described earlier, but they usually use CI to generate only molecular ions.

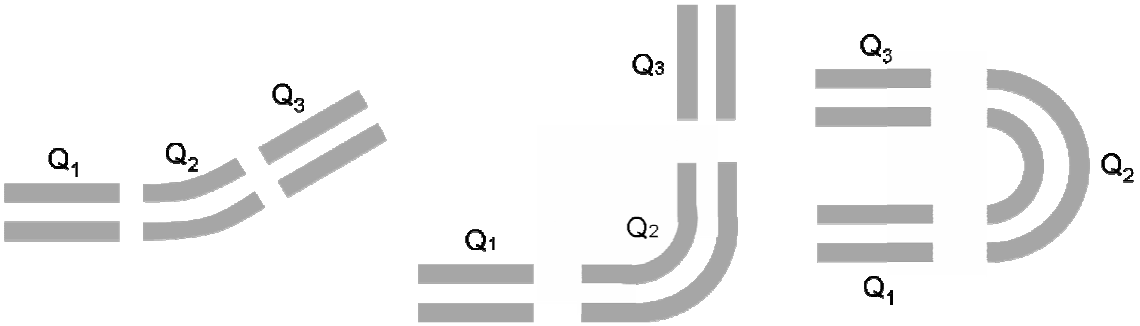


Figure 15. Different QQQ geometries available

The most common form of MS/MS analysis using these instruments involves setting the first quadrupole to allow only ions corresponding to the m/z of the ion of interest to pass. Once the ion has passed through the first quadrupole, it enters a second quadrupole, where fragmentation takes place. This is typically accomplished by putting a collision gas into this area, generating frequent collisions between these gas molecules and the ion that has been selectively allowed to pass through the first quadrupole. This process leads to collisionally activated dissociation of the ion. The single ion passed through the first analyzer is referred to as the precursor ion (or parent ion). The ions formed from the fragmentation of precursor ions are called product ions (or daughter ions). The third quadrupole can then be set to scan or selectively allow one or more of these product ions through to the detector. This method of MS/MS is referred to as MS/MS in space (see figure 16).

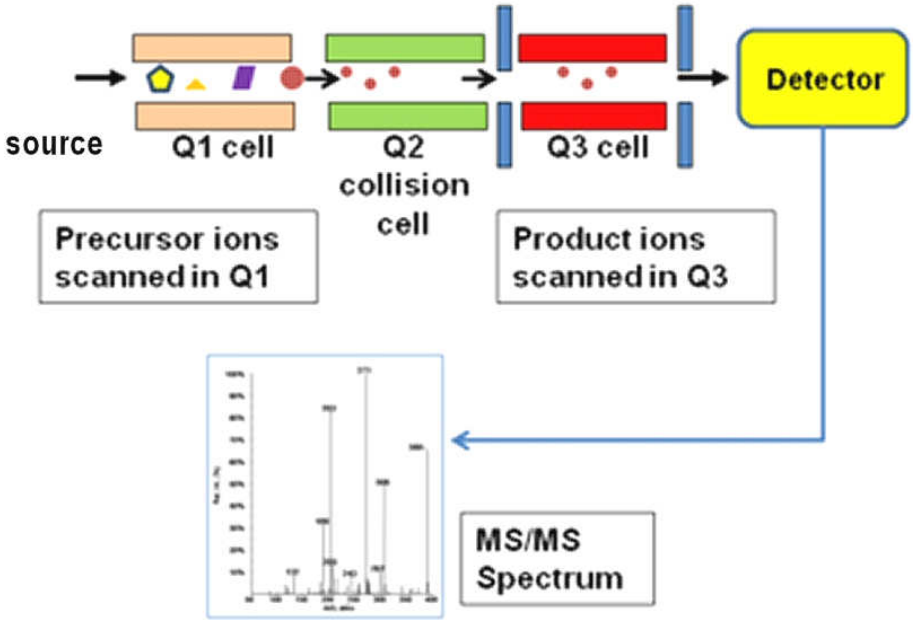


Figure 16. Scheme of QQQ instrument

MS/MS in time is accomplished instead with ion trap mass spectrometers (figure 17).

Ion traps [38] were (and still are!) very attractive devices because they being able to confine and to store in a well defined region of space ions of interest on which perform fundamental studies.

To do MS/MS or MS^n experiments in the ion trap, all ions are ejected from the trap except for the selected precursor ion. A voltage is then applied to the endcap electrode, 180 degrees from the field generated by the rf on the ring electrode. When the voltage applied to the endcap resonates with the energy of a particular m/z value, ions with that value are destabilized and fragment. The amount of energy used can be varied, yielding varying degrees of fragmentation. Following this, the productions are scanned out of the tap to the detector. All this is accomplished within the confines of the trap; thus it represents MS/MS in time.

The ability to trap ions can be a significant advantage in these experiments. When a single ion is trapped and then fragmented, the experiment can proceed to a third level by first isolating a single product ion in the trap by scanning out all others, and then fragmenting the remaining trapped ion. This represents MS/MS/MS (MS^3). Theoretically, this can be repeated over and over again giving fragments that are generated as MS^4, MS^5, etc. However, realistically going beyond three or four is unlikely, and unnecessary, in most applications.

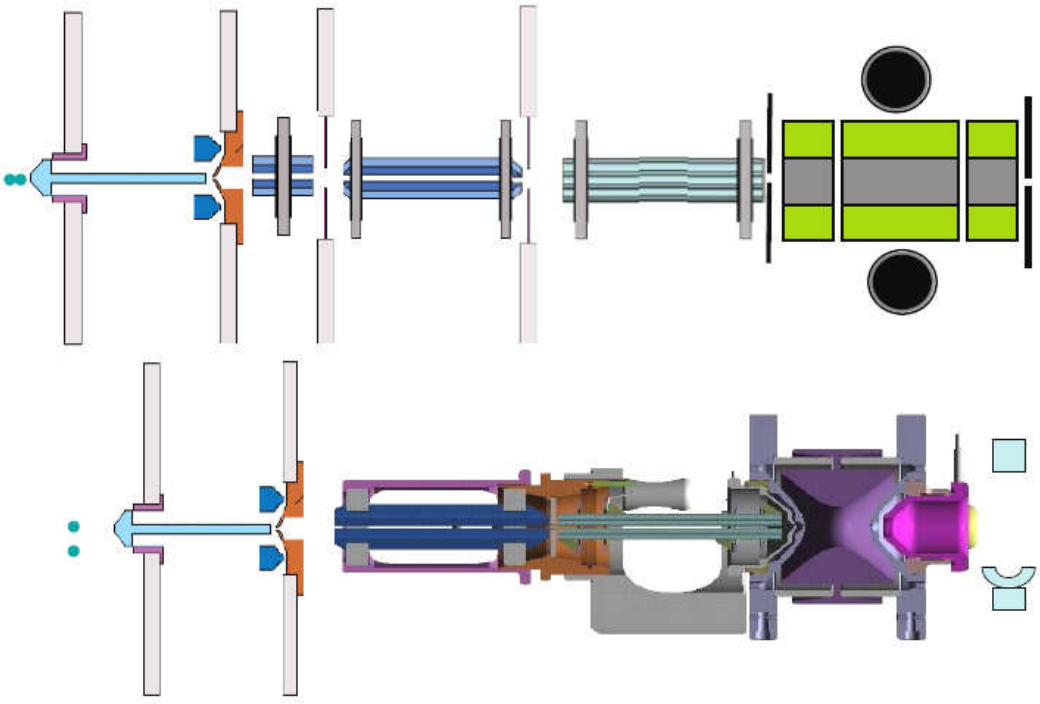


Figure 17. Scheme of mass spectrometers with 2D ion trap (upper) and 3D ion trap (lower)

In general, MS/MS has tremendous advantages in the analysis of compounds. Because the first ion isolated can be the molecular ion, the likelihood of interference from other compounds that may also be in the source at the same time (i.e., chromatographically co-eluting peaks) is all eliminated.

This raises the confidence of identification and depends less on the ability of the chromatographic method to provide a single pure compound to the mass spectrometer. In addition, these methods enhance the ability to elucidate the structure of a molecule. Rather than seeing the total spectrum formed from the fragmentation of a molecule, individual ions can be isolated and their fragmentation evaluated. This ability to determine which ions come from which other fragments can be a powerful tool in the determination of chemical structure. In the case of MS^n analysis, multiple levels of fragmentation can enhance the capacity to elucidate chemical structure, or in the case of identification of a compound, can provide very strong analytical evidence for the presence of a compound. The elimination of other interfering ions makes the use of these techniques more sensitive and allows for more rapid analysis of samples.

Another advantage of MS/MS analysis is the monitoring of neutral loss for all compounds entering the mass spectrometer. This can be a very powerful tool in the search for metabolites of a compound or identification of structurally related compounds.

1.3 DIRECT ANALYSIS SYSTEMS

The application of mass spectrometry (MS) to the identification of chemical compounds in a mixture, often, shows a main limitation in getting the sample of interest into the vacuum environment of the spectrometer in the form of ions suitable for mass analysis. This problem was solved, for the case of samples in the solution phase, with the introduction of ESI. For the analysis of condensed-phase samples, a critical development factor was the desorption/ionization (DI) methods, where molecules embedded in a substrate and introduced into the vacuum system are rapidly desorbed and ionized using energetic charged particles or laser beams. [39] High-energy sputtering methods such as SIMS (secondary ion MS) [40] can be used to produce intact molecular ions. Larger molecules such as proteins are also amenable to DI methods if they are embedded in a frozen solvent (typically ice) or in an ultraviolet (UV)-absorbing matrix that can be rapidly volatilized with a laser pulse, as in MALDI (matrix-assisted laser desorption/ionization) [41]. Although vacuum conditions are a simple choice for creating and maintaining ions, this environment is not absolutely necessary. Ions can in fact be generated in air; an atmospheric pressure version of the MALDI experiment [42] was an important progenitor of ambient MS experiments.

Ambient mass spectrometry, the ionization and mass spectrometric characterization of analytes directly from their natural matrices via a sample preparation-free procedure under atmospheric pressure and at room temperature, is one of the most-welcomed advances in modern mass spectrometry. These unique features also greatly facilitate the on-site application of mass spectrometry. [11] This 'high vacuum-to-real world' transition of mass spectrometry (MS) started in 2004 with the introduction of desorption electrospray ionization (DESI), [6] which was followed by direct analysis in real time (DART), [7] desorption atmospheric pressure chemical ionization (DAPCI),

electrospray-assisted laser desorption/ionization (ELDI) [39] and analysis of samples at atmospheric pressure (ASAP). [8]

In the DESI method, a fine spray of charged droplets hits the surface of interest, from which it picks up small organic molecules and large biomolecules, ionizes them, and delivers them—as desolvated ions—into the mass spectrometer. DESI can be considered an atmospheric pressure version of SIMS. In the DART method, an electrical potential is applied to a gas with a high ionization potential (typically nitrogen or helium) to form a plasma of excited-state atoms and ions, and these desorb low–molecular weight molecules from the surface of a sample. Other closely related methods are Desorption atmospheric pressure chemical ionization (DAPCI) and Electrospray-assisted laser desorption/ionization (ELDI). DAPCI is a variant of DESI that uses gas-phase projectile ions generated by an atmospheric pressure corona discharge in the vapor of toluene or another compound, produces ions by a heterogeneous (gaseous ion/adsorbed analyte) charge-transfer mechanism; ELDI uses a laser for the desorption of neutral molecules from an ambient surface and uses charged droplets produced by electrospray for post-desorption ionization of the ablated neutral molecules. Finally, atmospheric solids analysis probe (ASAP) is another variant on atmospheric-pressure DI methods for solids analysis: in this method a heated gas jet is directed onto the sample surface, and desorbed species are ionized by corona discharge in the gas phase. [39, 43]

1.3.1 DESORPTION ELECTROSPRAY IONIZATION (DESI)

DESI allows MS to be used to record spectra of condensed-phase samples under ambient conditions; it is applicable to solid samples, including complex biological materials, but it can also be applied to liquids, to frozen solutions, and to adsorbed gases. The method has high sensitivity, is virtually

instantaneous in response time, and is applicable to small-molecule organic compounds as well as to proteins and other biological molecules.

The DESI experiment combines features of ESI with those of the family of DI methods. An electrospray emitter is used to create gasphase solvent ions, ionic clusters, and charged microdroplets, which are directed at the sample surface. An electrical potential of several kilovolts is applied to the spray solution and pneumatic nebulization is used to assist in desolvation. With the important exception of the physical state of the sample, the DESI experiment closely resembles the ESI experiment. In fact, in DESI the electrospraying of solvent droplets, assisted by auxiliary gas flow, is a fundamental step and the electrostatic field present in the surface environment has been considered responsible for the micro droplets (sub-μm) ejected from the thin solvent layer covering the surface of sample. [44] Thus, the proposed mechanism shows that ionization is effected by spraying the sample with an electrically charged aqueous mist, achieved by directing a pneumatically assisted electrospray at the surface to be analyzed. The ions released from the surface are transported through air at atmospheric pressure for some distance before they reach the atmospheric interface of the mass spectrometer (figure 18).

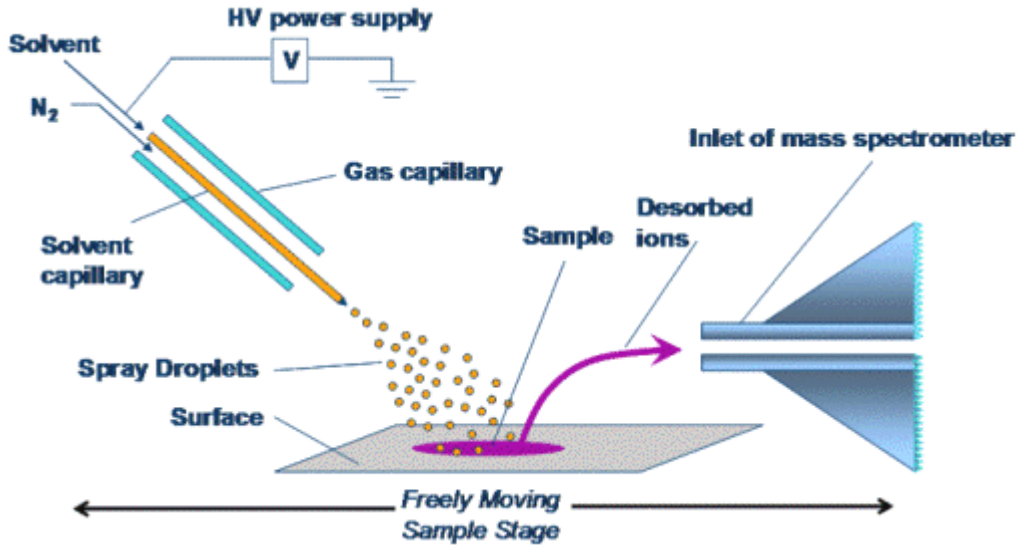


Figure 18. Schematic of DESI

The relationship of DESI with the DI methods is also close, at least at the phenomenological level. All the DI methods (plasma desorption, PD; laser desorption ionization, LDI; matrix assisted laser desorption ionization, MALDI; secondary ion mass spectrometry, SIMS; fast atom bombardment, FAB) involve the impact on condensed phase samples of projectiles, which include photons (laser desorption, including MALDI), translationally excited atoms (FAB), and energetic ions (SIMS). The projectiles used in SIMS include polyatomic ions and it is well established that their use significantly increases the efficiency of sputtering. DI experiments are conducted in a high-vacuum environment, while DESI is performed in air, which is the principal feature that distinguishes it from other DI methods.

DESI allows analysis of sample surfaces by MS without requiring, in most cases, any sample pre-treatment. In an important variant on the DESI method, selected chemicals can be added to the spray solution to provide specificity for the ionization of particular types of analytes. In all cases, the sample remains fully accessible to observation as well as additional physical and chemical processing during analysis (see figure 19).

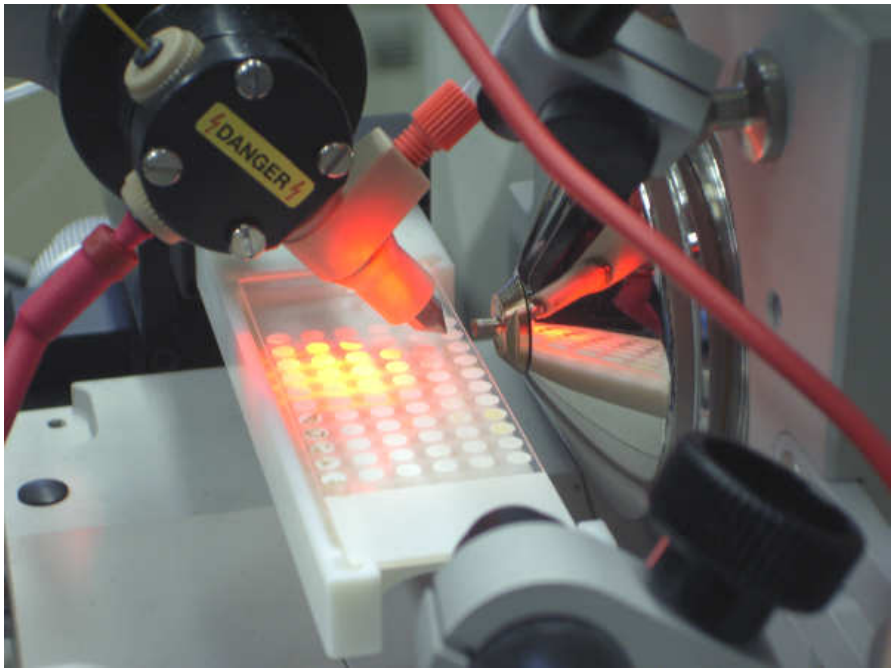


Figure 19. DESI source implemented on LTQ Orbitrap Mass Spectrometry

Implemented applications are the identification of natural products in plant material, highthroughput analysis of pharmaceutical preparations, drugs and drug metabolite identification, and quantitation in blood and other biological fluids, as well as the direct monitoring of biological tissue for biomarkers and in vivo analysis. The DESI method is applicable to the analysis of proteins and protein complexes, carbohydrates, and oligonucleotides, as well as industrial polymers and small organic molecules. Another focal point of DESI applications is forensics and public safety. Explosives, toxic industrial compounds, and chemical warfare agents are detected with high sensitivity and specificity on surfaces of a variety of common types of materials including paper, plastics, luggage, etc. as the pure compounds, in formulated mixtures and in complex solid and liquid matrixes. [43]

1.3.2 DESORPTION SONIC SPRAY IONIZATION (DeSSI)

A new method, at first sight similar to DESI, but actually substantially different, has been developed by Haddad et al. [11] in 2006: it has been called desorption sonic spray ionization and no voltage is applied to the sprayer, as schematized in figure 20.

This approach is based on sonic spray ionization (SSI) [45, 46], where the use of polar (typically methanol/water) solutions of the analyte, sprayed from a fused-silica capillary with a supersonic nebulizing gas flow coaxial to the capillary, guarantees the ion formation. SSI is unique, since neither heating nor voltage is used for ion formation. Charged droplets and consequently gaseous ions are produced at atmospheric pressure due to statistical (unbalanced) charge distribution during droplet formation in the supersonic pneumatic spray. [11]

It has been reported[43] that the DESI-MS signal intensity does not drop to zero in the absence of an electrospray voltage; DeSSI, whit out any voltage applied to the sprayer, has been proven to be a convenient method to create a dense cloud of charged droplets by action of a sonic spray of 1:1 acidic (0.01 % formic acid) water/methanol mixture. It was shown that under these conditions many protonated solvent clusters are produced, and these species can be considered responsible for the protonation of the analyte present on the surface.

Then, Haddad et al. proposed, for surface analysis of drugs in tablets, the use of DeSSI; the system is able to provide an adequate supersonic cloud of charged droplets for the efficient desorption and ionization of analytes directly from tablet surfaces, providing an alternative and more friendly (high) voltage-free environment for ambient MS. DeSSI is also found to provide more homogenous sampling and cleaner, more stable and longer lasting mass spectra, with ion abundances sufficient for tandem mass spectrometric (MS/MS) investigation. The presence of formic acid is retained essential to obtain valid results. [11]

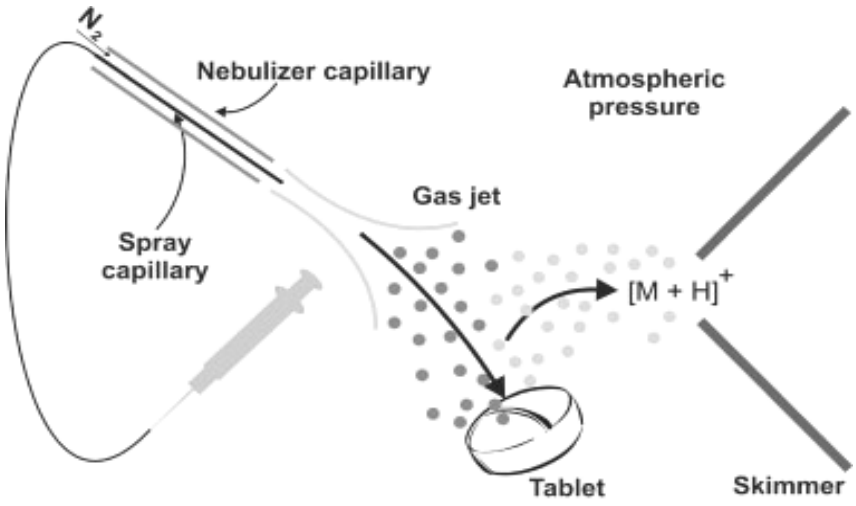


Figure 20. Schematic of DeSSI

2 DEVELOPMENT A NEW DESORPTION/IONIZATION INTERFACE

Desorption Electrospray Ionization is surely a method of wide interest, allowing the direct analysis of organic compounds present on a surface. As recently emphasized by Van Berkel et al. [47], its development has been the catalyst of a series of new investigations for ambient surface sampling and ionization and a wide number of ambient desorption/ionization techniques have been proposed.

In an extensive study on droplet dynamics[48] it has been shown that in DESI conditions impacting water droplets have velocities in the order of 100 m/s (figure 21a) and diameter less than 10 µm (figure 21b) and these data support the hypothesis that a droplet-pick-up mechanism operates for ionization under most circumstances. Alternatively it has been proposed[49] that, considering that the surface is pre-wetted by the initial droplets, the analyte desorption originates by the off-spring droplets generated by the bombardment of the solvent layer (in which the analyte is dissolved). These droplets (of sub-µm dimensions) are charged by the action of the ESI electrical field and the analyte ions are generated through the classical ESI mechanism.

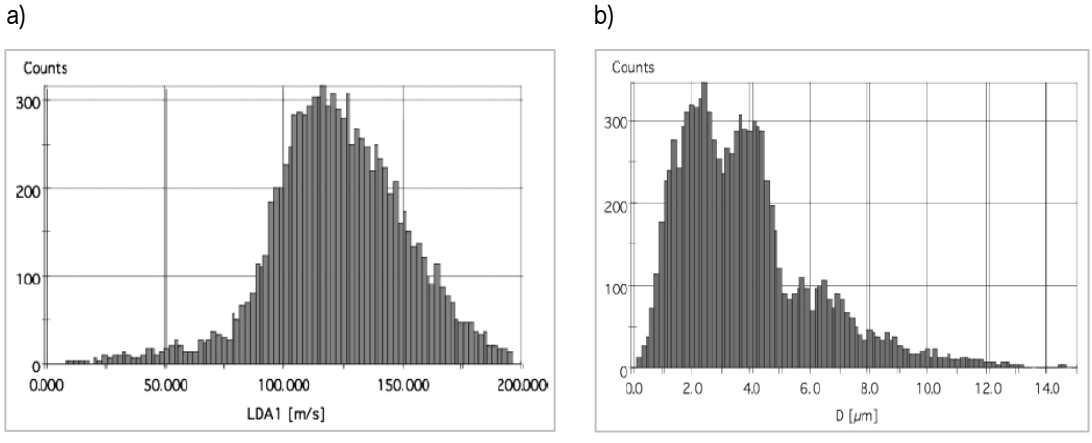


Figure 21. a) Velocities of droplets at 2 mm from the sprayer tip when a 50% methanol/water solution was sprayed using a supply pressure of 1130 kPa and a liquid flow rate of 5 µL/min. b) Diameters of droplets at 5 mm from the sprayer tip when a 50% methanol/water solution was sprayed using a supply pressure of 1130 kPa and a liquid flow rate of 2 µL/min.

However Haddad et al. [11] developed a desorption sonic spray ionization method where no voltage is applied to the sprayer, and the charged droplets were created by action of a sonic spray of 1:1 acidic (0.01 % formic acid) water/methanol mixture.

DeSSI is interesting for three main reasons:

i. the complete absence of any voltage allows its use for any instrumental configuration (e.g. for positive ion production in some instruments the sprayer is placed at high positive voltage, while the entrance capillary is placed at low positive voltage; in some other instruments the sprayer is grounded and the entrance capillary is placed at high negative voltage);

ii. the absence of any voltage allows its use on live objects;

iii. it seems to work properly either in positive or in negative ion mode.

Considering positive aspects of either DESI and DeSSI we built a DESI source and performed some experiments to investigate the intimate mechanism of ionization present in these approaches. We report here the results obtained by them, to be considered propaedeutic for the extensive use of the method in the analytical field.

2.1 PADI EXPERIMENTAL

2.1.1 SPRAYER ARRANGEMENT

We built up a new DESI-like source for the LCQ Deca (ThermoFisher) instrument.

The deployed device is the result of the models developed, the source used is the third generation, obtained by changing and improving all the individual aspects and critical characteristics identified in earlier versions. (see figure 22)

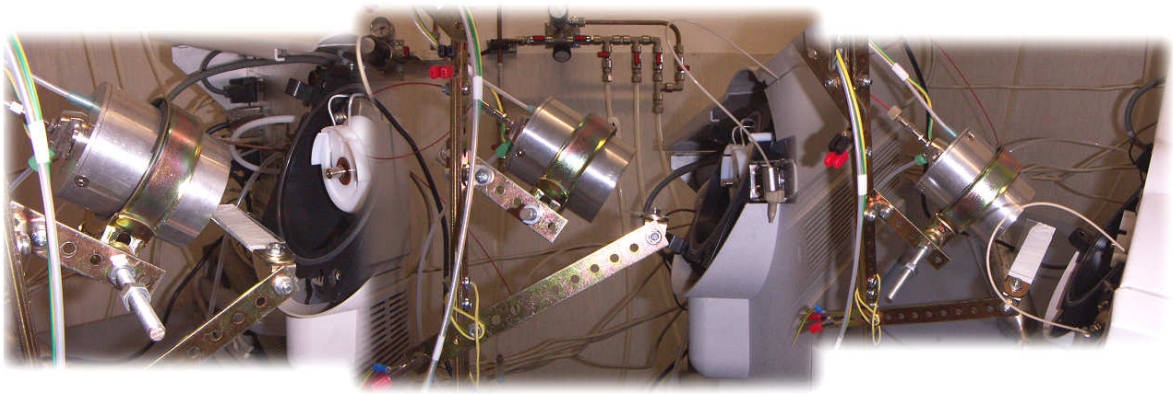


Figure 22a. The first version of PADI

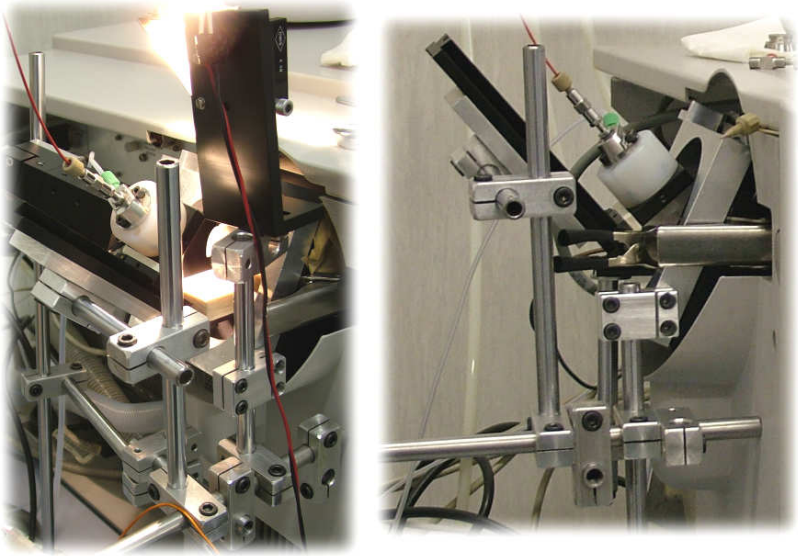


Figure 22b. The second version of PADI

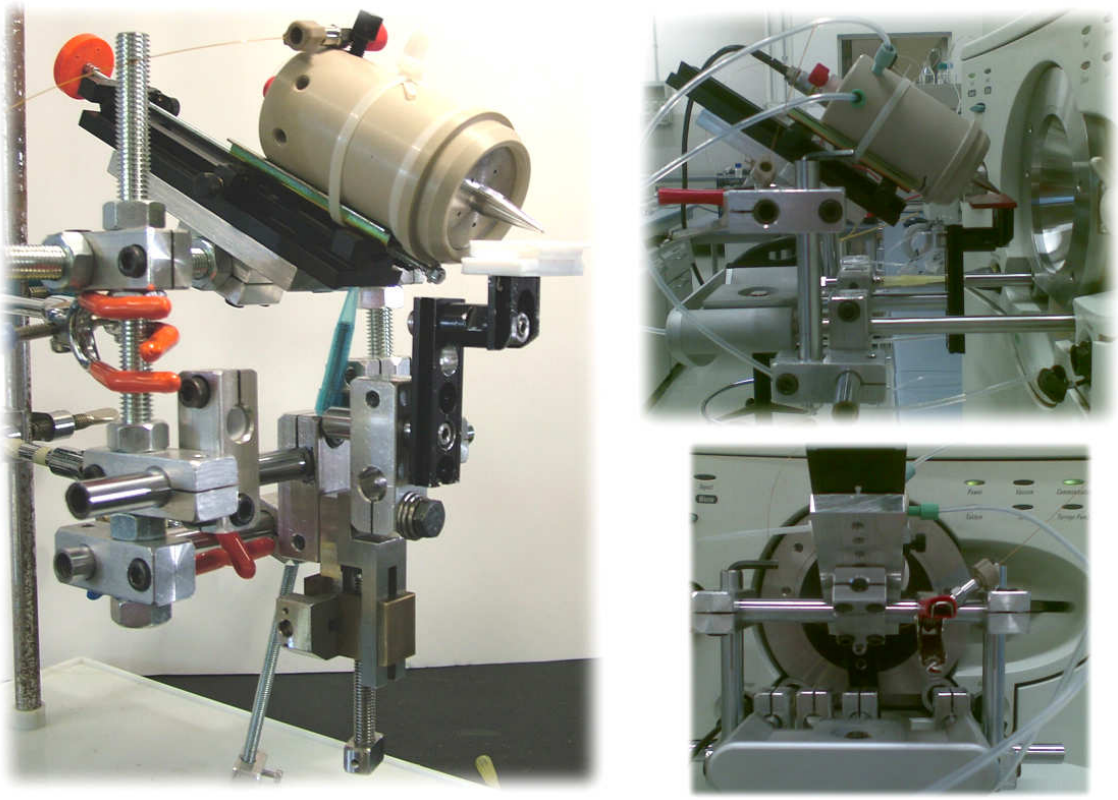


Figure 22c. The third version of PADI

The source can be easily assembled and disassembled from the supports of the mass spectrometer, thus ensuring a very easy use.

The source PADI consists of a sprayer of ThermoFisher, originally mounted on a LCQ Deca instrument, and a sample holder.

In detail, the sprayer is mounted on a carriage slide, coupled to a screw with nuts furniture that allows quick and precise movement along the horizontal axis perpendicular to the line of the capillary entrance of the mass spectrometer. The same screw is the pivot on which the sprayer can rotate to change the angle of incidence to the surface of the sample analyzed. A similar system, consisting of two screws with couples of mobile nuts allows quick and precise movement along the vertical axis perpendicular to the line of the capillary entrance of the mass spectrometer: this allows to easily adjust the angle of incidence and the

distance of sprayer to capillary entrance and sample surface. The result device is shown in figure 23: allowing movements in the J, K and L directions.

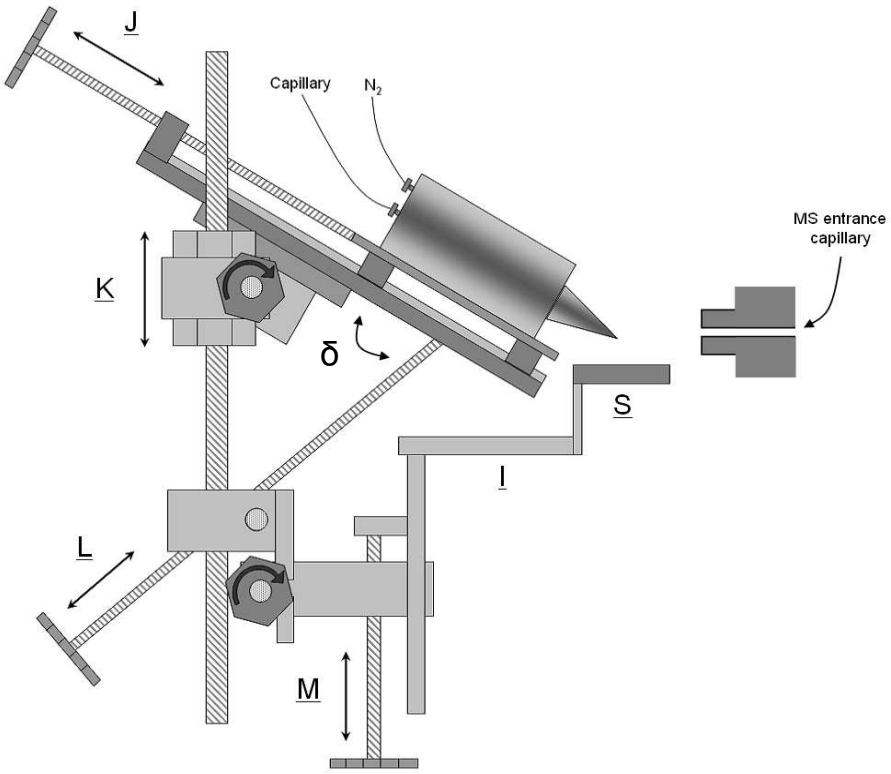


Figure 23. Scheme of the spraying system employed in PADI, allowing movements in the J, K, L and M directions with the consequent variation of the δ angle. S = sample holder; I = insulator

The sample holder is mounted on a separate 3D moving stage where a set of bolts and rocker arms allow to adjust finely the position of the sample and moves along the three axes (x, y and z) independently of the sprayer position and inlet capillary of the mass spectrometer.

In one version, the surface holder S is made with a thin PTFE block fixed and insulated from the system. In a different surface holder design, the surface holder is an aluminium block, to grounded the potential of sample surface.

The sample was placed on the surface, 2 mm close to the entrance capillary of instrument, whose position can be changed in the all direction. The surface was

insulated from the moving device. In the experiment different voltages were applied to it.

The sprayer, entrance capillary, sample holder and the sheath gas and solvent flows have been varied as described in detail in the "Results and discussion" section.

2.1.2 CHEMICALS

To proceed to a detailed study of the source, was chosen to use an analyte that would guarantee good reproducibility and a structure of the sample easy to manipulate and use. Following a preliminary phase of testing, for all experiments zopiclone tablets (7.5 mg tablet; Aventis Pharma, Milan, Italy)) were employed.

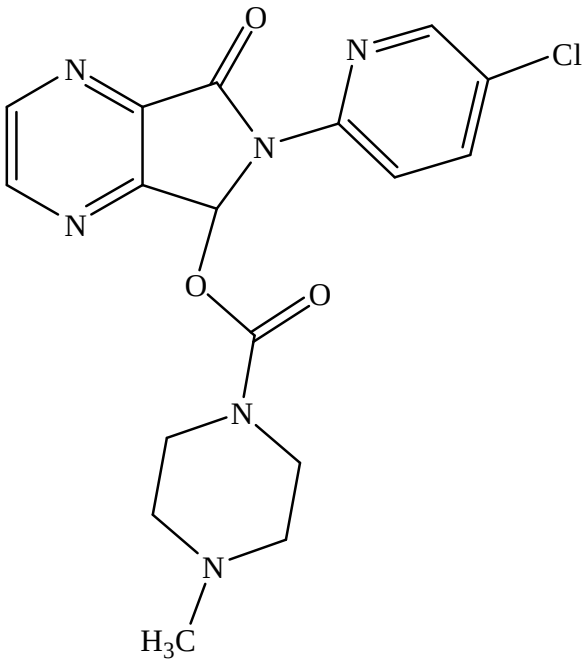


Figure 24. Zopiclone structure

The sprayed solvents (water, methanol, chloroform, acetonitrile) were analytically pure samples purchased from Sigma-Aldrich (Milan, Italy).

For preliminary application of PADI source, it was used a different type of sample: 4-metyl-7-hydroxycoumarin crystals were purchased from Sigma-Aldrich, Milan, Italy; Aspirin (Acetylsalicylic Acid), 500 mg tablets, were purchased from Bayer S.p.a., Milan, Italy; MDMA amphetamine drug tablet was conceded from judicial seizure; powder of Reserpine was purchase from Fluka Sigma-Aldrich, Milan, Italy; Furosemide, 25 mg tablets, were purchased from Sanofi-Aventis S.p.a., Milan, Italy; Ramipril, 5 mg tablets, were purchased from Sanofi-Aventis S.p.a., Milan, Italy; Pantoprazolo, 40 mg tablets, were purchased from Actavis Italia S.p.a., Nerviano (MI), Italy; Roipnol (Flunitrazepam), 1 mg tablets, were purchased from Roche S.p.a., Monza, Italy; peptide Glycyl-Glycyl-Arginin was purchased from CNR – ISTM, Padua, Italy. Cinnamon flavor of Cinnamomum zeylanicum specie, flavor of anise of Pimpinella anisum specie and radix of Echinachea angustifolia were purchase from laboratory of Plant Biology and Pharmaceutical Botany, Faculty of Pharmacy, Padua, Italy.

2.1.3 INSTRUMENTAL PARAMETERS

PADI consists in a simple spray of pure solvent, without acid contribution or high voltage. During the experimental all source and MS parameters were investigated. The operating parameters of the source PADI described below are the final results from the optimization of all variables and operational parameters that will be discussed more fully later.

The parameters of this desorption/ionization approach, listed in table 1, achieve an optimal mass spectrum for all kind of sample surfaces tested.

Table 1. Operating parameters of PADI source

Parameter	*Optimized value*	*Description*
Sprayer voltage	0V	The electrospray voltage (ES) is the voltage setting that is applied to the solvent
Sprayer Solvent flow	600-1000 µL/h	The rate at which solvent is pumped out of the solvent capillary
Sprayer Sheath Gas flow	9-16 mL/sec	The regulated flow of the gas applied to the spray head.
Distance from tip to surface	2-5 mm	The vertical distance from the end of the solvent capillary to the top of the disposable surface
Distance from MS inlet to surface	0-2 mm	The vertical distance from the end of the solvent capillary to the top of the disposable surface
MS entrance capillary voltage	0 V	The MS capillary voltage is the current applied to the mass spectrometer's inlet capillary
MS inlet temperature	260-280°C	The MS inlet temperature is the temperature setting of the mass spectrometer's inlet heated capillary
Spray Impact angle (α)	10°-60°	The angle at which the sprayed droplets from the outlet of the solvent capillary are directed towards the sample on the surface.
Collection angle (β)	5°-10°	The angle at which the secondary droplets leave the sample surface to enter the inlet of the mass spectrometer. This angle is calculated by approximating both the vertical distance from the center of the mass spectrometer inlet to the disposable surface and the horizontal distance from the inlet of the mass spectrometer to the spot on the sample being analyzed.
Solvent	ACN	The optimal solvent used to create the sprayed droplets is dependent on the composition of the sample being analyzed and its chemical-physic's characteristics.

Surface material (for liquid sample deposition)	TLC (glycerol)	In case of analyte in liquid state or in solution, the sample to be analyzed must be placed on a surface prior to being interrogated; different chemical composition and texture
Sample holder surface type	PTFE	The surface where the sample (or deposition surface) was placed.
Sample holder voltage	Floating	The surface where the sample was placed, is possible to set grounded or isolated (at specific voltage or floating itself).
Analysis time	5-60 s	The amount of time necessary to interrogate the sample in order to obtain a quality spectrum.

To obtain this values, initially preliminary tests were performed to optimize instrumental and all operative settings, to identify the most suitable conditions. During this phase of testing, for all experiments Zopiclone tablets were employed. Acetonitrile was used as spray solvent.

Comparative studies on **Sprayer voltage** were performed in duplicate, the acquisition was in MS or multiple mass spectrometry (MS/MS) mode, varying in the range 0 – 4 kV.

Comparative studies on **MS entrance capillary voltage** were performed in duplicate, the acquisition was in MS or multiple mass spectrometry (MS/MS) mode, varying in the range -100 – +100 V.

Comparative studies on **MS inlet temperature** were performed in duplicate, the acquisition was in MS or multiple mass spectrometry (MS/MS) mode, varying in the range 25 – 300° C.

Comparative studies on **Sprayer Solvent flow** were performed in duplicate, the acquisition was in MS or multiple mass spectrometry (MS/MS) mode, varying in the range 300 – 1500 µL/h.

Comparative studies on **Sprayer Sheath Gas flow** were performed in duplicate, the acquisition was in MS or multiple mass spectrometry (MS/MS) mode, varying in the range 5,88 – 26,06 mL/sec.

Comparative studies on **Sample holder voltage / Sample holder surface type** were performed in duplicate, the acquisition was in MS or multiple mass spectrometry (MS/MS) mode, varying in the range 0 (grounded) – 1000 V and floating state.

Comparative studies on **Surface material (for liquid sample)** were performed in duplicate, the acquisition was in MS or multiple mass spectrometry (MS/MS) mode, testing glass, polycarbonate sheet, filter paper, thin layer chromatography (TLC) plate and aluminium sheet.

Comparative studies on **Solvents** were performed in duplicate, the acquisition was in MS or multiple mass spectrometry (MS/MS) mode, testing water, methanol, chloroform and acetonitrile.

Other parameters (Distance from tip to surface, Distance from MS inlet to surface, Spray Impact angle (α), Collection angle (β) and Analysis time) have been developed and optimized depending on the type of surface analyzed. The reported values are the average of all test for all kind of sample.

2.2 RESULTS AND DISCUSSION

A key aspect of PADI method, applied to several complex matrix, is the number of controllable operating parameters that can be investigated and optimized to obtain an efficient surface analysis. The most important variables are taken in consideration were the source geometry (the spray angle and the ion uptake angle, as well as the various distances in aligning the spray, sample and mass spectrometer), characteristic of sprayer (contents of the solvent spray and gas flow rate, voltage) and surface-sample parameters (composition, texture, temperature, potential and possible solvent or matrix used to deposition of sample)

In the desorption/ionization approach we have verified the importance to study and optimise all variables to achieve an optimal mass spectrum for all kind of sample surfaces. Distance from tip to surface, Distance from MS inlet to surface, Spray Impact angle (α), Collection angle (β) and Analysis time have been developed and optimized depending on the type of surface analyzed, because both the chemical composition and the texture of surface dramatically affect the efficiency and the specific characteristics of each survey.

With regard to geometric parameters, they are direct related to the efficiency on the collection of analyte sample from surface and, hence, on the sensitivity of the method. The exact mechanism of ionization of the analyte has not been completely understood, however, in our opinion, the pneumatical contribution act for desorption/ionization.

We can schematize the general desorption/ionization mechanism as follows:

1. the droplet impacts on the surface and the liquid is spread on an area 3-10 times larger than the droplet original diameter;
2. the "capture" of the analyte takes place in the spreading phase of the droplet on the surface itself;
3. during this phase droplets are emitted in the region of the expanding liquid film (jetting);

4. the offspring droplets could be transferred into the mass spectrometer by various effects: the gas jet spray, the electrostatic field and the reduced pressure interface close to the entrance capillary.

This model is surely valid considering the fate and mechanisms related to a single droplet. In our opinion it is important to emphasize that hundred of droplets per second are sprayed onto the surface and, consequently, different mechanisms can be operative. The charge of the different droplets are all transferred to the surface and, even if the kinetic energy of a droplet is not enough to promote the ion sputtering, it is reasonable to assume that the impact on the liquid solvent surface promotes the formation of elastic waves, the top of which can act as a sprayer leading to the release of the analyte containing micro-droplets.

2.2.1 ROLE OF HIGH ELECTRICAL FIELDS

As emphasized in the paper of Takas et al. [43] the DESI-MS spray parameters also influence the spectral characteristics of DESI. These parameters determine the size distribution, average charging, and velocity of impacting droplets and ions.

The DESI method developed by Cooks is carried out by directing an electrospray of droplets onto the surface to be analyzed at atmospheric pressure. It foresees the use of a classical pneumatically assisted ESI source, with a high voltage applied to the needle, to generate charged microdroplets of pure solvent that bump onto the sample surface. Impact of the primary droplets at the sample surface creates secondary droplets which carry with them analyte molecules that are fully desolvated to form gas-phase ions. The resulting mass spectra are similar to normal ESI mass spectra. In this instrumental configuration, two strengths acts for desorption/ionization: electrical and

pneumatical. In figure 25 is reported a general scheme of DESI and the Applied Voltage on the device.

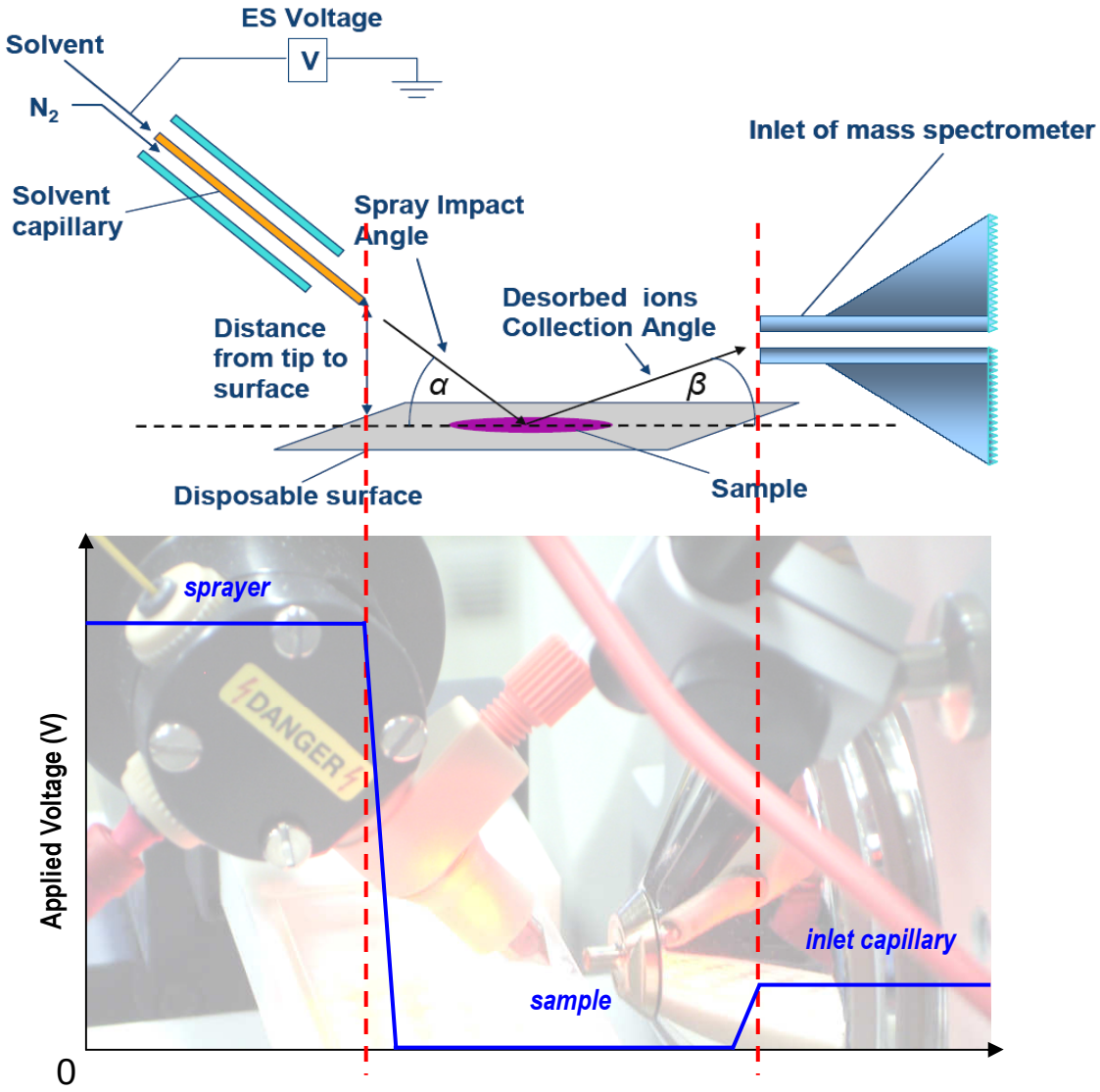


Figure 25. General scheme of DESI (upper) and potential configuration profile

Interestingly, the MS signal intensity does not drop to zero in the absence of an electrospray high voltage. It is known that the sprayer used here also works in the sonic spray mode, generating charged droplets without application of a high voltage. This phenomenon suggested to the Eberling group[11] that the DESI source can operate also in the sonic spray ionization conditions, even if at a

lower sensitivity, the DeSSI. In absence of any electrical field, the analyte protonated molecule was proposed to be formed in the presence of organic acid in the sprayed solvent.

This aspect was the stimulus for a series of investigations.

First of all we tested the signal intensity decrease related to MH^+ of zopiclone contained in a drug tablet by varying the sprayer voltage: this experiment have used a mixture of H_2O and CH_3OH 1:1 (v/v) as solvent sprayer . Quite surprisingly, as shown in figure 26, the intensity changes from 4×10^5 to 1.2×10^5 passing from 4 kV to 0 kV sprayer voltage. Then the sprayer voltage has surely an effect, but in its absence a signal with intensity of the same order of magnitude (10^5) is obtained, with a decrease to 30% of the original intensity.

In this case, in contrast with DeSSI method, the solvent sprayer not have organic acid contribution.

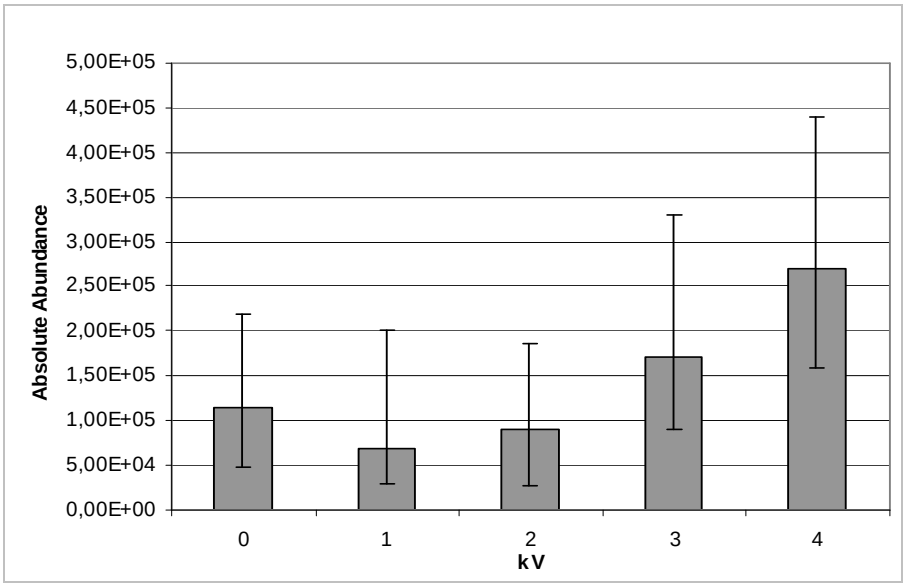


Figure 26. Intensity of MH+ of zopiclone obtained by varying the sprayer voltage in the range 0-4 kV

Then, the question is: why the analyte molecule was protonated?

Under these conditions there are no charge on solvent droplets produced by the nebulizer or acid contribution and then no charge is present on the surface. Could be suggested a possible generation of charges related to dissociation constant (K) of the solvent. To verify the presence of charges on droplets, with the sprayer at ground potential, has made a simple experiment as shown in figure 27.

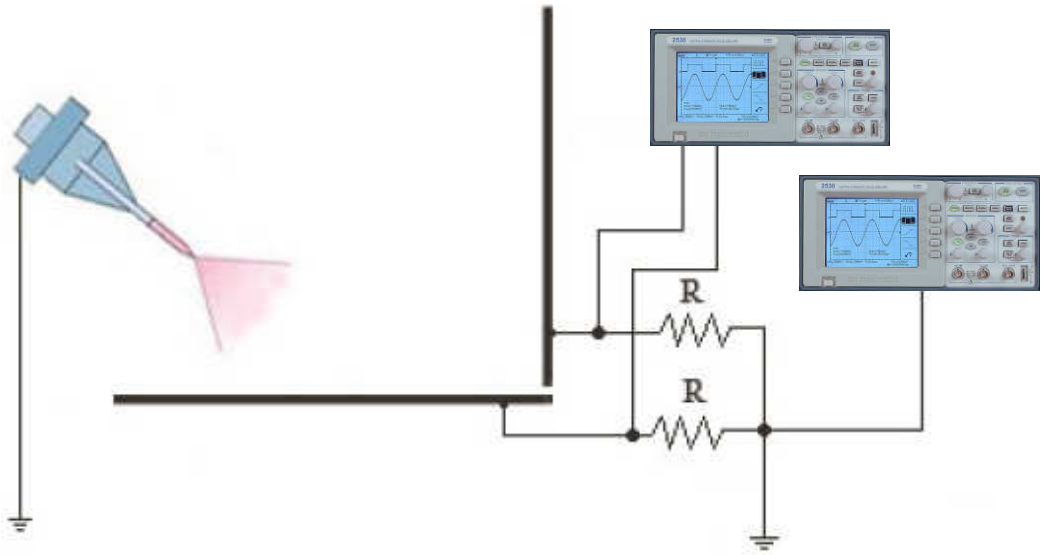


Figure 27. Experiment setup to verify the presence of charges on droplets with sprayer at ground potential

The sprayer was activated on a conductive surface connected to ground through a resistance of 10 KΩ, then the voltage across the resistance has properly measured by an oscilloscope. Whereas no signals are observed on the oscilloscope, it follows that any current due to charged droplets are less than 1uA, and virtually non-existent (it must be remembered that in the current ESI conditions is of the order of 10 to 102 mA).

Now, considering that signal intensity related to MH+ of zopiclone tested decrease is of the same order of magnitude also in absence of any field, these data indicate that the ion production is mainly governed by mechanism(s) other than those due to the presence of high electrostatic fields (as, for example, charge deposition on either impacting or secondary droplets) and consequently all the further measurements were performed at 0 kV sprayer voltage.

Then, different voltages (in the range 0 – 1000 V) were applied to the surface of sample holder and the best results in term of signal intensity and stability were obtained for V = 0. For these reasons all the further measurements were performed by keeping the surface at ground potential.

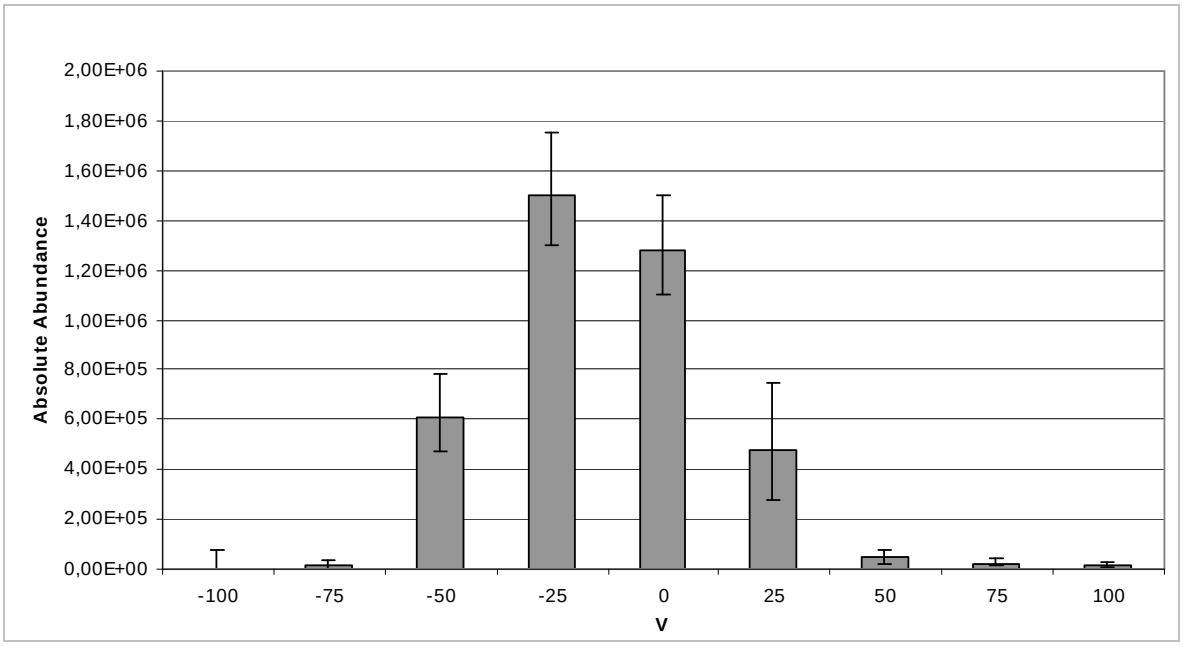


Figure 28. Intensity of MH+ of zopiclone obtained at 0 V sprayer voltage by varying the entrance capillary voltage in the range -100 ÷ +100 V

The entrance capillary voltage (varied in the range -100 – +100 V) affects the signal intensity (figure 28). At 0 V a signal intensity of 1.2×10^6 a.u. is obtained and for positive voltages it shows a dramatic decrease, dropping practically to 0 a.u. for values higher than +50 V. On the contrary for negative values of entrance capillary voltage a small increase is observed at – 25V (passing from 1.2×10^6 to 1.5×10^6), while for lower values a trend analogous to that observed for positive voltage values is present. The effect, even if moderate, observed for Vcapillary = -25 V could be explained by a "lens effect" with respect to positive ions and/or positively charged droplets emitted from the surface, before their entrance into the capillary. However all the further measurements were performed by keeping Vcapillary = 0.

In other words all the components of the system (sprayer, surface, entrance capillary) were kept at ground potential.

Hence, the potential profile related to PADI configuration is reported in figure 29.

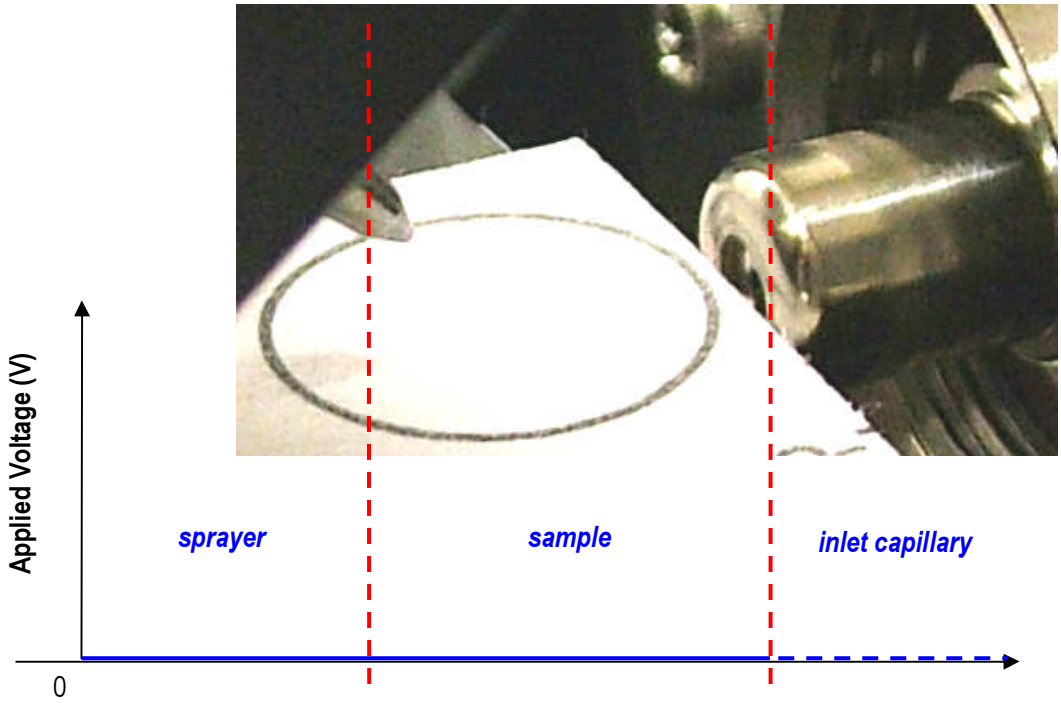


Figure 29. Potential profile related to PADI configuration

Finally, the entrance capillary temperature was varied in the range 25-300°C. In this range the best results were obtained for temperatures between 200-280 ° C, with increased signal almost four times the value obtained at 100 ° C (see figure 30). For the MS inlet heated capillary, we decided to use the temperature between 260-280°C, because this range provides the best results of reproducibility and stability of signal intensity.

The increase in temperature leads to an increase in kinetic energy. This promotes the phenomenon of desolvatation, similarly to ESI mechanism, related to the increase in kinetic energy of molecules (gas phase, solvent and analyte) present in the capillary entrance of the mass spectrometer. This increase of kinetic energy also influence the mechanism of ionization, as discussed below in section "Ionization Mechanism(s)". Probably for high temperature (greater than 280 ° C) the excess of kinetic energy prevents the formation of ion species.

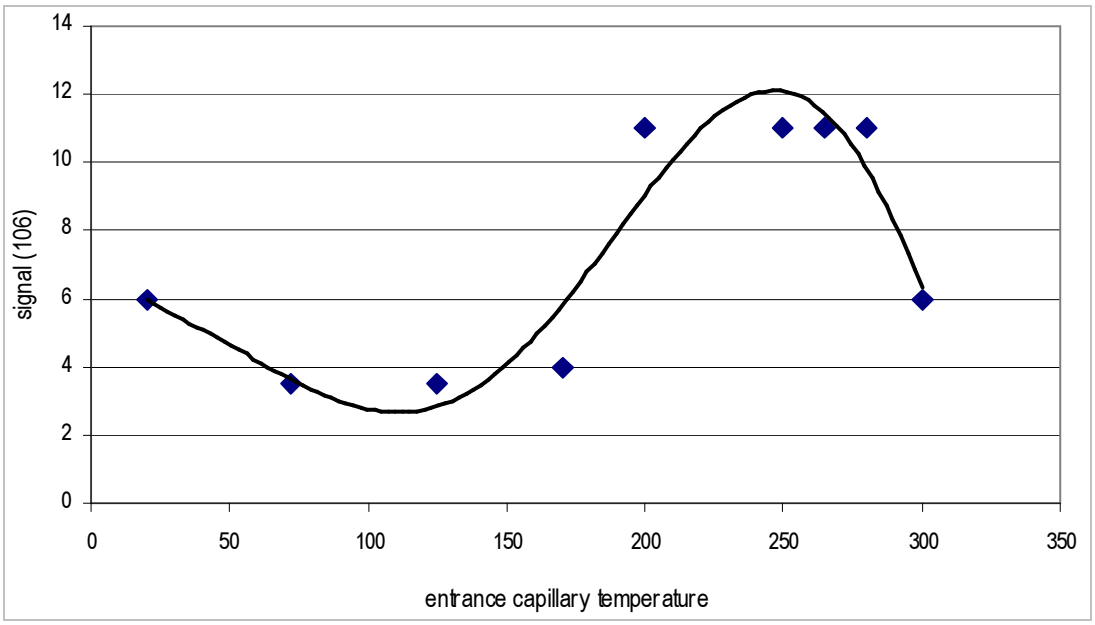


Figure 30. Intensity of MH^+ of zopiclone obtained by varying the entrance capillary temperature in the range 25 - 300 °C

2.2.2 SPRAYER & SURFACE WETTING

The first effect of the bombardment with the solvent sprayed droplets on the surface is the formation of a thin solvent layer which solubilizes the analytes present on the surface itself.

The formation of this layer depends on many different factors, that must be considered to achieve valid and well reproducible experimental conditions.

First of all it must be taken into account that the surface interacts with a high nitrogen flow (typically of 75 a.u., corresponding to 26 mL/s), essential to perform DESI and DeSSI experiments. Then the presence and thickness of the solvent layer will critically depend on:

1. the sprayed solvent and nitrogen flows. The latter is essential to obtain the appropriate spray density and droplet (either primary or secondary) kinetic energy and dimensions;
2. the physico-chemical characteristics of the solvent (mainly boiling point, viscosity, polarity) which reflect on its interaction ("bonding") with the surface; its evaporation rate from the surface under the nitrogen stream and on the solubility of selected analytes present on the surface, mainly related to its polarity;
3. the physico-chemical properties of the surface. Experiments performed on smooth surfaces (e.g. stainless steel, glass, polymers) lead to very weak and unstable signals, due to the instability of the solvent layer under the high nitrogen flow. Surfaces with high affinity with solvents (e.g. silica, eccipients present in drug tablets) generally lead to more intense and stable signals.

The different phenomena can be schematized as follows: a solvent thin layer is formed by the splashing of the sprayed solvent droplets on the surface. Then, in the surface-liquid interface the analyte solubilization takes place. In the liquid-gas interface a critical equilibrium between solvent deposition by

droplets and solvent evaporation by action of the nitrogen stream exists and this equilibrium is strongly perturbated by the emission of secondary microdroplets originating by the splashing of primary solvent droplets on the solvent thin layer.

2.2.2.1 SPRAYED SOLVENT AND NITROGEN FLOWS

Solvent flow rate and gas flow rate have a dual role in PADI, as well as DESI.

Both are essential to determine the drop size and velocity of impact on the surface. This determines how it will wet the surface and then how efficient will be the process of analyte solubilizing and subsequently pick-up mechanism from the surface, more extensively explain in the later section "Droplets – Solvent Layer Interaction".

As expected, different results are obtained for different values of solvent flow and sheath nitrogen flow. First of all, a calibration curve relating the arbitrary units (a.u.) given by the instrument software with the real nitrogen sheath gas flow was calculated, allowing the evaluation of the real nitrogen flow value interacting with the surface (table 2 and figure 31).

Table 2. Calculation of real nitrogen sheath gas flow

instrumental unit	*psi*	*atm*	*flux (mL/sec)*	*flux (µL/min)*
10	**10**	0,68	**2,71**	1,63E+05
20	**20**	1,36	**5,88**	3,53E+05
25	**25**	1,70	**7,58**	4,55E+05
30	**30**	2,04	**9,25**	5,55E+05
40	**40**	2,72	**12,57**	7,54E+05
50	**50**	3,40	**16,03**	9,62E+05
75	**75**	5,10	**26,06**	1,56E+06

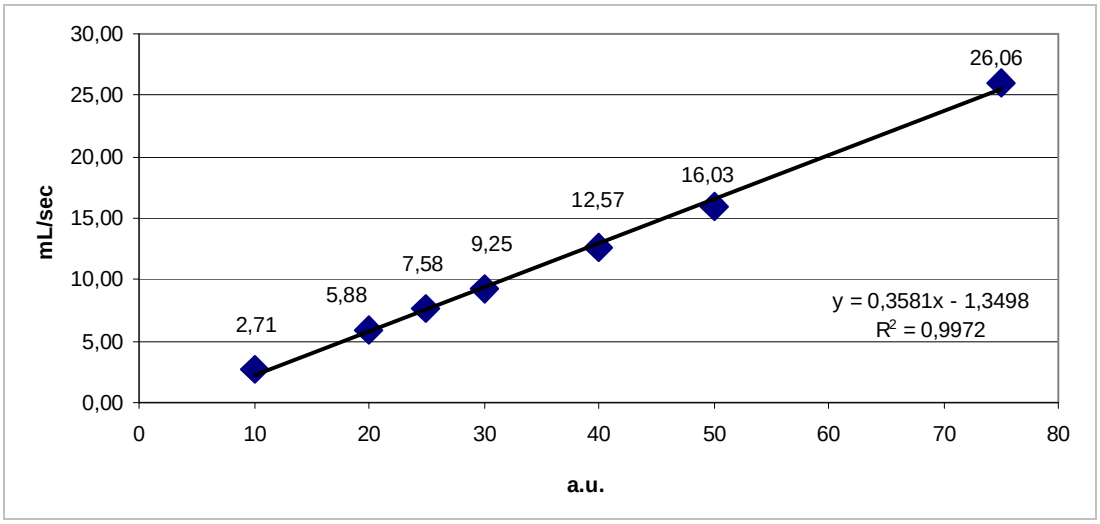


Figure 31. Calibration curve relating the arbitrary units (a.u.) to real nitrogen sheath gas flow and pressure

The studies on Sprayer Solvent flow were performed varying in the range 300 – 1500 µL/h. For each value of flow of solvent, the flow of Sprayer Sheath Gas flow was varied in the range 5,88 – 26,06 mL/sec; the studies was performed by monitoring the signal intensity of the molecular ion of zopiclone.

As shown in figure 32, for a solvent flow of 300 µL/h the most intense signal (~ 7×10^5) is obtained for N_2 flow of 75 a.u., corresponding to 26 mL/s. With this flux intensities detected were extremely variable and not reproducible as absolute abundance. Even in the case of flow at 600 µL/h the signal strength of the molecular ion is small, although the reproducibility of results is better. Substantially different results in the case of flow 1000 µL/h. The signal intensity increases gradually with increasing the flow of carrier gas. With the greatest flow of solvent (1500 µL/h) the best results are obtained by the gas flows between 9 mL/s and 12 mL/s: this range guaranteed the acquisition of a constant and highly reproducible signal, comparable to that achieved by flow of solvent 1000 µL/h, at the same gas flow rate.

In the case of the best intense signal (~ 2.5 × 10^6), with Sprayer Solvent flow at 1000 µL/h and Sprayer Sheath Gas flow at 26,06 mL/sec, the molecular ion abundance is highly variable and does not guarantee good reproducibility. The apparent gain of approximately twice the signal in absolute value, not an actual increase in performance of their method in relation to decreased reproducibility of the same.

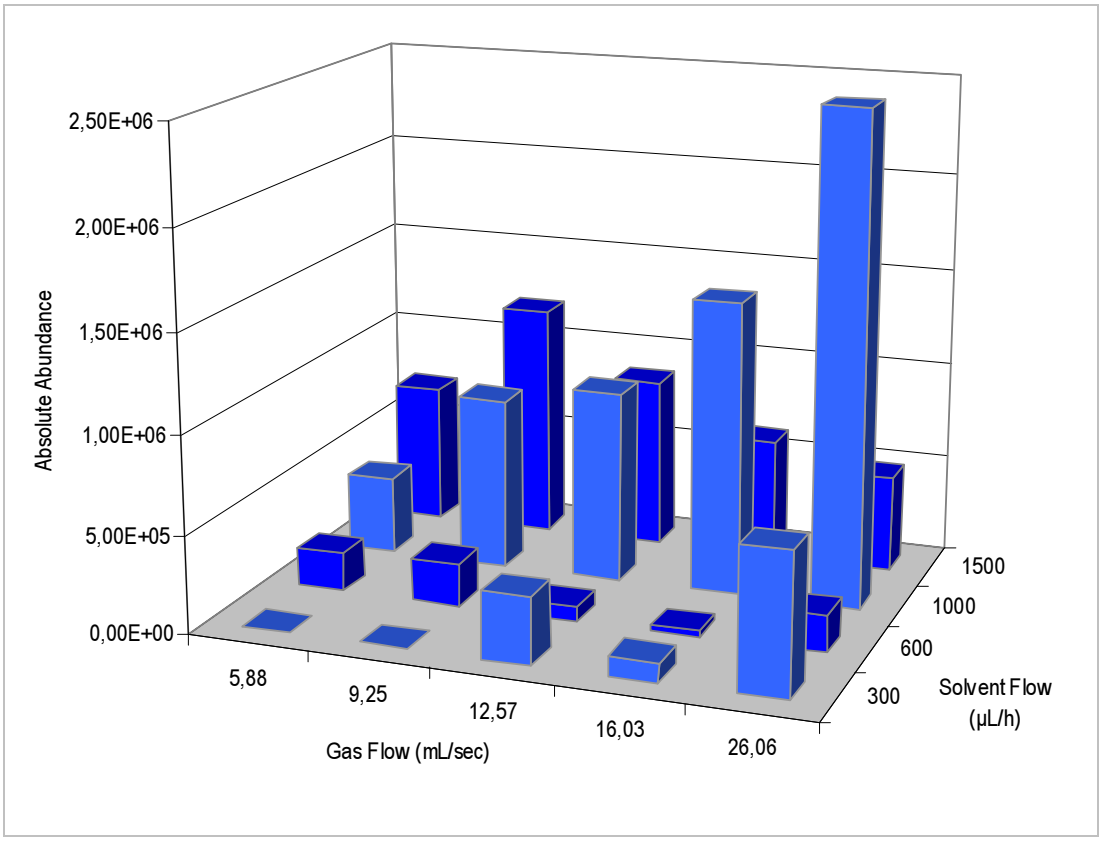


Figure 32. Variation of the MH^+ intensity of zopiclone by varying both solvent flow and sheath gas (nitrogen) flow and keeping sprayer, entrance capillary and surface at ground state.

In all cases, high gas flow rates decrease the initial droplet size and increase the velocity of impacting droplets. These phenomena are advantageous up to a point; because smaller droplet sizes favour enhanced desolvation efficiency and droplets having higher velocity produce more offspring droplets upon impact. Above a certain limit, however, the small size and high velocity will cause droplet evaporation before impact with the surface, a result that appears to correlate with the decrease of ion formation and MS revelation: the high gas velocity decreases the sampling efficiency of the mass spectrometer.

Solvent flow rate is also expected to have an effect on droplet size distribution in relationship with gas flow rate.

At low solvent flow rates, the droplet size may be too small for survival of the droplets from the spray tip to the surface, a phenomenon similar to that described in the case of high gas flows.

High solvent flow rates result in the formation of larger droplets, which might cause inefficient desolvation and, in extreme cases, accumulation of liquid on the surface. These constraints define a clear optimal working range for solvent flow rates which is summarized, together with optimum values of gas flow rate, in figure 33.

The best results in terms of reproducibility and absolute abundance were obtained for N_2 flow of 9-16 mL/sec and solvent flow of 600-1000 μL/h.

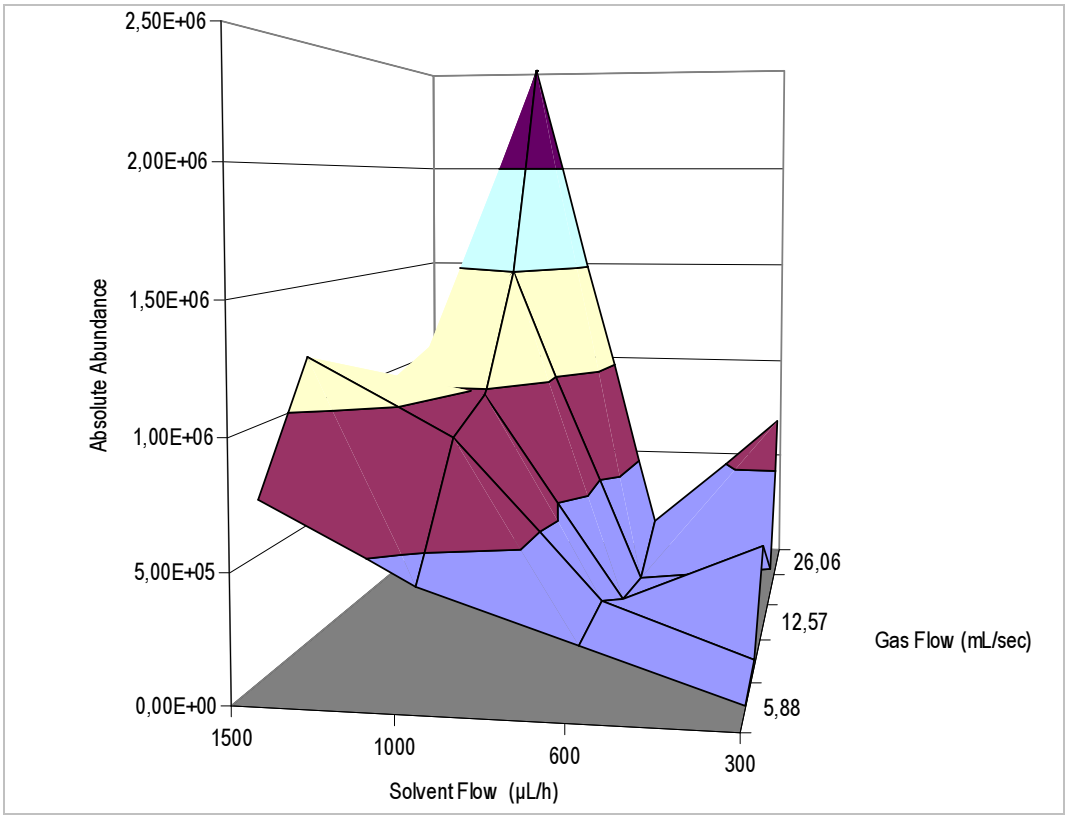


Figure 33. 3D representation of results in varying both solvent flow and sheath gas (nitrogen) flow

The observed differences can be explained by the different equilibria for solvent layer production, solvent evaporation rate from the layer, number and kinetic energy of the impinging solvent droplets. It must be emphasized that the N_2 flow is higher than those usually employed for ESI direct infusion (12 mL/s) and HPLC coupling (20 mL/s).

These data indicate that the desorption phenomena is mainly pneumatically driven.

2.2.2.2 PHYSICO-CHEMICAL CHARACTERISTICS OF SOLVENT

The choice of solvent is essential to ensure that the analyte or analytes sought to dissolve in the thin layer of solvent sprayed on the surface of the sample analyzed. Moreover, the solvent must ensure the proper balance in the evaporation phase, the gas-phase, so it is vital to its surface tension.

The solvent usually sprayed in DESI and DeSSI experiments is a 1:1 mixture of methanol and water, in the latter case always acidified with formic or trifluoroacetic acid.

The use of this solvent mixture necessarily leads to the solvation of the polar compounds present on the surface and water, methanol and the organic acid have been invoked as protonating agents, leading to the formation of protonated molecules of the analyte. However, due to their physico-chemical proprieties (as, for example, density, viscosity, evaporation rate) this solvent mixture results also in the formation of quite stable solvent layer on the surface and its removal by the nitrogen stream becomes more difficult than that observed with more volatile solvents.

The spray solvents tested are water, methanol, chloroform, acetonitrile.

In the case of hydrophilic surfaces the spraying of water, as well as water-methanol mixtures, leads to a severe degradation of the surface itself (see below). The use of acetonitrile ensured the preservation of the matrix, for example a pharmaceutical tablet of zopiclone, and allow even up to one hour test on the same surface sample. In any case it has proven to be effective in dissolving all organic analytes of interest.

The solubility of the analytes, however, is not the only characteristic to consider. In fact, the zopiclone is soluble in chloroform, also. Now, the same experiment carried out with chloroform has not yielded any results, indicating that the process of desorption / ionization consists of several different phases.

Then Acetonitrile was chosen as the reference solvent.

Acetonitrile (figure 34) compared with water (figure 35), methanol (figure 36) and methanol/water (1:1 v:v) (figure 37), for the same sample of zopiclone tablet, has a mass spectrum cleaner with less noise, with a decided increase in the signal of molecular ion, from 10^4 to 10^7. This indicates a better ionization of the analyte compared to other solvents. This will be discussed in detail later.

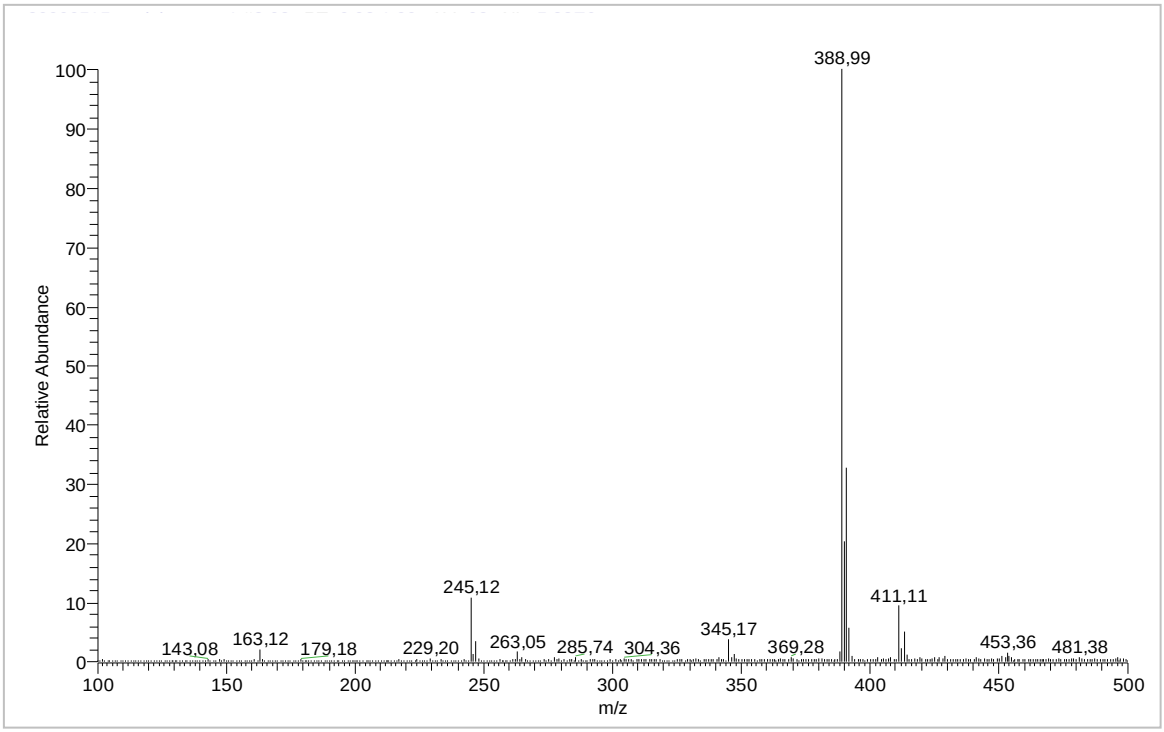


Figure 34. Positive ions mass spectra obtained by spraying a zopiclone tablet with acetonitrile

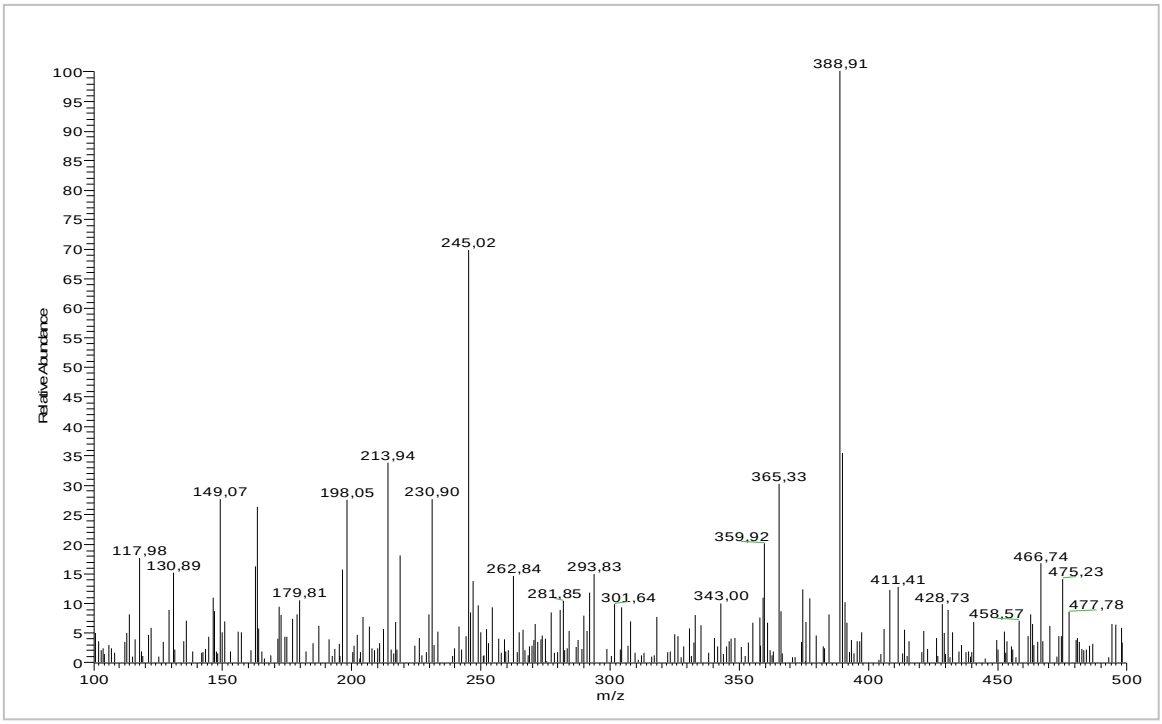


Figure 35. Positive ions mass spectra obtained by spraying a zopiclone tablet with water

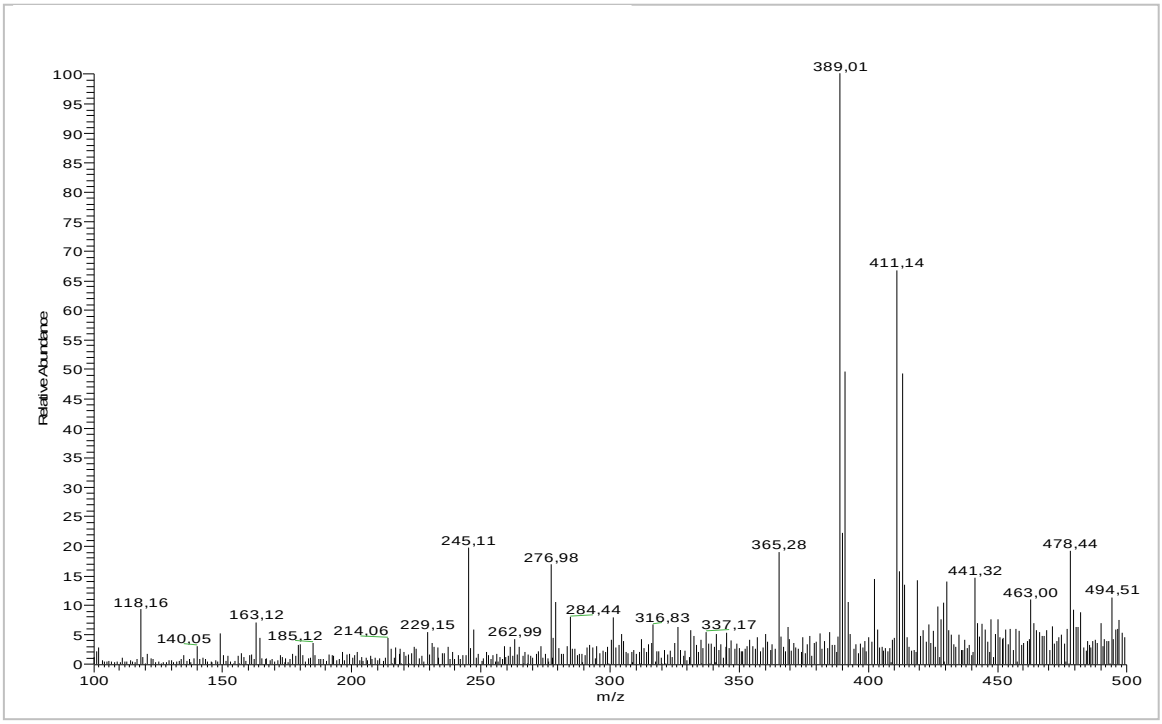


Figure 36. Positive ions mass spectra obtained by spraying a zopiclone tablet with methanol

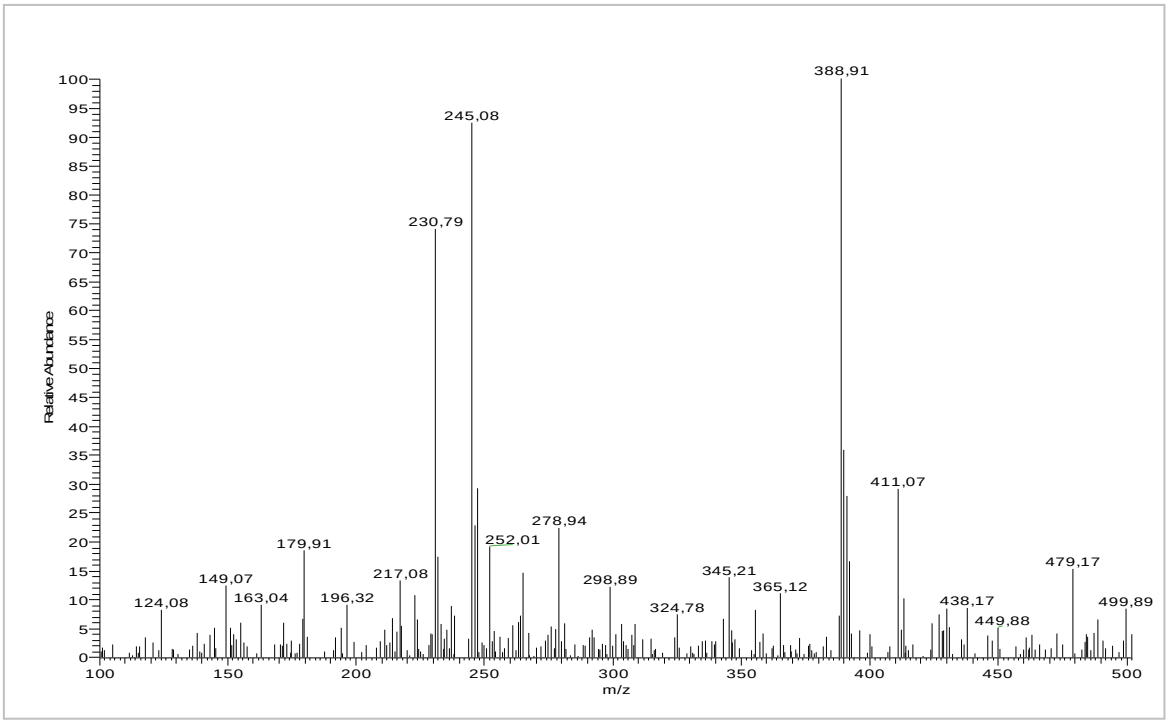


Figure 37. Positive ions mass spectra obtained by spraying a zopiclone tablet with methanol/water (1:1; v:v)

2.2.2.3 PHYSICO-CHEMICAL PROPERTIES OF SURFACE

Both the chemical composition and the texture of a surface dramatically affect the desorption process. In DESI technique, particularly important in ionization is the electrical conductivity of the surface[43]. In fact, since the DESI mechanism involves the landing and release of charged particles at a surface, neutralization at the surface must be avoided. Neutralization is most likely in the case for conductive materials, such as metals or graphite, which must either be carefully isolated or floated at a potential that is equal to or lower than the spray voltage. Signal stability depends strongly on whether the surface prefers the polarity of the spray or not. In PADI, PTFE as highly isolation and inert polymer gives excellent signal stability in the positive and negative ion mode, as reported later.

The choice of solvent type and sprayer conditions is strictly related to the physico-chemical properties of the surface. A fundamental point that must be considered is the critical evaluation of the nature of the surface under investigation.

In the case of hydrophilic surfaces the spraying of water, as well as water-methanol mixtures, leads to a severe degradation of the surface itself. As an example the photos of a tablet of zopiclone before and after 1 min H_2O spraying (1000 µL/h) are reported in the inset of figure 37b. The spectra obtained by spraying water at t = 5 s and t = 1 min are reported in figure 37a. While in the former case the protonated zopiclone molecule is detected at m/z 389, in the latter (figure 37b) any trace of this ion is no more present, indicating that the morphology of the surface is a major factor.

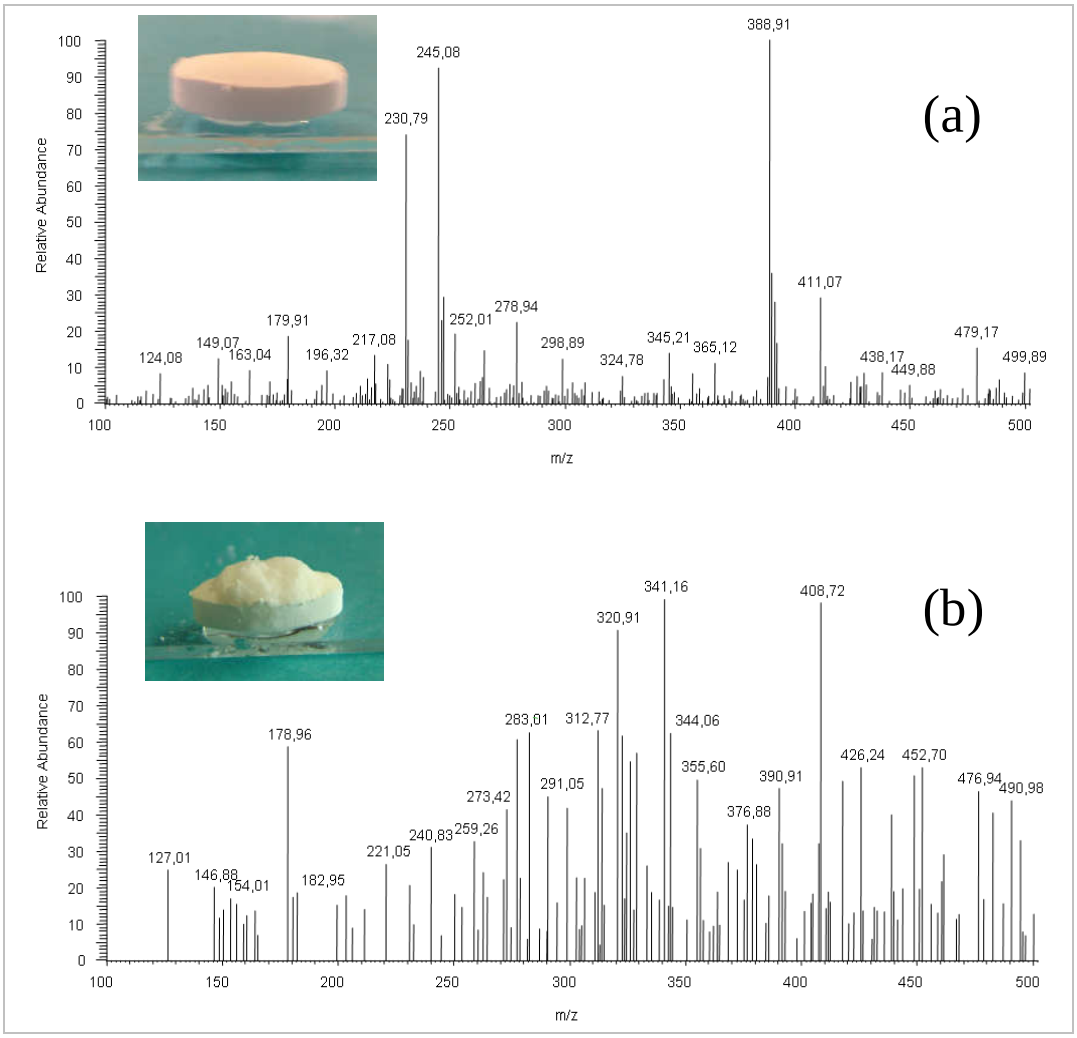


Figure 37. Positive ions mass spectra obtained by spraying water on a zopiclone (MH^+ at m/z 389) tablet: (a) after 1 min (signal intensity: 2.1 x 10^5) and (b) after 5 min (signal intensity: 1 x 10^4). The photographs of the tablet are reported in the inset.

When the sample is liquid or in solution, the chemical nature of the surface of sample holder is also an important parameter, because it is necessary to deposit the sample. The nature of the surface may affect crystallization of the analyte, causing uneven distribution of the analyte on the surface. Higher affinity of analyte molecules to the surface results in a loss of sensitivity. Moreover, higher porous or irregular surface would adsorb or redistribute the

solvent sprayed and inhibit the formation of thin solvent layer upon the sample surface, necessary to perform the pick-up mechanism.

The surface tested were PTFE, glass, polycarbonate sheet, filter paper, thin layer chromatography (TLC) plate and aluminium sheet. All surfaces tested gave the same background ions with similar signal intensities. The background ions were mainly thought to result from compounds in the room atmosphere and possible instrument and solvent impurities. Thus, none of the surfaces was observed to suppress the ionization of the analytes.

In PADI, the best results was obtained with PTFE; its inert polymers and highly isolation propriety gives excellent signal stability in the positive and negative ion mode. In other hand, when the sample is liquid or in solution, the nature of the surface seemed to have a significant effect on the signal stability of the analytes. With PTFE, glass and polycarbonate sheet, the distribution of the analyte would seemed to be more uneven, causing very unstable analyte signals and 'sweet spot' effects, together with a rapid loss of the analyte signal. In any cases, PTFE surfaces could also be used several times after washing with acetonitrile or other organic solvents without any memory effects from previous sample applications.

TLC plate, instead, showed stability thanks to the even spreading of the solution on it, which also resulted in a longer duration of the sample signal than with the other surfaces. For testing, 25 µL soluzion of zopiclone, obtained dissolving a thin amount of tablet in acetonitrile, was placed on TLC plate and sprayed with acetonitrile at solvent flow of 1000 µL/h; the MS spectrum obtained, are reported in figure 38. We obtained a mass spectrum cleaner with less noise, with an absolute abundance of molecular ion of about 10^5.

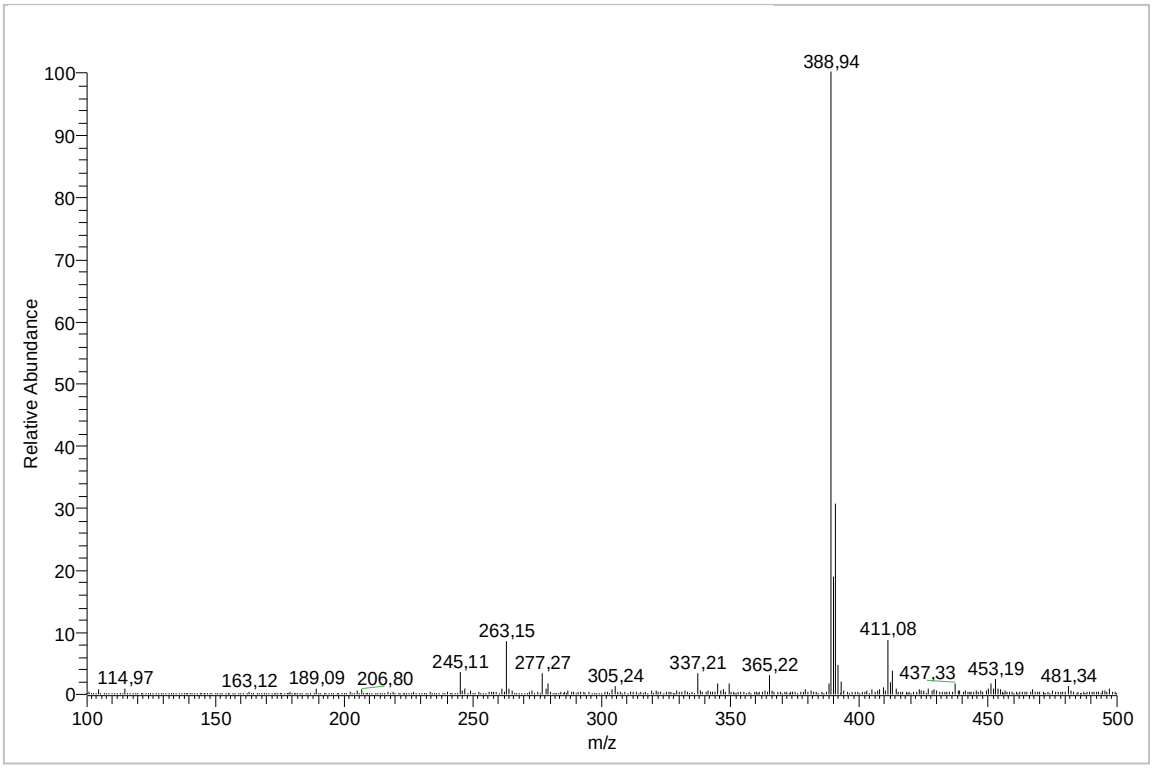


Figure 38. Positive ions mass spectra obtained after deposition of zopiclone solution on a TLC plate

One problem in using the TLC plate is related to its intrinsic propriety. In case of large quantities of samples or a very complex matrix (rich in components), it is possible that the deposition of the sample produces a true chromatography of constituents, such that the analysis is not as uniform and the result varies depending on the position of the sample surface where the desorption is carried out. This may be also positive, for example, where the separation of its constituents can make a complex matrix easier to interpret.

The use of filter paper is possible but it does not give a good sensitivity, probably due to the thicker adsorbent layer, because the high porosity prevents the proper formation of the layer of solvent on surface, even with extremely fine filter papers.

If analytes are immiscible or slightly miscible in solvents, it is not possible to make a efficient and reproducible deposition on sample holder. Now, it was

tested the use of glycerol, as wettable agent, to form a suspension or permit properly moisten the surface. Moreover, in using the TLC plate the glycerol was allowed to obtain good results in terms of analytical MS response. However, the signal was not constant over time but tended to decrease gradually in relation to the elimination of the layer of glycerol by the action of pneumatic spray.

2.2.2.4 DROPLETS – SOLVENT LAYER INTERACTION

In the case of DESI with high voltage on the sprayer, we fully agree with the mechanism given by Venter et al. [49]:

1. the surface is wetted by initial droplets;
2. the analyte is solubilized in the solvent layer;
3. later-arriving droplets impact the solvent layer, creating a high number of off-spring droplets (secondary droplets) (analogous to what can be easily seen in larger scale on the road during summer storm!) of the analyte solution present on the surface;
4. the off-spring droplets interact with the strong electrical field present in the DESI source, and the ionization follows the ESI mechanism.
5. In the case of absence of any electrical field points 1) – 3) can be still considered, being mainly governed by pneumatic effects.

Recently some interesting studies have been published in order to explain the secondary droplet formation and the ionization phenomena occurring in DESI conditions, i.e. in presence of electrostatic fields. In the first paper [50] by Costa and Cooks numerical multiphase fluid dynamics simulations were used to describe the droplet-wet surface interaction leading to the production of secondary drops from solutions. In the second paper [51] experimental data (based on current measurement showing charge-discharge phenomena) demonstrate that the DESI source behaves as a capacitor with a well defined

time constant, and that the surface can be considered an electrical active element of the circuit. Consequently the dielectric constant of the surface material is an important factor: PTFE exhibits the greatest time constant. The experimental data showed that hydrophobic materials are excellent DESI substrates and that the surface charge can cause electrochemical oxidation of the analyte. Of course the dielectrical constant of the sprayed solvent will also play a role.

To compare what happens in PADI, a further experiment was performed by placing a zopiclone tablet either on a grounded stainless steel surface or a dielectric (Teflon), electrically floating, surface. If some charges (to the extend so limited to be undetectable by the millivoltmeter of the above described experiment) would be carried by the sprayer droplets in absence of any field, some differences in the abundance of MH^+ ions would be present. But, spraying the tablet for 10 s in the two different conditions, identical spectra with identical ion abundances were obtained.

Thus the ion production in PADI method without any voltage cannot be related to extra charges brought by sprayer droplets on the surface. In PADI any charge is not present on the surface and the mechanism just discussed above cannot be operative for the ion production. Consequently the analysis of what happens when uncharged droplets interact with the solution can be surely of interest.

What are the origin of the charges? In particular, what are the protonating agents responsible for the MH^+ production?

Venter et al. [48] have calculated that in a DESI experiment a 3 μm droplet reaches the surface with a speed of 100 m/s. Its kinetic energy is consequently in the order of 500 MeV.

It is worth of noting that an "old guard" ionization method, called ^{252}Cf plasma desorption, is based on the interaction of fission fragments of ^{252}Cf (usually $^{142}Ba^{+18}$ and $^{106}Tc^{+22}$) with kinetic energy in the MeV range, i.e in an energy regime analogous to that present in the DESI experiments. These

fragments interact with the surface of a thin (~ 1 μm) nickel foil. The sample is deposited on the other side of the foil and ions are generated by the fission fragment bombardment on the Ni foil, primarly through electronic exitation.

In the present case, even considering that this energy is distributed across a relatively large contact area, a portion of this kinetic energy must be necessarily transformed in heat. Once can imagine a series of events as those schematized in figure 39.

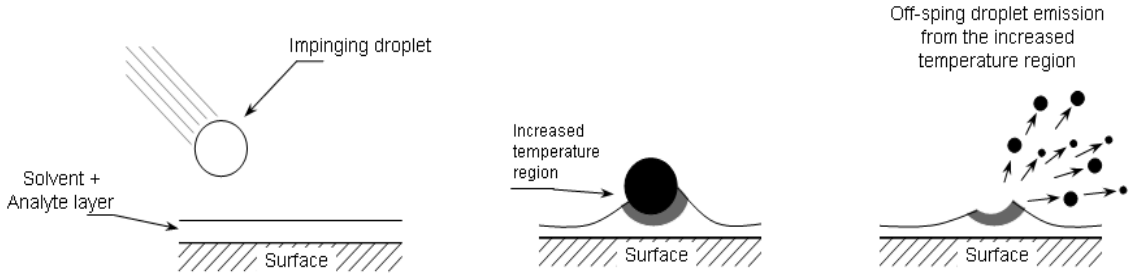


Figure 39. Interaction of an impringing droplet with the solvent+analyte thin layer showing the local heating in the collision region and the release of secondary "hot" droplets.

Once the droplet is splashed on the surface, its high kinetic energy (transferred as heat in the solvent layer) induces the local heating of the surface and promotes the formation of an elastic wave with the consequent emission of off-spring droplets. This heating can be easily calculated. In fact, considering an impacting droplet of 3 μm diameter, its volume is

$$V = 4/3\ \pi\ r^3 = 4/3\ \pi\ (1.5 \times 10^{-6})^3\ m^3 = 14.11 \times 10^{-18}\ m^3$$

and its mass is (considering that 1 $m^3 = 10^3$ kg and a density = 1)

$$m = 14.11 \times 10^{-15}\ kg$$

As calculated by Venter et al. [48], the droplet will reach the surface with a speed of 100 m/s. Consequently its kinetic energy will be

$$\varepsilon_k = \tfrac{1}{2}\, mv^2 = \tfrac{1}{2}\, 14.11 \times 10^{-15} \times 10^4 = 7.5 \times 10^{-11}\ \text{J}$$

And, considering that 1 J = 6.24×10^{18} eV

$$\varepsilon_k = 468\ \text{MeV},$$

in agreement with the Venter et al. [48] evaluation.

Now, considering that 1 J = 0.239 cal, it results that

$$\varepsilon_k = 1.79 \times 10^{-11}\ \text{cal}$$

The number of droplets reaching the surface every second will depend on the solvent flow. If we consider a flow of 10 µL/min = 0.167 µL/s and that

$$1\ \mu\text{L} = 10^{-6}\ \text{L} = 10^{-9}\ \text{m}^3$$

the number of droplets (N) reaching the surface every second for a solvent flow of 10 µL/min will be

$$N = \frac{0.167 \times 10^{-9}}{14.11 \times 10^{-18}} = 11.83 \times 10^6\ \text{droplets/s}$$

and the total kinetic energy deposited on the solvent thin layer every second will be (in calories)

$$\varepsilon_k = 11.83 \times 10^6 \times 1.79 \times 10^{-11} = 2.12 \times 10^{-4}\ \text{cal.}$$

Considering that the heat (Q) and the temperature (T) are related by the classical equation

$$Q = c_s\, m\, \Delta T$$

where c_s is the specific heat capacity and m is the mass, it follows that the temperature increase, ΔT, can be calculated by

$$\Delta T = Q/c_s\, m.$$

If we consider that:

1. only a portion (reasonably in the order of 10%) of the kinetic energy of the impacting droplets will be transformed in heating of the thin solvent layer covering the surface (inelastic/elastic impact ratio of 0.1);
2. water as solvent with density = 1;
3. a thin layer volume of 3.0×10^{-3} cm^3 (as due to a 6 mm diameter drop with 100 μm thickness);
4. the specific heat capacity of water is 1 cal g^{-1} $°C^{-1}$;

it follows that:

$$\Delta T = \frac{2.12 \times 10^{-4} \times 10^{-1}}{1 \times 0.3 \times 10^{-3}} = \frac{2.12 \times 10^{-2}}{0.3} = 0.07\ °C$$

that represents a moderate increase of the solvent thin layer temperature.

If we consider a local temperature increase, as due to a completely inelastic impact of a single droplet with an equal volume of thin layer solution, we obtain

$$\Delta T = \frac{1.79 \times 10^{-11}}{1 \times 14.11 \times 10^{-12}} = 1.27\ °C$$

This local heating of solution could lead to a decrease of the pKa of the solvent present in the original layer, increasing the yield of the analyte protonation, but the small temperature increase suggests that this effect can play only a minor role.

2.2.3 IONIZATION MECHANISM(S)

The hypothesis just reported could be in agreement with the most of published results and, over all, could give account for the MH^+ formation even in absence of any electrical field. However, as hypothesis, it needs to be confirmed by a series of experiments.

For this aim, purely pneumatic desorption experiments (without any voltage applied to the source) were performed with solvent exhibiting different pk_a: water (pk_w = 14.17 at 20°C), methanol (pk_a = 15.5) acetonitrile (pk_a = 24), tetrahydrofuran (pk_a > 50) and chloroform (pk_a = 25), using a zopiclone tablet as test bench.

Considering that pk_a decrease by increasing the temperature (as an example pk_w = 14.17 at 20°C, 13.02 at 60°C and 12.42 at 90°C), it follows that H^+ concentration increases by increasing the temperature. The calculation above reported indicate that the increase of H^+ concentration will be small.

However some differences should be observed by using different solvents, even if the data must be critically evaluated: in fact if on one hand the MH^+ ion intensity should be related to the thermodynamics of the protonation reaction, on the other the analyte can exhibit a different solubility in different solvents, thus leading to microlayers of solution at different analyte concentrations.

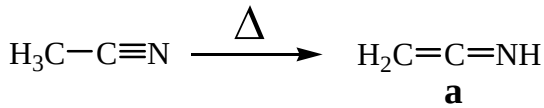
As a matter of fact the MH^+ of zopiclone was not detectable by using tetrahydrofuran (THF) and chloroform. It is to emphasize that zopiclone is soluble in $CHCl_3$ and CH_2Cl_2. Thus the results obtained by spraying $CHCl_3$ cannot be ascribed to solubility aspects but, at first sight, to the relative high volatility of chloroform that does not allow the formation of a stable thin layer of the analyte solution.

The spectra obtained by using acetonitrile, H_2O, methanol, methanol/H_2O (1:1 v:v) (in all cases without the addition of organic acids) are reported in figure 34, 35, 36 and 37. Signal intensities (a.u.) of MH^+ ions of 5.99×10^7, 1.5×10^4, 6.39×10^5 and 2.9×10^4 have been obtained for acetonitrile, H_2O, methanol,

methanol/H_2O (1:1 v:v) respectively. As can be seen, the best results in term of both MH^+ ion intensity and chemical background suppression is obtained with acetonitrile.

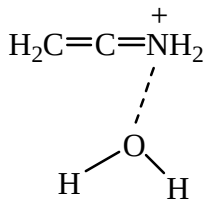
These results could be justified by a high solubility of the analyte in acetonitrile, but, on the other hand, it is difficult to consider acetonitrile as an highly effective protonating molecule, able to protonate zopiclone in DeSSI conditions with a yield two-three order of magnitude higher than those achieved with water and methanol.

It could be hypothesized that, as observed in APPI conditions and confirmed by theoretical calculation [52], acetonitrile could undergo a thermally-induced isomerization



a

Compound **a** exhibits an ionization energy of 9.5 eV, much lower than that of acetonitrile (IE_{ACN} = 12.5 eV) and can be considered surely more reactive than acetonitrile. In the same investigation [52] it was shown that structure **a**, when protonated, can easily lead to a complex with H_2O, with the possible structure



b

Interestingly, looking at the low m/z region of the spectra (see figure 40) any trace of the corresponding ions (m/z 41 and 59) was undetectable but two abundant ions are detected at m/z 64 and m/z 105, corresponding to [ACN +

Na]$^+$ and [2ACN + Na]$^+$ respectively. They could be invoked as responsible for the production of [M + Na]$^+$ ions of zopiclone, but not for [M + H]$^+$.

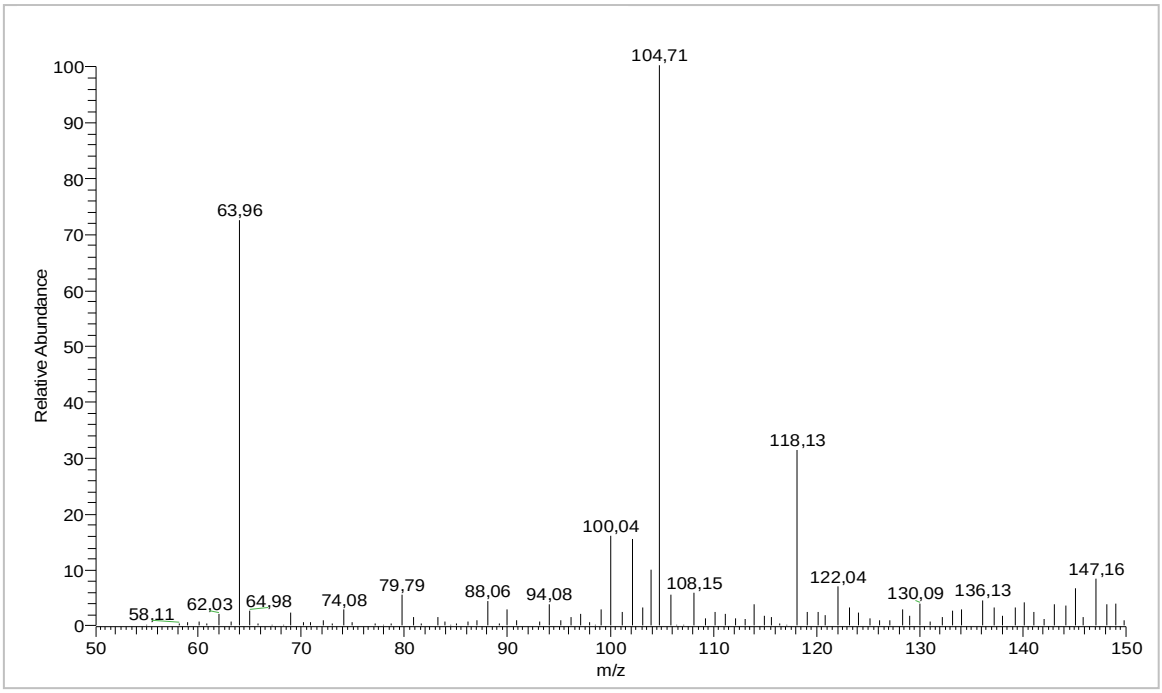


Figure 40. Low m/z region of the positive ions mass spectra obtained by spraying a zopiclone tablet with acetonitrile

.

In order to establish the possible role of acetonitrile as protonating agent, a further experiment was carried out by spraying deuterated acetonitrile, CD_3CN, on zopiclone tablet. In these conditions MH^+ and not MD^+ ions were observed, proving that acetonitrile cannot be considered responsible for the production of protonated molecules. The spectra obtained by using CD_3CN is reported in figure 41, molecular ion [M+H]$^+$ at m/z 389 and related sodium adduct [M+Na]$^+$ at m/z 411 are visible. Underlying these signals it is easily visible the isotope pattern of chlorine.

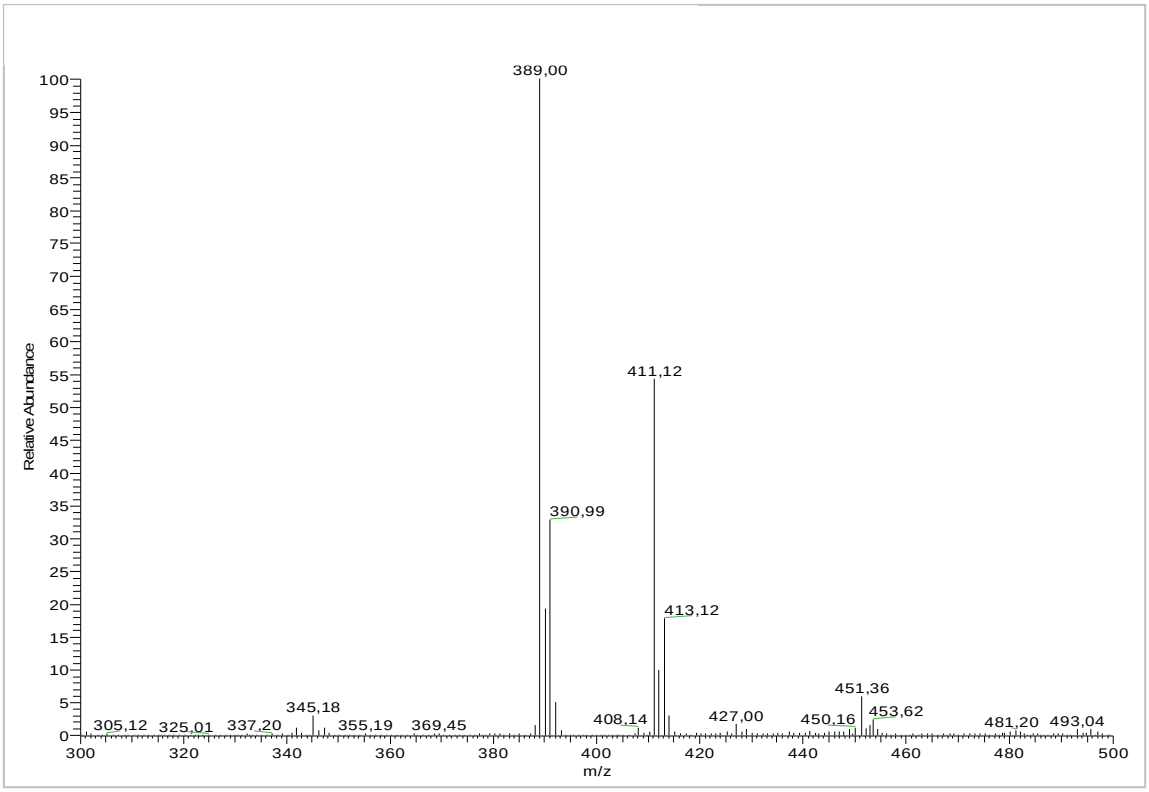


Figure 41. Positive ions mass spectra obtained by spraying a zopiclone tablet with deuterated acetonitrile CD_3CN

Then, from where is the proton coming?

Surely, the presence of humidity in the tablet as well as in the atmospheric pressure ion source environment cannot be excluded, but if these water molecules would be the proton origin, the best signal intensity would have to be obtained using water as sprayed solvent, contrary to what shown by figures 34 - 37.

The other hydrogen atoms available in the acetonitrile-analyte system are those of analyte itself and the protonation could originate through thermally induced decomposition of analyte dimers with the formation of an ion pair:

$$2\,M \xrightarrow{\Delta} MH^+ + [M - H]^-$$

This hypothesis can be immediately excluded, due to the absence of $[M - H]^-$ anions in the negative ion mass spectrum.

However, looking at the high mass region of the positive ion mass spectrum, a quite abundant ion at m/z 799 is detected (see figure 42). Its mass value, as well as the isotopic cluster, indicates for it a structure of $[2M + Na]^+$ species of zopiclone. The less abundant ion at m/z 777 corresponds to the proton bound dimer $[2M + H]^+$. These processes cannot be related to electrochemically driven mechanisms, due to the complete absence of any electrostatic field in the experimental set-up.

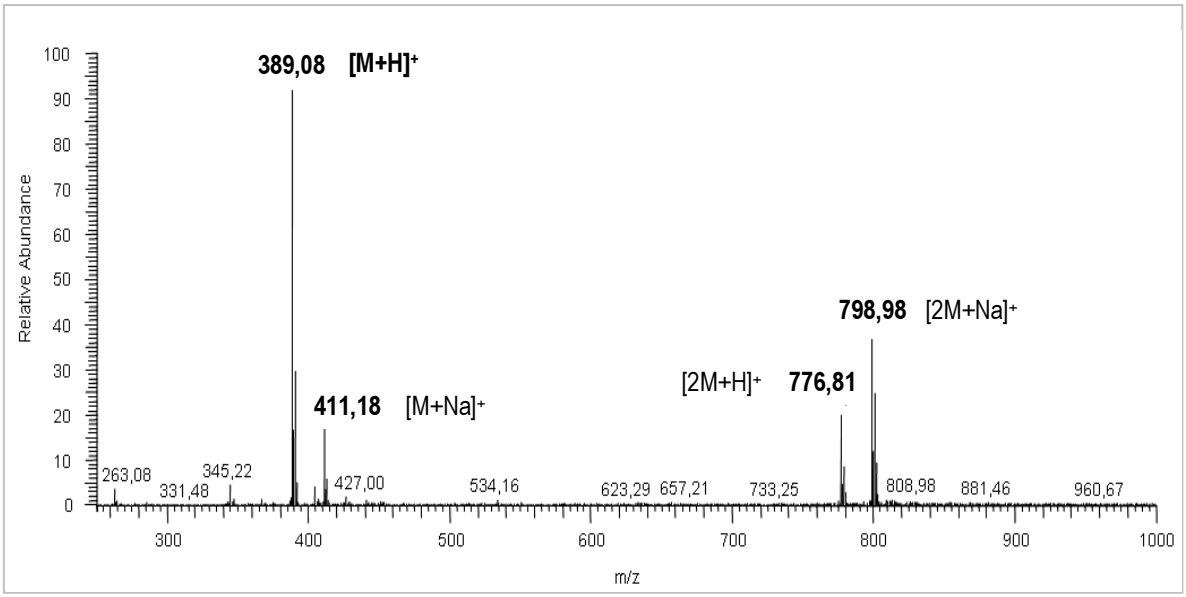


Figure 42. Enlarged view of the positive ions mass spectra obtained by spraying a zopiclone tablet with acetonitrile, showing the presence of $[M_2H]^+$ (m/z 777) and $[M_2Na]^+$ (m/z 799) ions

The pickup of analytes from the surface observed in both DESI and DeSSI conditions can be somehow related to the experiments performed by Gebhardt et al. [53] which described a new ionization mechanism, called *clusterelectric effect* (schematized in figure 43). They observed that neutral cluster of water (as well

as SO_2) molecules lead, by impact with a solid surface, to the production of a series of positive and negative fragment ions, even if the kinetic energy of the neutral species is lower than its ionization energy. All the positively charged species carry an alkali ion. The yield of charged species can be significantly enhanced by treating the surface with alkali atoms. An analogous mechanism could be invoked to justify the results shown in figure 42. The pickup from the solution of the analyte and Na^+ (or other alkali) ions would lead to an easy formation of either $[M + Na]^+$ or $[2M + Na]^+$ ions. We emphasize that the presence of Na^+ ions would enhance the acidity of the secondary droplet solution, making available H^+ for the analyte protonation.

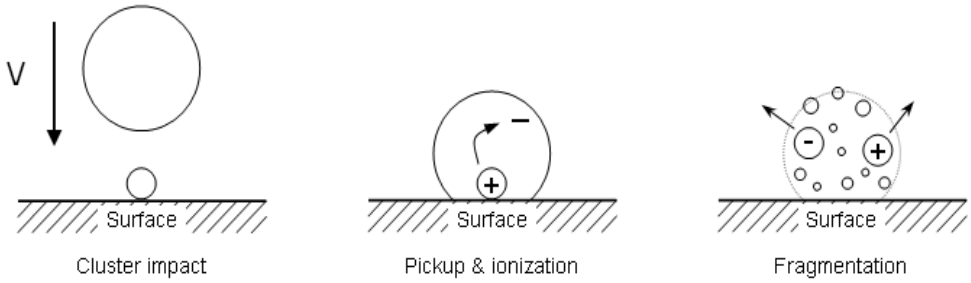


Figure 43. Scheme of the cluster effect mechanism

A further thought was stimulated by the behaviour observed by varying the sheath gas (N_2) flow above discussed, showing that the ion intensity increases by increasing the flow. This behaviour can be explained by the production of droplets with high kinetic energy, more effective for the splashing phenomenon but, in our opinion, this behaviour seemed to be quite strange. In fact high N_2 flows would reflect in a higher solvent evaporation rate, making the solvent thin layer formation on the surface quite unstable. Apart from these effects,

mainly of pneumatic nature, an active role of N_2 could be taken into account, considering the possibility of N_2 ionization by collision.

The velocity of nitrogen molecules can be easily calculated by

$$v = \frac{\dot{V}}{s}$$

where V° represents the nitrogen flow and *s* is the section of the channel through which nitrogen is flowing.

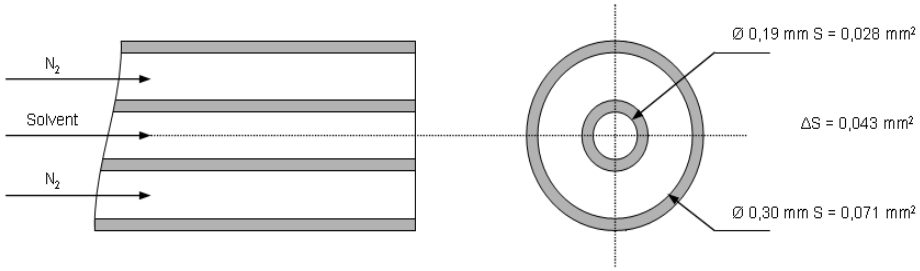


Figure 44. Dimension of the sprayer device employed to calculate the N_2 speed

Considering the pneumatically assisted sprayer dimensions reported in figure 44, it follows that s = 0.043 mm^2 = 43 × 10^{-5} cm^2. Consequently, for a nitrogen flow of 26 cm^3/s

$$v = 26 / 43\times10^{-5} = 0.604 \times 10^5 \text{ cm/s} = 0.604 \times 10^3 \text{ m/s}$$

The molar kinetic energy of nitrogen can be calculated as

$$\varepsilon_k = \tfrac{1}{2}\, mv^2 = \tfrac{1}{2}\,(28 \times 10^{-3}) \times (0.604 \times 10^3)^2 = 5.11 \times 10^3 \text{ J}$$

and, considering that 1 J = 6.241 × 10^{18} eV, it follows that:

$$\varepsilon_k = 31.45 \times 10^{21} \text{ eV/mol.}$$

Then the kinetic energy of a nitrogen molecule is

$$\varepsilon_{k\ \text{Nitrogen}} = \frac{\varepsilon_{KM}}{N_A} = \frac{31.45 \times 10^{21} \text{ eV/mol}}{6.022 \times 10^{23} \text{ mol}^{-1}} = 5.22 \times 10^{-2} \text{ eV}$$

where N_A is the Avogadro number.

It must be considered that this kinetic energy represents the mean value. In fact it can be assumed that the gas follows, inside the capillary, a laminar regime, for which a speed with parabolic distribution is present.

The mean collision energy is orders of magnitude lower than the nitrogen ionization energy ($IE_{nitrogen}$ = 14.53 eV) but, considering the high gas density present in the source environment, the N_2 ionization induced by collision cannot be excluded, due to the occurrence of multiple collisions. In fact, considering the kinetic theory, the mean free path *l* for a particle of high velocity relative to an ensemble of identical particles with random location, can be calculated as $l = (n\sigma)^{-1}$, where n is the number of target particle for unit volume and σ is the effective cross sectional area. It has been calculated that for ambient pressure (1013 mbar, corresponding to 2.7×10^{19} molecules/cm^3) the mean free path is 68 nm. If we consider a pathway of a fast nitrogen molecule from the sprayer exit to the entrance capillary of 1 cm, it follows that the nitrogen molecule will experiment

$$N_c = 1 \text{ cm} / 68 \times 10^{-7} \text{ cm} = 0.0147 \times 10^7 = 1.4 \times 10^5$$

collisions with target molecules inside the source, mainly N_2 and the sprayed solvent.

Then, through sequential, multiple collisions the internal energy of N_2 could increase and reach the IE value, leading to the production of $N_2^{+\cdot}$.

In this view N_2 could play an active role in DESI and DeSSI experiments. $N_2^{+\cdot}$ could undergo a charge-exchange reaction with analyte molecules leading to a short-lived odd electron ion

$$N_2^{+\cdot} + M \rightarrow N_2 + M^{+\cdot}$$

which behaves as protonating media for neutral molecules

$$M^{+\cdot} + M \rightarrow [M - H]^{\cdot} + [M + H]^{+}$$

The former process will be highly favoured from the thermodynamic point of view, considering the IE of N_2, surely higher than that of possible analytes.

An analogous behaviour could be present inside the entrance capillary, in the transfer of desorbed species, solvent and N_2 molecules from the source to the mass analyser, which exhibit velocities in the same order of magnitude of the N_2 molecules above calculated.

This hypothesis could be applied also in the case of solvent and analyte molecules, which, because of their higher mass, will necessarily exhibit a higher kinetic energy, a shorter mean path (for their higher value of σ) and, consequently, undergo a higher number of collisional events. However experimental data exclude the occurrence of this phenomenon in massive way for acetonitrile; in fact in the related spectrum the $M^{+\cdot}$ ion (m/z 41) is absent. Analogously the odd electron molecular ion of the analyte is detected in small abundance. These results can be explained by the stoichiometry of the reaction environment, in which the N_2 concentration is orders of magnitude higher than those of solvent and analyte.

3 PADI PRELIMINARY APPLICATIONS

In the final phase of the trial subject of this thesis, the focus has been placed in preliminary analysis of samples consisting of active principle chemically different from zopiclone. This to check, quite preliminary, the fairness of the system beyond standardized conditions. Moreover, to first verify the quality of technical skills, were also analyzed other organic compounds of different origin and use than drugs. We carried out tests on samples of medicinal preparations (tablets) by which it was possible to identify immediately the presence of biologically active molecules. Finally, given the positive results obtained by this approach in tablets and solutions analysis, it was investigated the potential for the in-vivo tests: we have been analyzed samples of plant origin, attempting to identify the active principle and highlighting different MS profiles characteristic of each specie.

Usually no sample treatment was necessary. Freshly cut surfaces of plant tissues, for example, were directly exposed to the spray.

For solid surface analysis, the sample is largely unused after the experiment and also that these are not extrapolated detection limits.

In experiments which deposed analyte solution was analysed, 25 μL of sample was deposited on the desired surface and allowed to dry.

In these experiments, the total time for analysis is typically 30 seconds.

All measurements and acquisition data have been performed in multiple mass spectrometry mode (MS^n). All analytes identified were confirmed by a rapid direct-infusion of a solution of each active principle, in electrospray mode. Spectral characteristics of PADI analysis are very similar to those of ESI for same analytes.

3.1 ANALYSIS OF TABLETS

Tablets were positioned respect to the spray emitter and the orifice extension firstly by hand and subsequently with the X-Y-Z stage sample holder. MS acquisition was started as soon as the spray was emitting and lasted long enough to attain relevant information, the begin of analytes desorption from surface. Depending on the spot desorbed on the tablet, it had to be moved in order to have analytes desorbed. This could take from a few seconds to around a half minute. When the signal was too low, after five minutes for example, the tablet was moved slightly to desorb on another position.

An interesting point is the fact that PADI permitted identification of different ions at different locations on the surface of the tablets. As visual inspection sometimes showed great inhomogeneity, imaging with PADI could be made. This spatial resolution is specific to these surface analysis techniques and that kind of information is lost with solution-based analysis.

As it is known that tablets are sometimes manufactured with protective films, their surfaces were usually scraped. In this case, in our experience no visual differentiation could be made between the intact surface of the tablet and the inside. This point shows a minor limitation of PADI for analysis of some tablets: surface scraping as a kind of sample preparation step can be mandatory in the case of tablets with some protective films. Nevertheless, if needed, this step, taking only a few seconds per tablet, is not time-consuming when analyzing small numbers of tablets. This made a different local surface roughness, with holes or peaks and microcrystals of analytes, and thus a tablet's inhomogeneity. In this case, it is needed to adjust the position of tablet on sample holder, to obtain the maximum MS response. This necessity shows that the state of a surface plays a major role in the signal obtained and that major differences in ion intensity can occur.

PADI spectra usually showed, as most abundant ion, the protonated or deprotonated ion of the active principle contained in the tablets, without background subtraction.

Zopiclone Tablet

Following a preliminary phase of testing, for all experiments Zopiclone tablets (figure 46: 7.5 mg tablet; Aventis Pharma, Milan, Italy) were employed as standard.

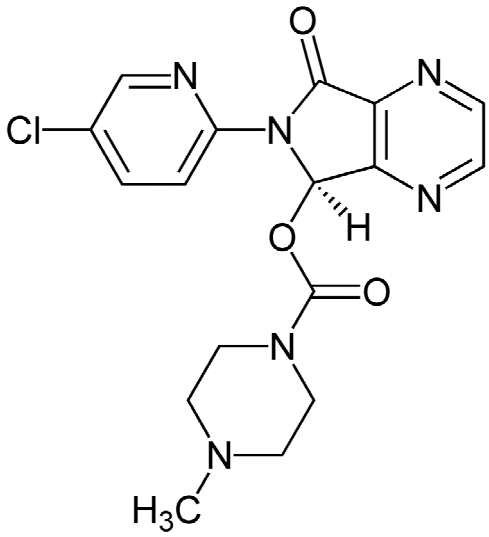


Figure 45. Formula of zopiclone

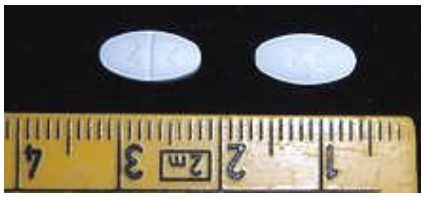


Figure 46. Zopiclone 7.5 mg tablets

Zopiclone (brand name Imovane in Italy) is a non-benzodiazepine hypnotic agent. Zopiclone was first developed and introduced in 1986 by Rhône-Poulenc S.A., now part of Sanofi-Aventis, the main worldwide manufacturer of the drug. Initially it was promoted as being an improvement on benzodiazepines. A recent meta analysis found that zopiclone had no superiority over benzodiazepines in any of the aspects assessed. [54] Zopiclone is indicated for the short term treatment of insomnia where sleep initiation or sleep maintenance are prominent symptoms. Long term use is not recommended as tolerance, dependence, addiction can occur with prolonged use. [55, 56]

Active principle: Zopiclone

Systematic (IUPAC) name: (RS)-[8-(5-chloropyridin-2-yl)- 7-oxo-2,5,8-triazabicyclo [4.3.0]nona-1,3,5-trien-9-yl] 4- methylpiperazine-1-carboxylate

Molecular Formula: $C_{17}H_{17}ClN_6O_3$

Exact Mass: 388.105066

In figure 34 is reported MS spectra obtained, in positive mode, for tablets containing zopiclone. Molecular ion $[M+H]^+$ at m/z 389 and related sodium adduct $[M+Na]^+$ at m/z 411 are visible. Underlying these signals it is easily visible the isotope pattern of chlorine. A quite abundant ion at m/z 799 is detected (see figure 42). Its mass value, as well as the isotopic cluster, indicates for it a structure of $[2M + Na]^+$ species of zopiclone. The less abundant ion at m/z 777 corresponds to the proton bound dimer $[2M + H]^+$. After isolation of molecular ion, as a result of fragmentation due to ionization potential applied, the fragment at m/z 345 was obtained and related to $[M –COO + H]^+$ transition (figure 47).

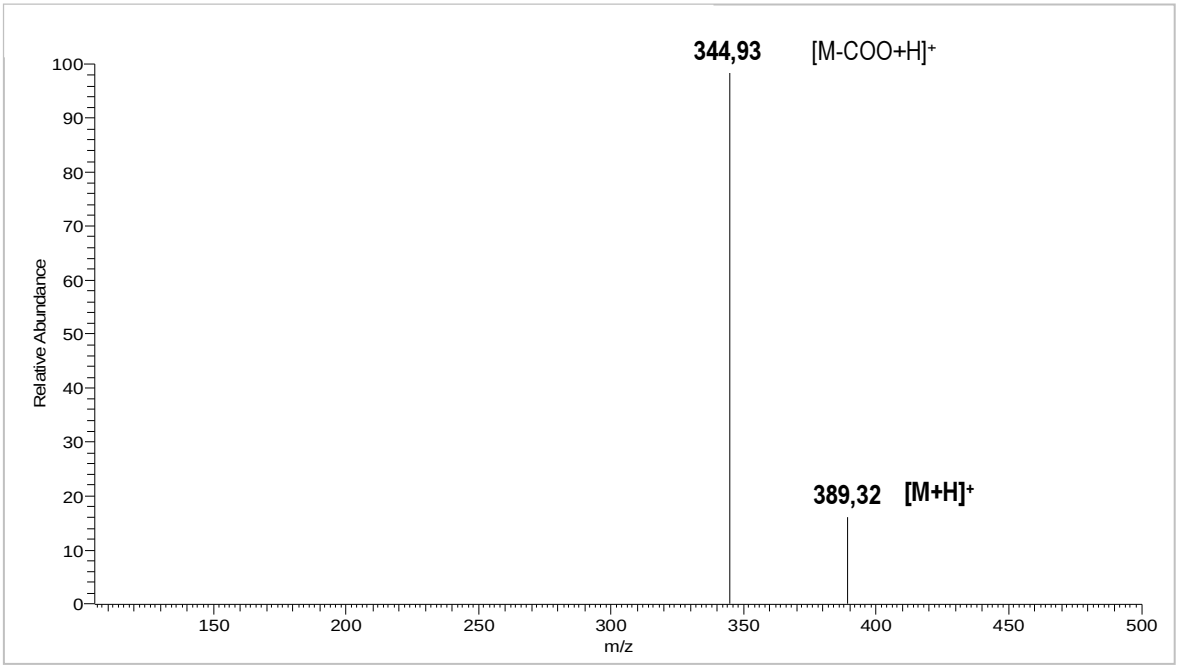


Figure 47. MS/MS positive ions mass spectra of molecular ion of zopiclone ($[M+H]^+$ at m/z 389)

Acetylsalicylic Acid Tablet

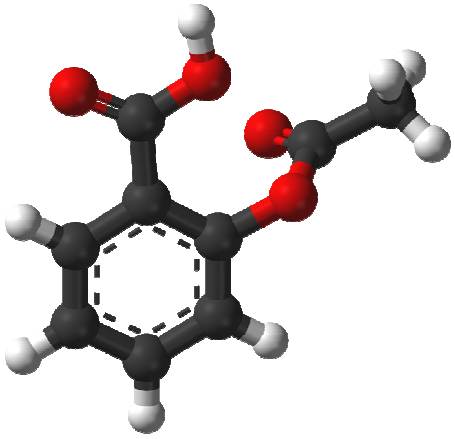


Figure 48. Acetylsalicylic Acid 3D formula

An acetylsalicylic acid tablet was processed. Known as Aspirin, acetylsalicylic acid is a salicylate drug, often used as an analgesic to relieve minor aches and pains, as an antipyretic to reduce fever, and as an anti-inflammatory medication. Acetylsalicylic acid also has an antiplatelet effect by inhibiting the production of thromboxane, which under normal circumstances binds platelet molecules together to create a patch over damage of the walls within blood vessels. Because the platelet patch can become too large and also block blood flow, locally and downstream, aspirin is also used long-term, at low doses, to help prevent heart attacks, strokes, and blood clot formation in people at high risk for developing blood clots. [57] It has also been established that low doses of aspirin may be given immediately after a heart attack to reduce the risk of another heart attack or of the death of cardiac tissue. [58, 59]

Active principle: Acetylsalicylic Acid

Systematic (IUPAC) name: 2-acetoxybenzoic acid

Molecular Formula: $C_9H_8O_4$

Exact Mass: 180.042259

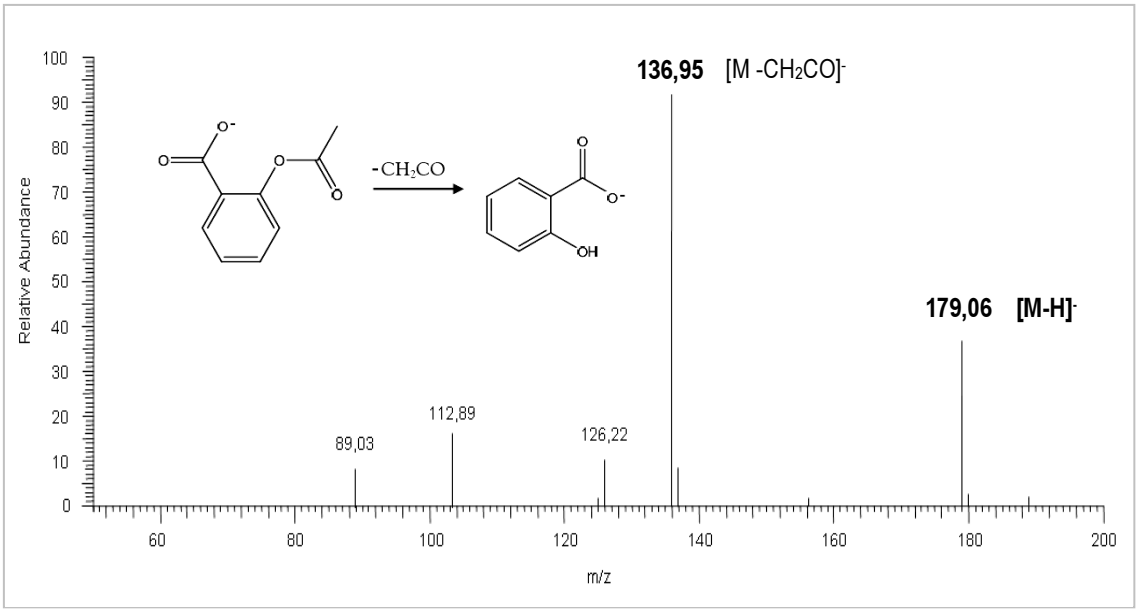


Figure 49. MS/MS negative ions mass spectra of molecular ion of Acetylsalicylic Acid ([M-H]- at m/z 179)

In figure 49 is reported MS/MS spectra obtained for tablets containing Acetylsalicylic Acid. In negative ionisation mode the deprotonated molecular ion ([M-H]-) is the most abundant specie at m/z 179 (acetylsalicylic).

After isolation of molecular ion, as a result of fragmentation due to ionization potential applied, the fragment at m/z 137 was obtained and related to [M - CH_2CO]- transition (salicilic).

Very interesting that, as shown in figure 50, the tablet of acetylsalicylic acid with a solution of water : methanol 50:50, was beginning to fall apart and break after about two minutes under the spray of solvent, in relation to the pharmaceutical formulation. This is a potential problem that must be carefully assessed in the process of choosing the most suitable solvent to be used in the PADI analysis.

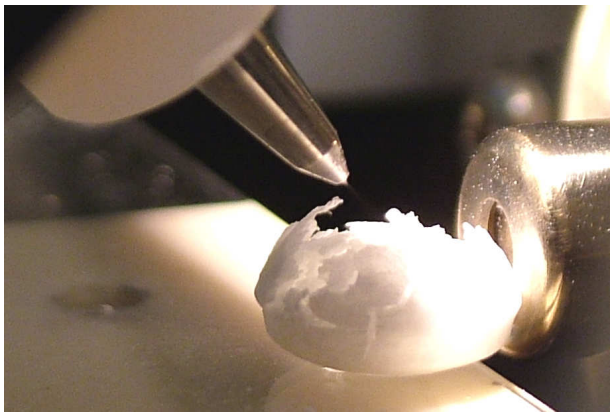


Figure 50. Fall apart and break of Acetylsalicylic Acid tablet after test with water/methanol mixture

Furosemide Tablet

Furosemide, is a loop diuretic and it is principally used in edema associated with heart failure, hepatic cirrhosis, renal impairment, nephrotic syndrom, hypertension and adjunct in cerebral/pulmonary edema where rapid diuresis is required. It is also sometimes used in the management of severe hypercalcemia in combination with adequate rehydration. It has also been used to prevent thoroughbred and standardbred race horses from bleeding through the nose during races. [60, 61]

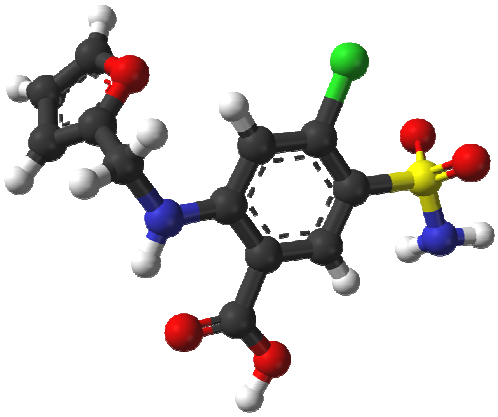


Figure 51. Furosemide 3D formula

It is most commonly marketed under the brand name Lasix. The name Lasix is derived from the phrase "*la*sts *six* (hours)" – referring to its duration of action.

Along with some other diuretics, furosemide is also included on the World Anti-Doping Agency's banned drug list due to its alleged use as a masking agent for other drugs.

Tablet was analyzed as previously described. No scraping was done on surface.

Active principle: Furosemide

Systematic (IUPAC) name: 4-chloro-2-(furan-2-ylmethylamino)- 5-sulfamoylbenzoic acid

Molecular Formula: $C_{12}H_{11}ClN_2O_5S$

Exact Mass: 330.00772

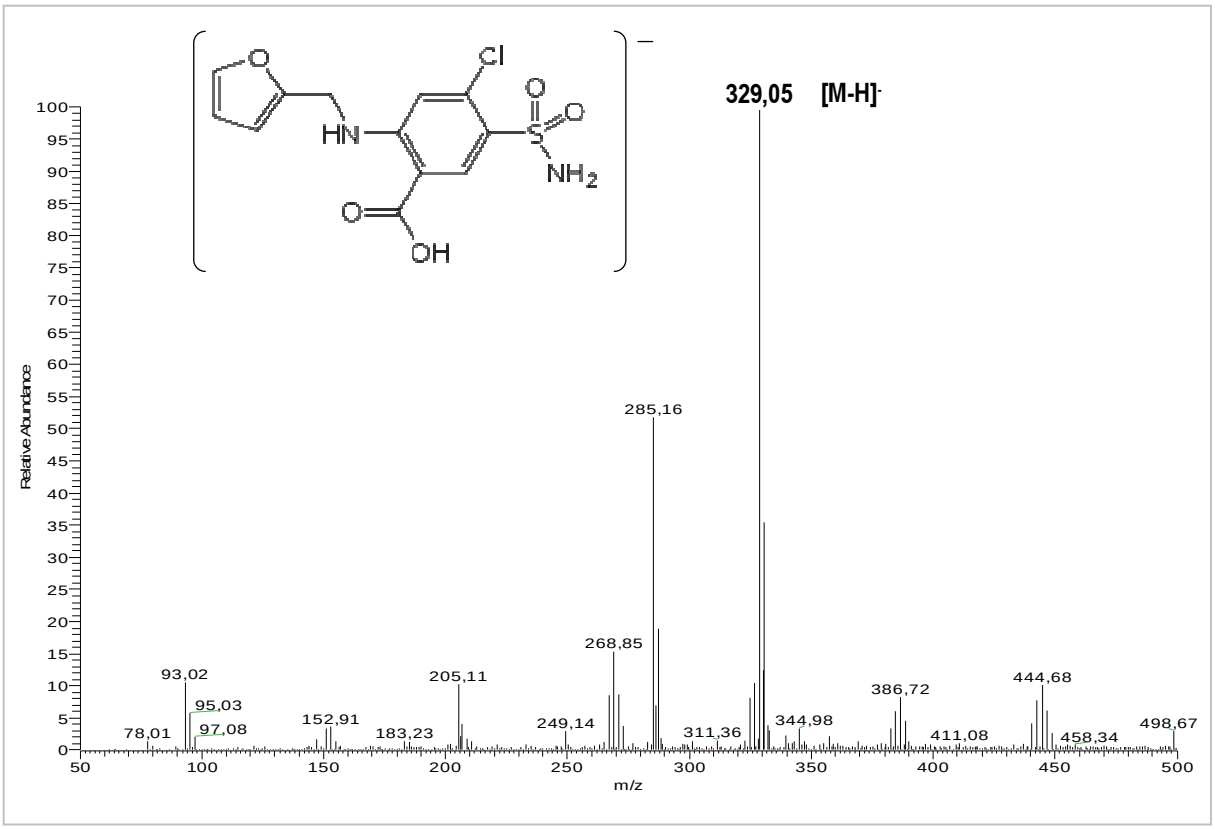


Figure 52. Negative ions mass spectra obtained by spraying a furosemide tablet with acetonitrile

In relation to its structure, the active principle molecule of furosemide (m/z 330) loses a proton and is revealed in negative mode as the predominant signal. In figure 52 is reported MS spectrum of tablet, deprotonated molecular ion $[M-H]^-$ at m/z 329 is indicated.

Underlying molecular signal can easily find the isotope pattern of chlorine. In the case of chlorinated analyte, this peculiar characteristic help its identification.

Then, isolate the molecular ion was found the appropriate energy fragmentation to obtain the best representative fragments of structure.

Fragmentation of isolated $[M-H]^-$ ion allows to identify other characteristic fragment at m/z 285, the $[M-COOH]^-$ ion, obtained from the loss of carboxylic group linked on aromatic ring (see figure 53).

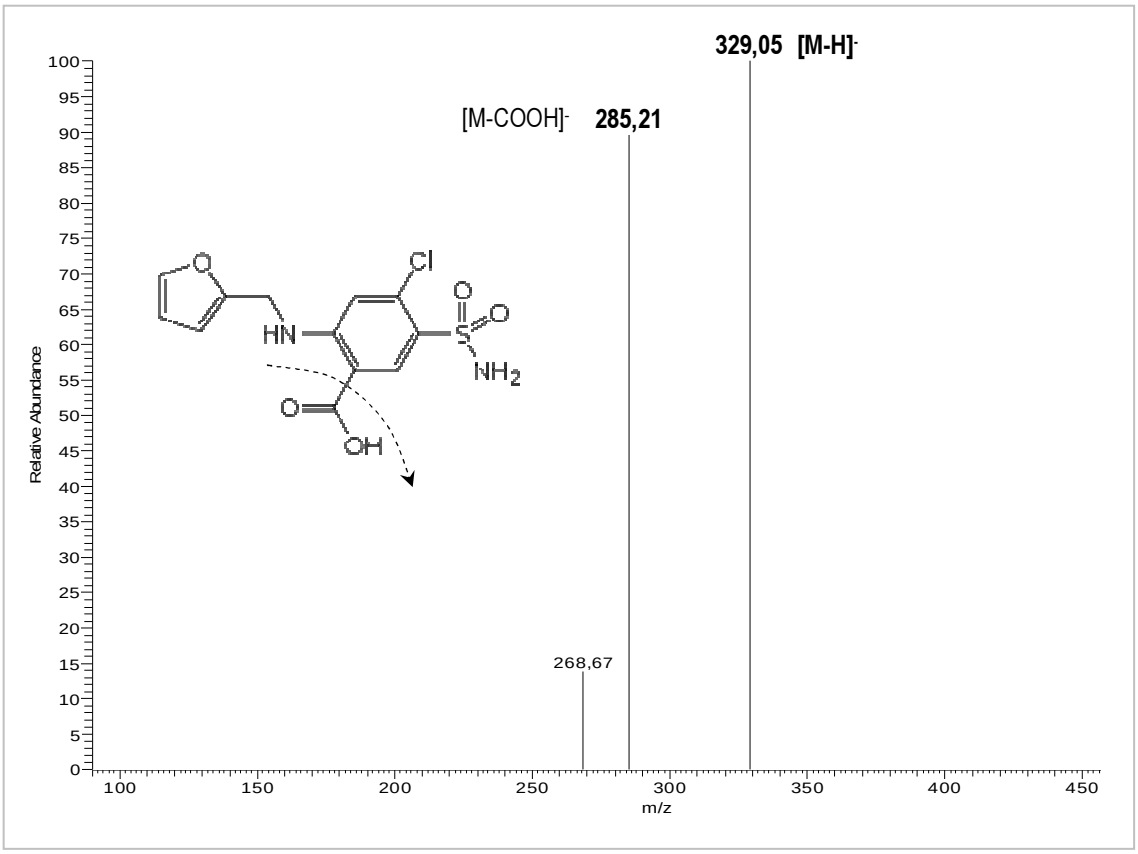


Figure 53. MS/MS spectra of m/z 329 molecular ion

Besides, MS^3 spectra were acquired manually while the tablet stayed under the spray, tuning the instrument. Figure 54 shows the manually acquired MS/MS spectra of m/z 285 isolated ion. Manual optimization of the compound-dependent fragmentation parameters was performed in around 30-60 s per experiment.

This second fragmentation of isolated $[M\text{-}COOH]^-$ ion allows to identify other characteristic fragment at m/z 205, the $[M\text{-}SO_2NH_2\text{-}COOH]^-$ ion, obtained from the loss of sulfur nitrogen group linked on aromatic ring.

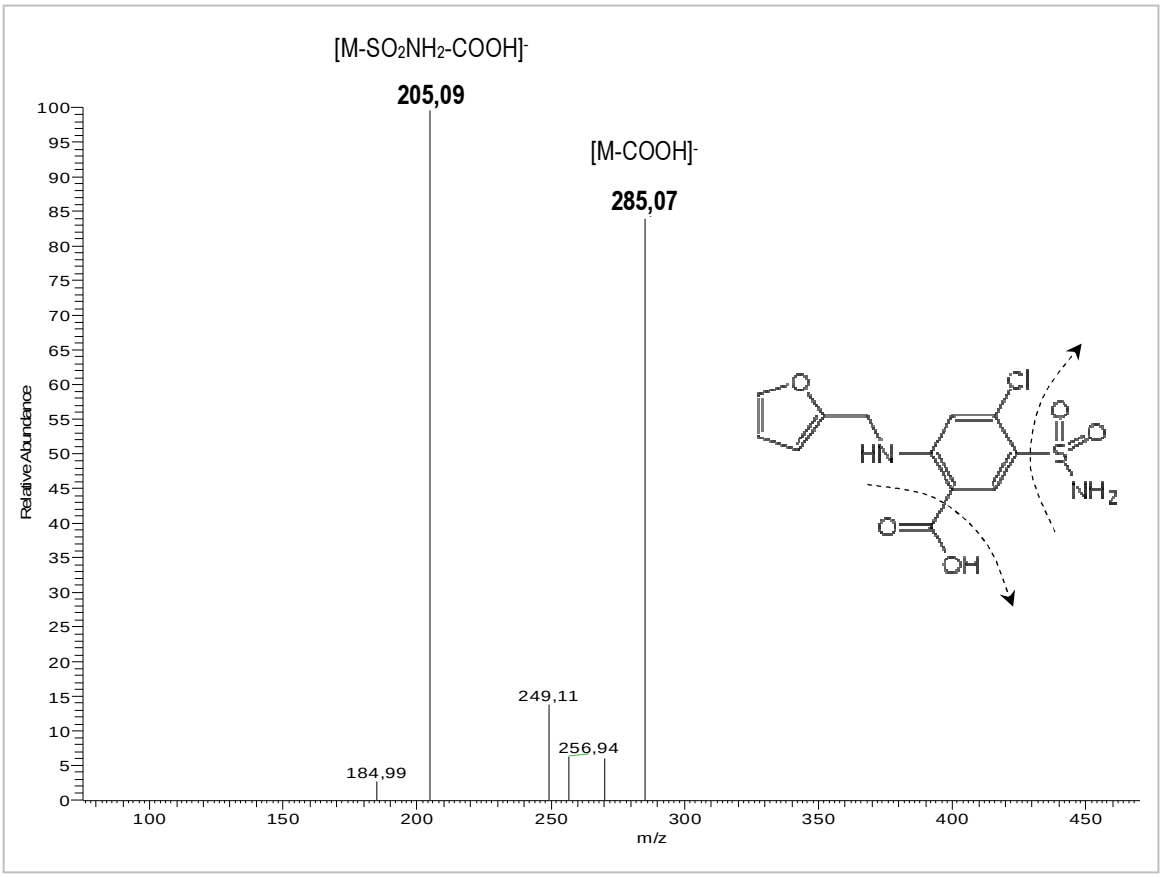


Figure 54. MS^3: MS/MS spectra of isolated ion at m/z 285

Ramipril Tablet

Ramipril is an angiotensin-converting enzyme (ACE) inhibitor. ACE inhibitors lower the production of angiotensin II, therefore relaxing arterial muscles while at the same time enlarging the arteries, allowing the heart to pump blood more easily, and increasing blood flow due to more blood being pumped into and through larger passageways. Ramipril is a prodrug and is converted to the active metabolite ramiprilat by liver esterase enzymes. [62]

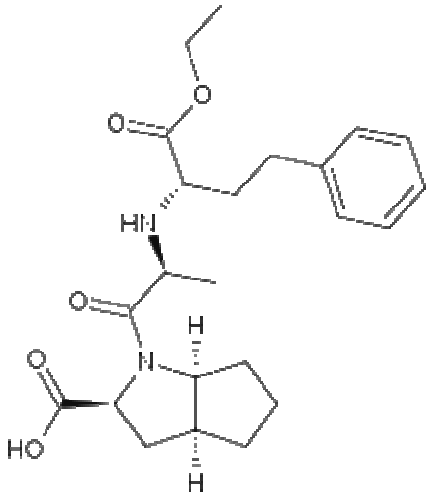


Figure 55. Ramipril formula

Indications for its use include hypertension, congestive heart failure, following myocardial infarction in patients with clinical evidence of heart failure and diabetic nephropathy with microalbuminuria. [63]

Active principle: Ramipril

Systematic (IUPAC) name: (2S,3aS,6aS)-1-[(2S)-2-{[(2S)-1-ethoxy-1-oxo-4-phenylbutan-2-yl]amino}propanoyl]-octahydrocyclopenta[b]pyrrole-2-carboxylic acid

Molecular Formula: $C_{23}H_{32}N_2O_5$

Exact Mass: 416.231122

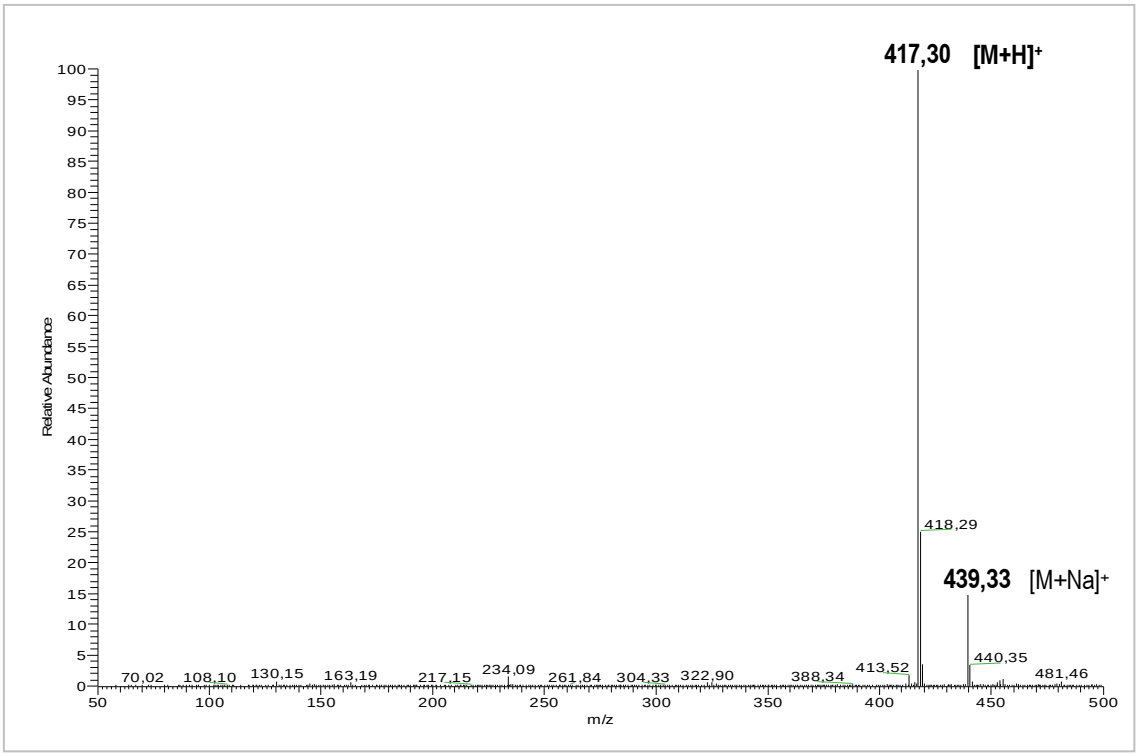


Figure 56. Positive ions mass spectra obtained by spraying a Ramipril tablet with acetonitrile

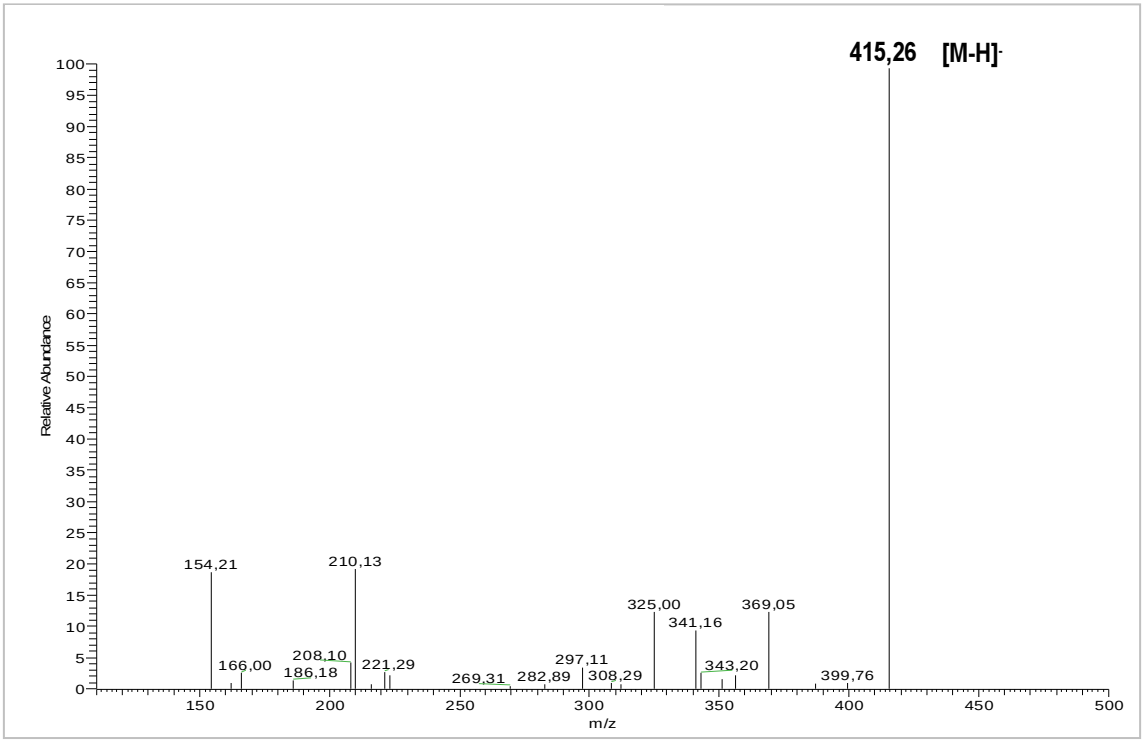


Figure 57. Negative ions mass spectra obtained by spraying a Ramipril tablet with acetonitrile

Ramipril molecule exhibits ionization capability in both negative and positive modes.

In figure 56 is reported MS spectra obtained, in positive mode, where molecular ion $[M+H]^+$ at m/z 417 and related sodium adduct $[M+Na]^+$ at m/z 439 are visible.

In figure 57 is reported MS spectra obtained, in negative mode: the deprotonated molecular ion ($[M-H]^-$) is the most abundant specie at m/z 415.

After isolation of negative molecular ion, as a result of fragmentation due to ionization potential applied, many specific fragments were obtained at m/z 154, m/z 210 and m/z 343 (see figure 58).

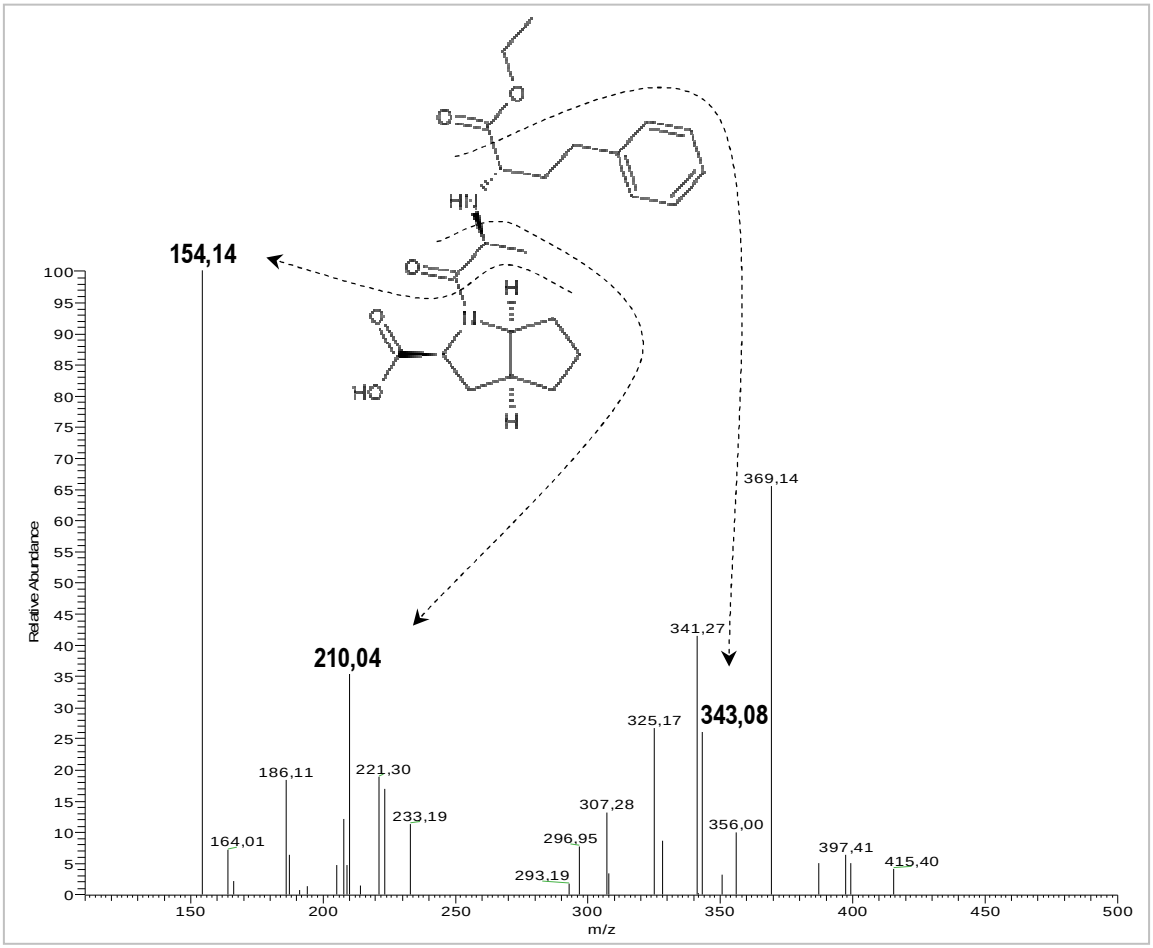


Figure 58. MS/MS fragmentation, in negative mode, of molecular ion of Ramipril at m/z 415

MDMA Illicit Tablet

MDMA (3,4-Methylenedioxymethamphetamine, commonly known as Ecstasy) is a synthetic, psychoactive drug that is chemically similar to the stimulant methamphetamine and the hallucinogen mescaline.

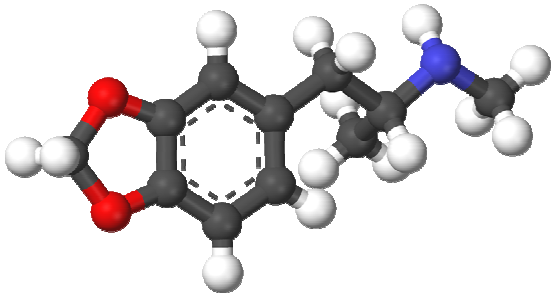


Figure 59. MDMA 3D formula

The effects of MDMA on the human brain and body are complex, interacting with several neurochemical systems. It induces serotonin, dopamine, norepinephrine, and acetylcholine release, and can act directly on a number of receptors, including a2-adrenergic (adrenaline) and $5HT_{2A}$(serotonin) receptors. MDMA promotes the release of several hormones including prolactin, oxytocin, dehydroepiandrosterone (DHEA) and the antidiuretic hormone vasopressin. [64]

Figure 60. Illicit MDMA tablets

MDMA shows tendency to induce a sense of intimacy with others and diminished feelings of fear, anxiety, and depression. Before the illicit use, it was made a controlled substance, as augmentation to psychotherapy, couples therapy, and to help treat clinical depression as well as anxiety disorders. MDMA has been used as recreational substance in most countries MDMA and first appeared as a street drug in the early 1970s.

Active principle: MDMA; 3,4-Methylenedioxymethamphetamine

Systematic (IUPAC) name: (RS)-1-(benzo[d][1,3]dioxol-5-yl)-N-methylpropan-2-amine

Molecular Formula: $C_{11}H_{15}NO_2$

Exact Mass: 193.110279

Tablet was analyzed as previously described. No scraping was done on surface.

In positive ion mode methamphetamine (m/z 150) was the predominant ion; the active principle MDMA (m/z 194) was detected. Other analytes identified in tablet were ions of caffeine (m/z 195), amphetamine (m/z 136) and N-methyl-1-(1,3-benzodioxol-5-yl)-2-butanamine (MBDB, m/z 208).

In figure 61 is reported MS spectrum of illicit tablet and all protonated molecular ions ($[M+H]^+$) are visible and detailed: in addition to the active ingredient we are able to indicate derivatives and substances thinners.

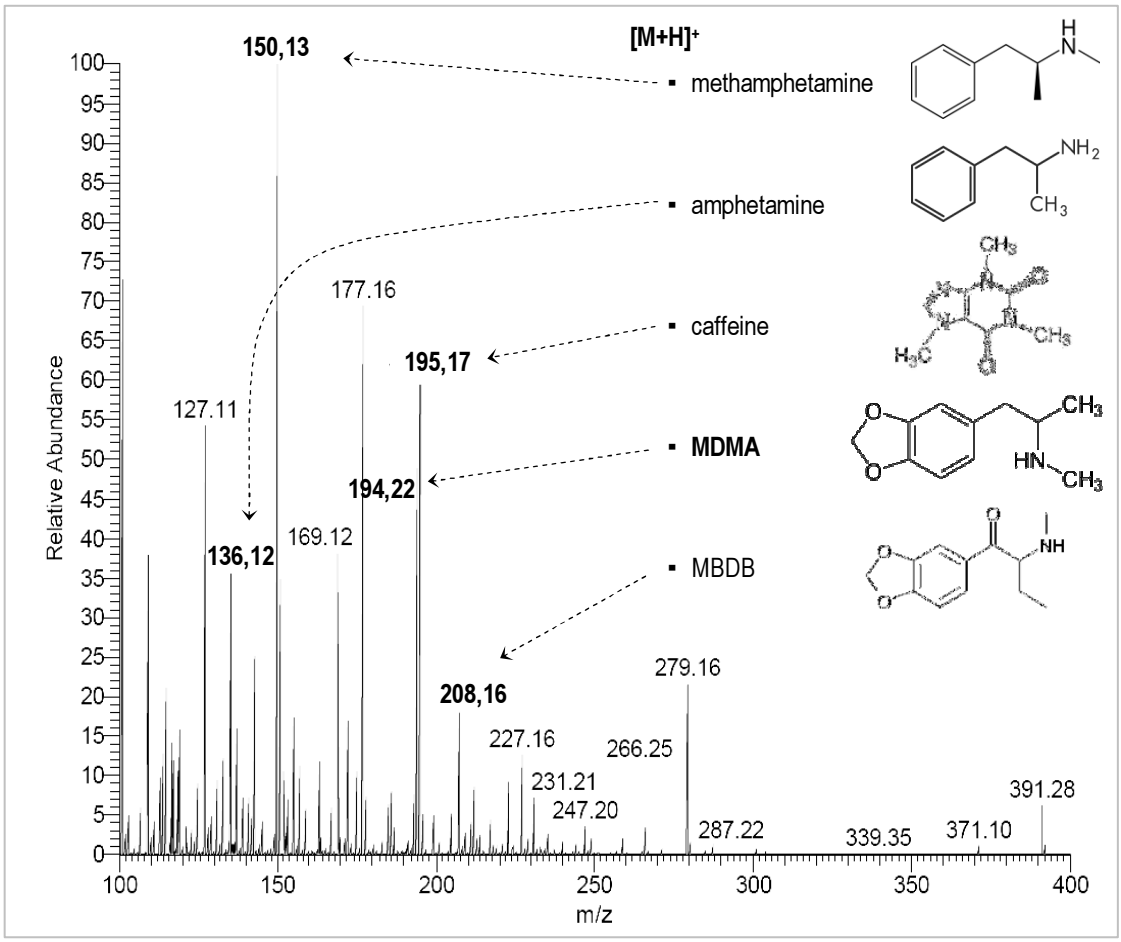


Figure 61. Positive ions mass spectra obtained by spraying a Illicit MDMA tablet

- **methamphetamine** Molecular Formula: $C_{10}H_{15}N$
  Exact Mass: 149.120449
- **amphetamine** Molecular Formula: $C_9H_{13}N$
  Exact Mass: 135.104799
- **caffeine** Molecular Formula: $C_8H_{10}N_4O_2$
  Exact Mass: 194.080376
- **MDMA** Molecular Formula: $C_{11}H_{15}NO_2$
  Exact Mass: 193.110279
- **MBDB** Molecular Formula: $C_{12}H_{17}NO_2$
  Exact Mass: 207.125929

Pantoprazole Tablet

Pantoprazole is a proton pump inhibitor drug used for short-term treatment of erosion and ulceration of the esophagus caused by gastroesophageal reflux disease. It is metabolized in the liver by the cytochrome P450 system. [65] This analyte may affect the results of immunochemical lab tests, such as drug screenings (pantoprazole can cause a false positive for THC).

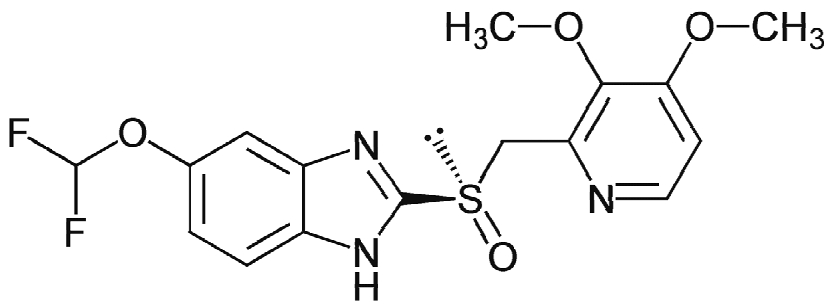


Figure 62. Pantoprazole formula

Active principle: Pantoprazole

Systematic (IUPAC) name: (RS)-6-(difluoromethoxy)-2-[(3,4-dimethoxypyridin-2-yl)methylsulfinyl]-1H-benzo[d]imidazole

Molecular Formula: $C_{16}H_{15}F_2N_3O_4S$

Exact mass: 383.075133

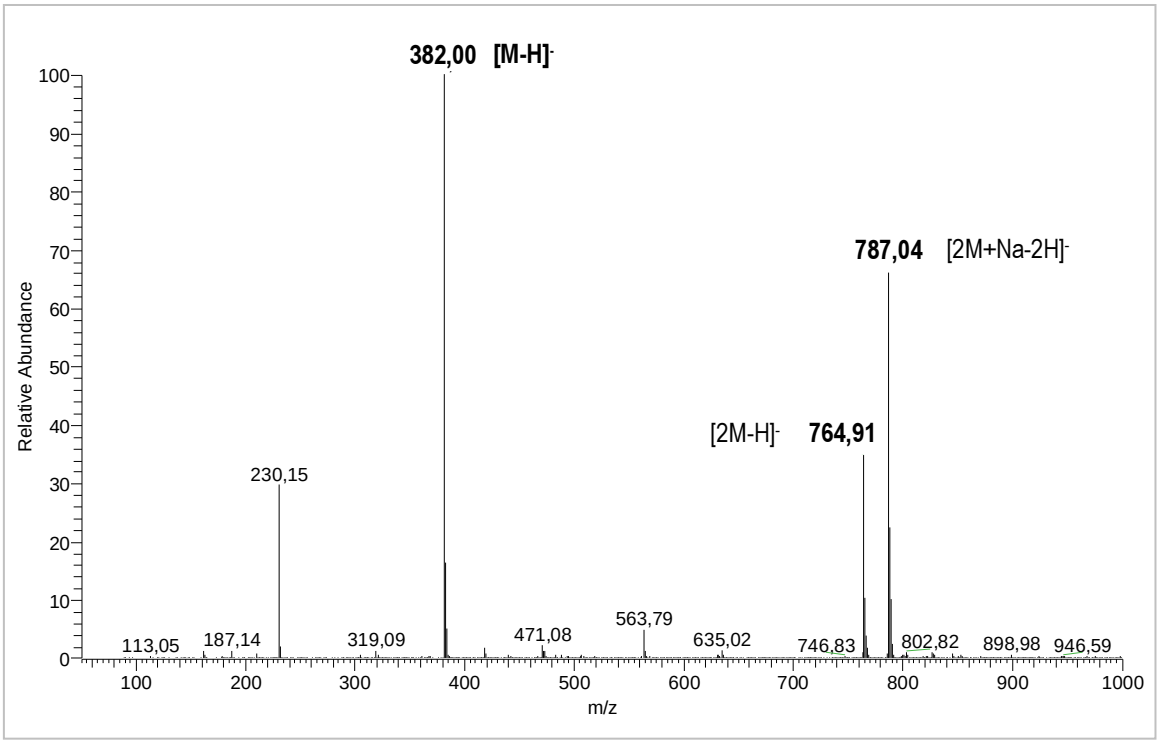


Figure 63. Negative ions mass spectra obtained by spraying a Pantoprazole tablet with acetonitrile

In figure 63 is reported MS spectra obtained, in negative mode, for tablets containing pantoprazole. Molecular ion $[M-H]^-$ at m/z 382 are visible as most abundant ion. A quite abundant ions at m/z 765 and m/z 787 is detected. Their mass value indicates that they are related to two pantoprazole molecules interaction. In detail, the less abundant ion at m/z 765 corresponds to the ionic specie $[2M–H]^-$, the ion at m/z 787 corresponds to sodium adduct specie $[2M+Na–2H]^-$.

3.2 ANALYSIS OF POWDERS AND SOLUTIONS

Some compounds are available in powder or crystal, in this case the PADI analysis is very difficult because the sample, under the influence of pneumatic sprayer, can easily reach the entrance of the mass spectrometer, clog the capillaries, blocking the instrumental functionality. The strong push air linked to the gas flow tends to remove the samples from the surface preventing the analysis of the sample of interest. Even some drugs were sold as capsules and cannot be directly analyzed by PADI: the powder is hermetically sealed by the capsule.

Have been identified three possible solutions to this problem: the first is to dissolve the crystals of the substance in an appropriate solvent and then depositing the solution obtained on a TLC plate. The second is to suspend the crystals of compounds in a viscous mixture of glycerol, as wettable agent, if analytes are immiscible or slightly miscible in solvents, as discussed previously. The last is to set the crystals of the substance on the surface of sample holder by cyanoacrylic resin, and then proceed direct analysis. The best results in term of sensibility and signal stability were obtained by depositing the sample on a cyanoacrylate resin, then left to dry under atmospheric conditions. Under these conditions the sample is effectively immobilized and subsequent analysis PADI allow to obtain stable signals for a time interval of the order of minutes.

In the case of solution's analysis, 25 µL of solution of each sample were placed on TLC plate, drop by drop, to allow proper absorption of the solution to the surface. In this way we tried to avoid as much as possible the chromatographic phenomenon on a wide surface, to focus the absorption and distribution of the sample on a well-defined area.

4-methyl-7-hydroxicoumarin crystals fixed on cyanoacrylic resin.

The crystals of -methyl-7-hydroxicoumarin was fixed on surface of sample holder by cyanoacrylic resin (figure 64).

Figure 64. 4-methyl-7-hydroxycoumarin crystals deposited on cyanoacrylic resin

Blank test, carried out with the resin alone, show that once polymerized the resin itself does not produce interfering species. In figure 65 is reported the PADI spectrum of the blank resin. After deposition of 4-methyl-7-hydroxycoumarin crystals, it was obtained the MS spectrum shows in figure 67.

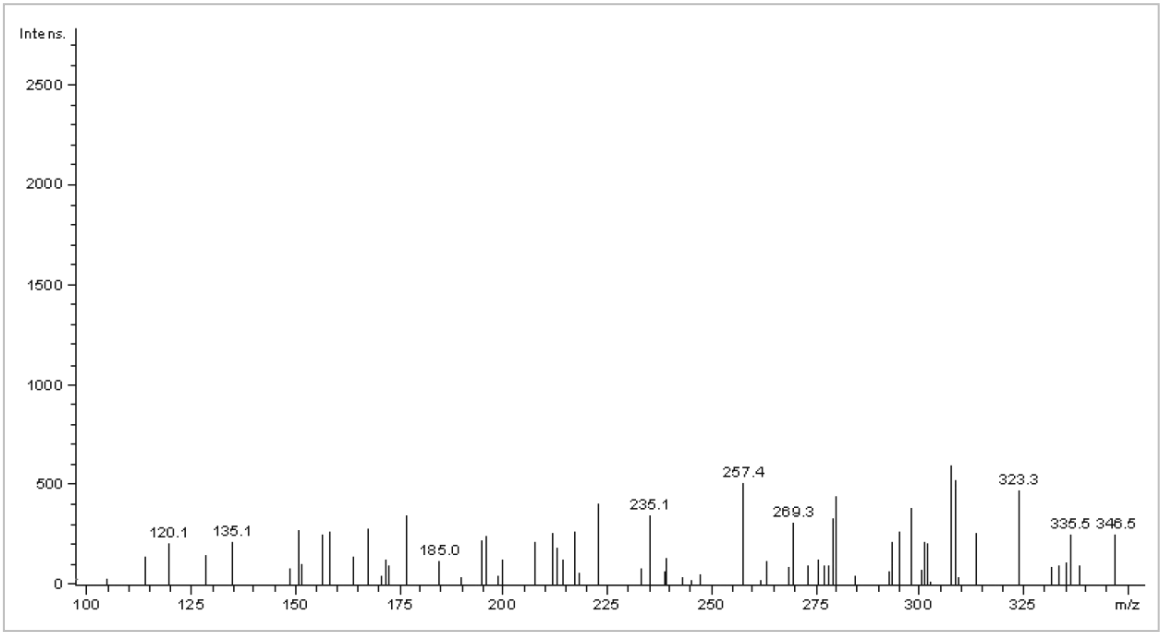


Figure 65. MS spectra of cyanoacrylic resin used in PADI experiments

Compound name: 4-methyl-7-hydroxicoumarin

Molecular Formula: $C_{10}H_8O_3$

Exact Mass: 176.047345

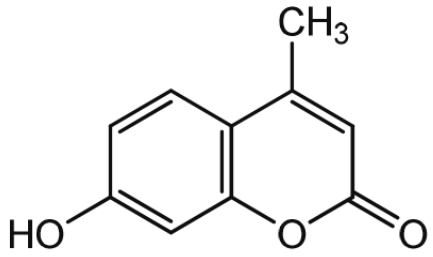


Figure 66. Formula of 4-methyl-7-hydroxicoumarin

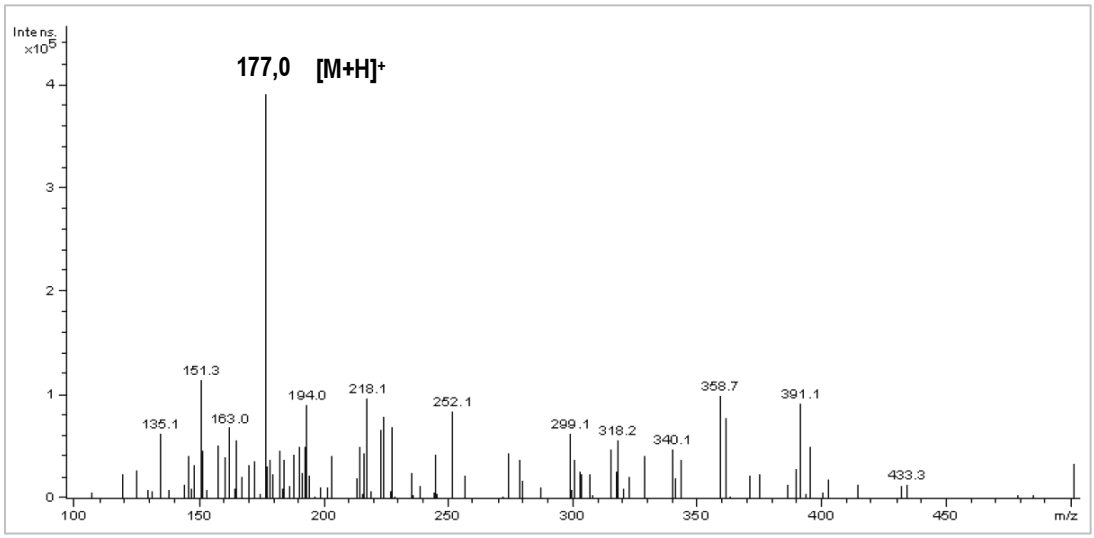


Figure 67. MS spectra of 4-methyl-7-hydroxycoumarin crystals, molecular ion in positive mode at m/z 177

The most abundant ion at m/z 177 was assigned to the protonated molecule of 4-methyl-7-hydroxy-coumarin. To obtain confirmation of this structural assignment was carried out a MS/MS experiment. The spectrum in figure 68, shows the fragmentation of a single fragment ion at m/z 149. This result is in agreement with the structure of the molecule in question, as described in the key of fragmentation (figure 68, lower).

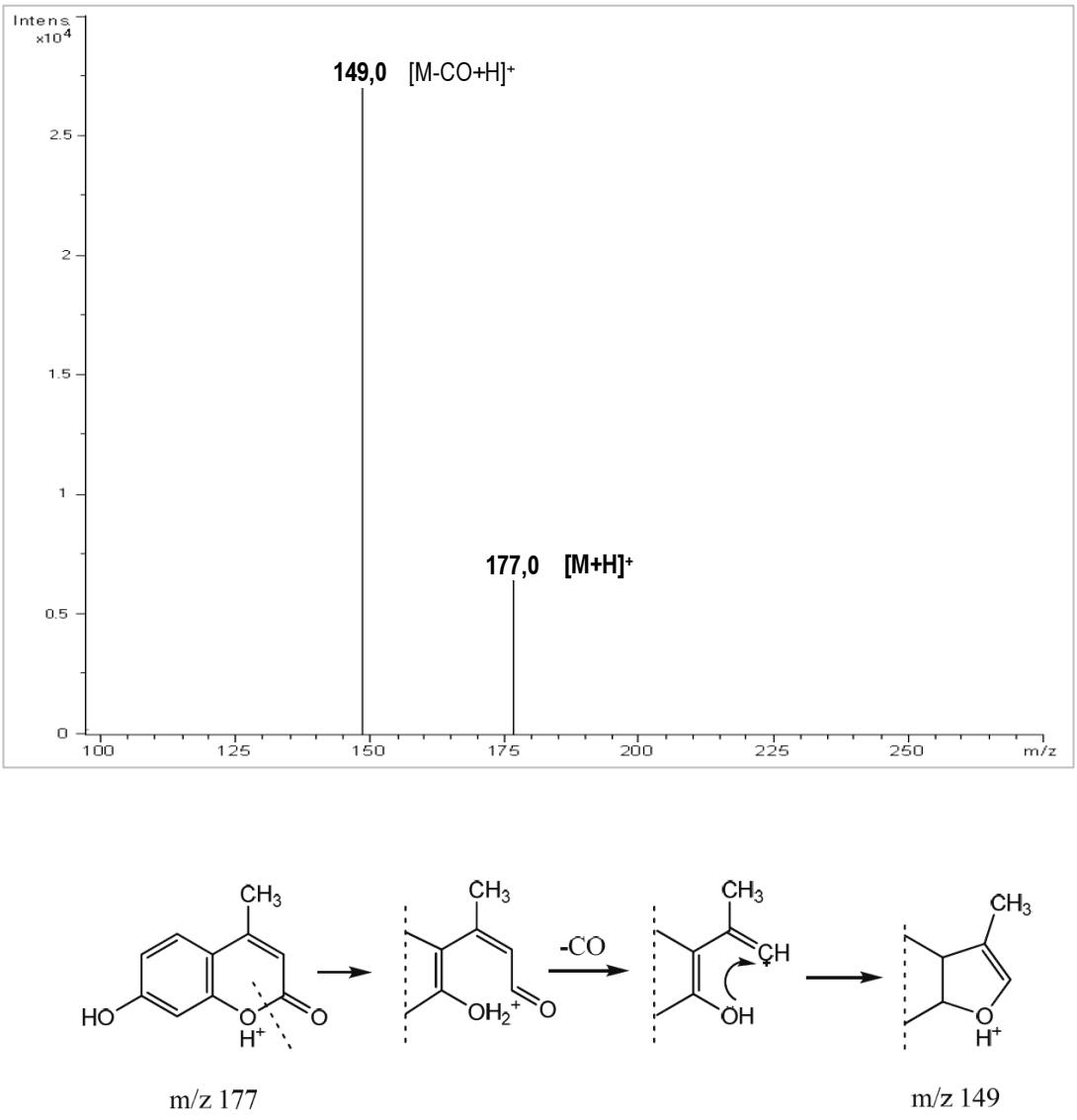


Figure 68. MS/MS experiment of molecular ion m/z 177. Lower, the scheme of fragmentation.

To give a rough assessment of the sensitivity of the method, we would calculate that the spectrum shown in figure 67 has been obtained by depositing 1 µg of sample on the resin before its polymerization. Is reasonable to assume that the compound is uniformly dissolved in the resin itself and then on the surface remains a quantity of the order of ng.

Zopiclone solution on TLC plate

To test the performance of TLC deposition as solutions sample holder, 25 µL soluzion of zopiclone, obtained dissolving a thin amount of tablet in acetonitrile, was placed drop by drop on TLC plate and sprayed with acetonitrile at solvent flow of 1000 µL/h. The MS spectrum obtained are reported in figure 38. We obtained a mass spectrum cleaner with less noise, with an absolute abundance of molecular ion of about 10^5. We identified the molecular ion at m/z 389 and the sodium adduct at m/z 411. For each ion it is easily visible the specific isotope pattern of chlorine.

Flunitrazepam solution on TLC plate

Flunitrazepam is a strong hypnotic and powerful sedative, anticonvulsant, anxiolytic, amnestic, reduction in anxiety and skeletal muscle relaxant drug. [66]

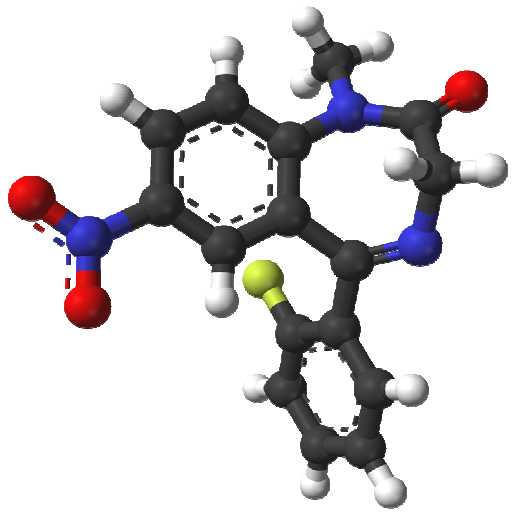


Figure 69. Flunitrazepam 3D formula

A short-intermediate acting benzodiazepine derivative, flunitrazepam is prescribed for the treatment of severe insomnia. The prescription of flunitrazepam as a hypnotic is generally intended to be for short-term treatment of chronic or severe insomniacs that are not responsive to other hypnotics, especially in inpatients. It is considered to be one of the most effective benzodiazepine hypnotics on a dose basis.

The main pharmacological effects of flunitrazepam are the enhancement of GABA at the $GABA_A$ receptor. [67] Like other benzodiazepines, adverse effects of flunitrazepam include dependence, both physical and psychological; reduced sleep quality resulting in somnolence, and overdose, resulting in excessive sedation, impairment of balance and speech, respiratory depression or coma and possibly death.

25 µL soluzion of flunitrazepam at 1 mg/mL was placed on TLC plate and analyzed as previously described.

Active principle: Flunitrazepam

Systematic (IUPAC) name: 6-(2-fluorophenyl)- 2-methyl- 9-nitro- 2,5-diazabicyclo [5.4.0] undeca- 5,8,10,12- tetraen- 3-one

Molecular Formula: $C_{16}H_{12}FN_3O_3$

Exact Mass: 313.086269

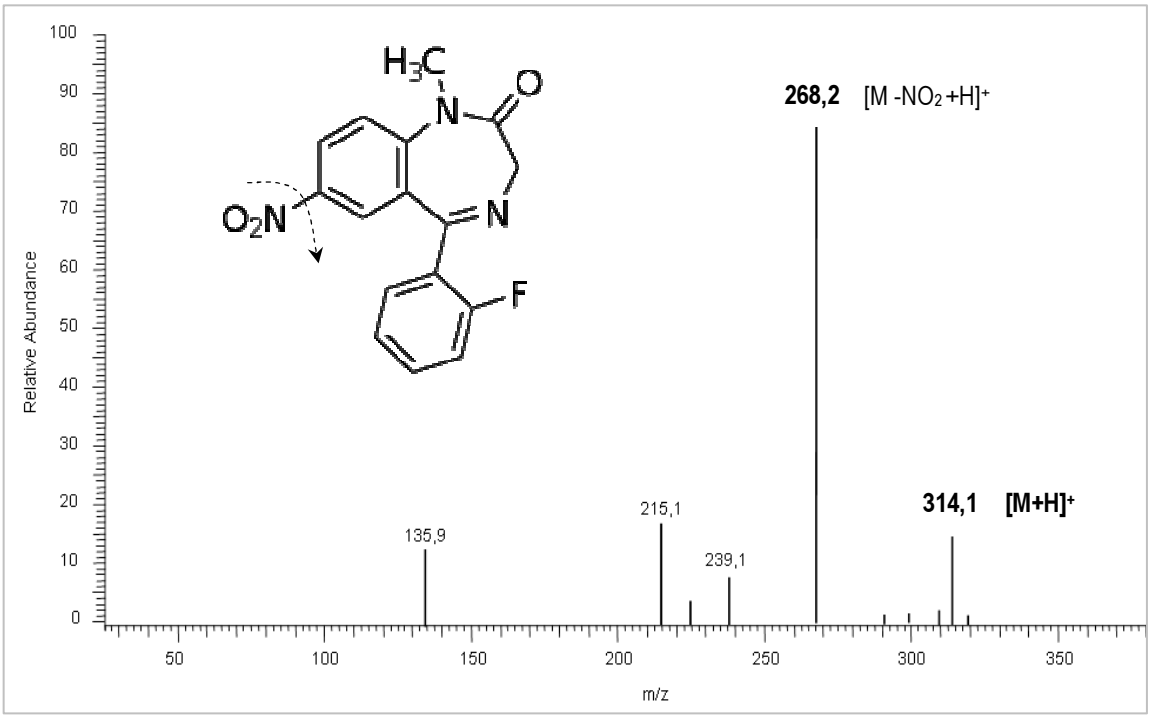
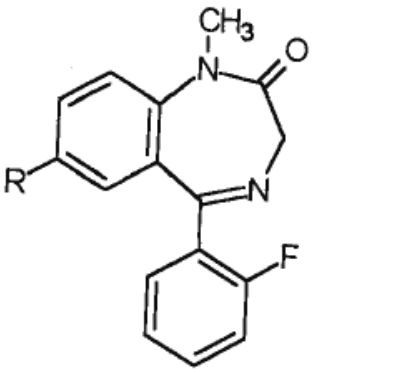


Figure 70. MS/MS spectra of molecular ion of flunitrazepam at m/z 314

In Figure 70 is reported MS/MS spectra obtained, in positive mode, for solution containing flunitrazepam. In positive ionisation the protonated molecular ion $[M+H]^+$ is identified at m/z 314. After isolation of molecular ion, as a result of fragmentation due to ionization potential applied, the fragment at m/z 268 was obtained and related to $[M-NO_2+H]^+$ transition.

7-aminoflunitrazepam solution on TLC plate



Flunitrazepam, $R = NO_2$
7-Aminoflunitrazepam, $R = NH_2$

Figure 71. Molecular formula of Flunitrazepam and its metabolite, 7-aminoflunitrazepam

7-aminoflunitrazepam is the main metabolite of Flunitrazepam in urine (figure 71). The detection of flunitrazepam in urine is complicated by the low doses administered in the case of 'date rape' and also complete metabolism. The detection of 7-aminoflunitrazepam, which is the major urinary metabolite, is vital for confirmation of the drug's administration. Several methods have already been reported for the analysis of flunitrazepam metabolites by gas chromatography/mass spectrometry (GC/MS). Has been proposed to use acid hydrolysis to convert urinary flunitrazepam metabolites to their corresponding benzophenone derivatives prior to analysis; derivatisation of 7-aminoflunitrazepam with bis- (trimethylsilyl) trifluoroacetamide (BSTFA) to form a trimethlysilyl (TMS) derivative has also been reported. [68]

25 µL soluzion of 7-aminoflunitrazepam at 1 mg/mL was placed on TLC plate and analyzed as previously described.

Metabolite: 7-aminoflunitrazepam

Systematic (IUPAC) name: (E)-7-amino-5-(2-fluorophenyl)-1-methyl-1H-benzo[e][1,4]diazepin-2(3H)-one

Molecular Formula: $C_{16}H_{14}FN_3O$

Exact Mass: 283.112089

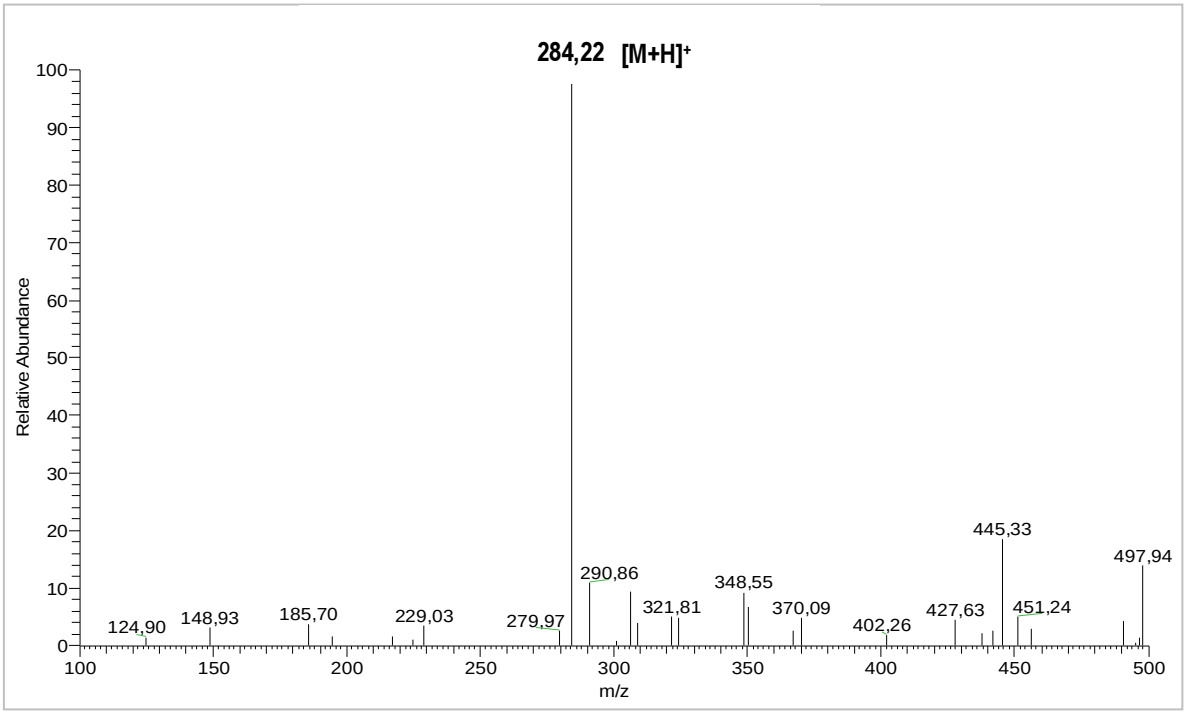


Figure 72. MS positive spectra of 7-aminoflunitrazepam, molecular ion at m/z 284

Figure 72 shows the MS positive spectra obtained for solution containing 7-aminoflunitrazepam. The protonated molecular ion $[M+H]^+$ is the most abundant ion identified at m/z 284. Isolated molecular ion was fragmentized

due to ionization potential applied, and the fragments obtained was indicate in figure 73.

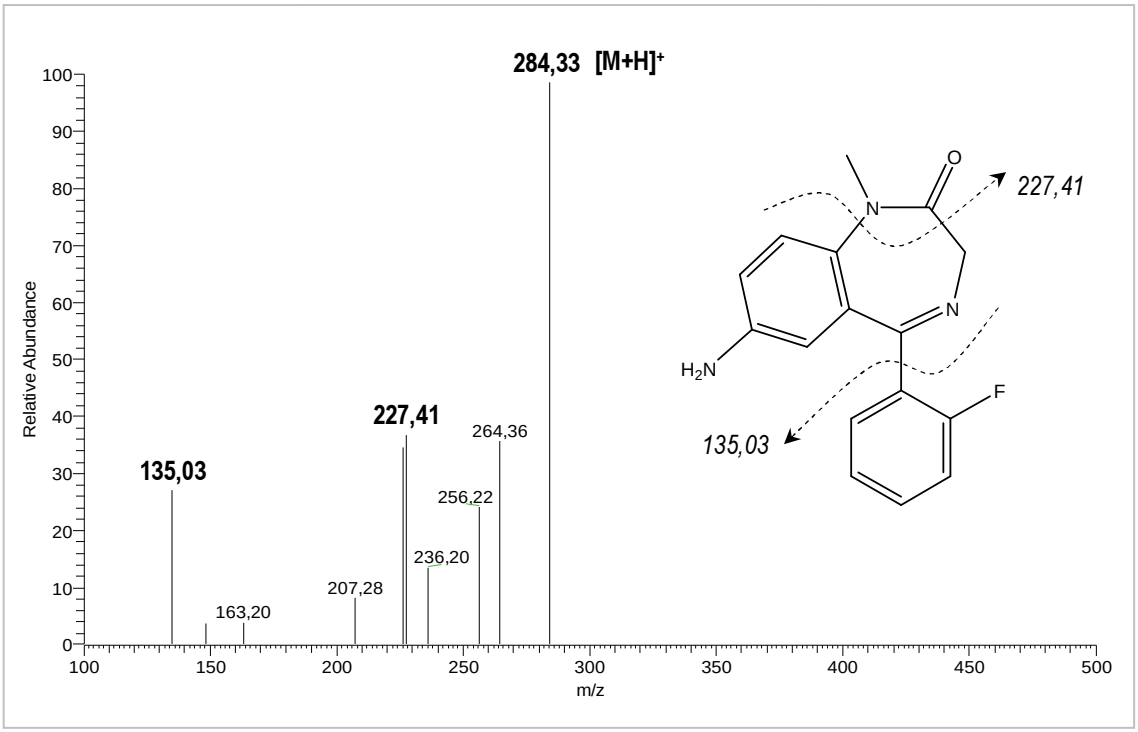


Figure 73. MS/MS spectra of molecular ion of 7-aminoflunitrazepam and related specific fragments

Reserpine solution on TLC plate

Reserpine is an indole alkaloid antipsychotic and antihypertensive drug that has been used for the control of high blood pressure and for the relief of psychotic behaviours. The antihypertensive actions of Reserpine are a result of its ability to deplete catecholamines from peripheral sympathetic nerve endings. Reserpine acts by blocking the vesicular monoamine transporter VMAT, which normally transports free norepinephrine, serotonin, and

dopamine. These substances are normally involved in controlling heart rate, force of cardiac contraction and peripheral resistance. [69]

For this test, we used glycerol, as wettable agent, to permit properly moisten the surface. After deposition on TLC plate of 25 µL Reserpine satured solution, we deposed one single drop of glycerol on TLC surface. Note that the signal obtained was tended to decrease gradually, in minutes, in relationship to the elimination of the layer of glycerol by the action of pneumatic spray.

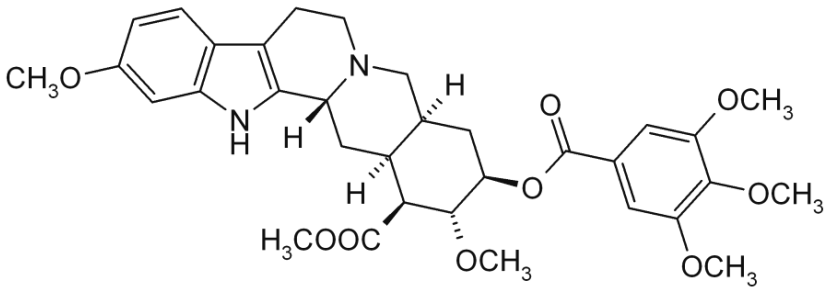


Figure 74. Reserpine molecular formula

Active principle: Reserpine

Systematic (IUPAC) name: methyl-11,17α-dimethoxy-18β-[(3,4,5-trimethoxybenzoyl)oxy]-3β,20α-yohimban-16β-carboxylate.

Molecular Formula: $C_{33}H_{40}N_2O_9$

Exact Mass: 608.273381

MS spectra obtained is reported in figure 75. In positive ion mode, molecular ion $[M+H]^+$ at m/z 609 are the most abundant signal. Some difficult to identify related sodium adduct $[M+Na]^+$ at m/z 631. After isolation of molecular ion, as a result of fragmentation due to ionization potential applied, related fragment was obtained and identified (figure 76).

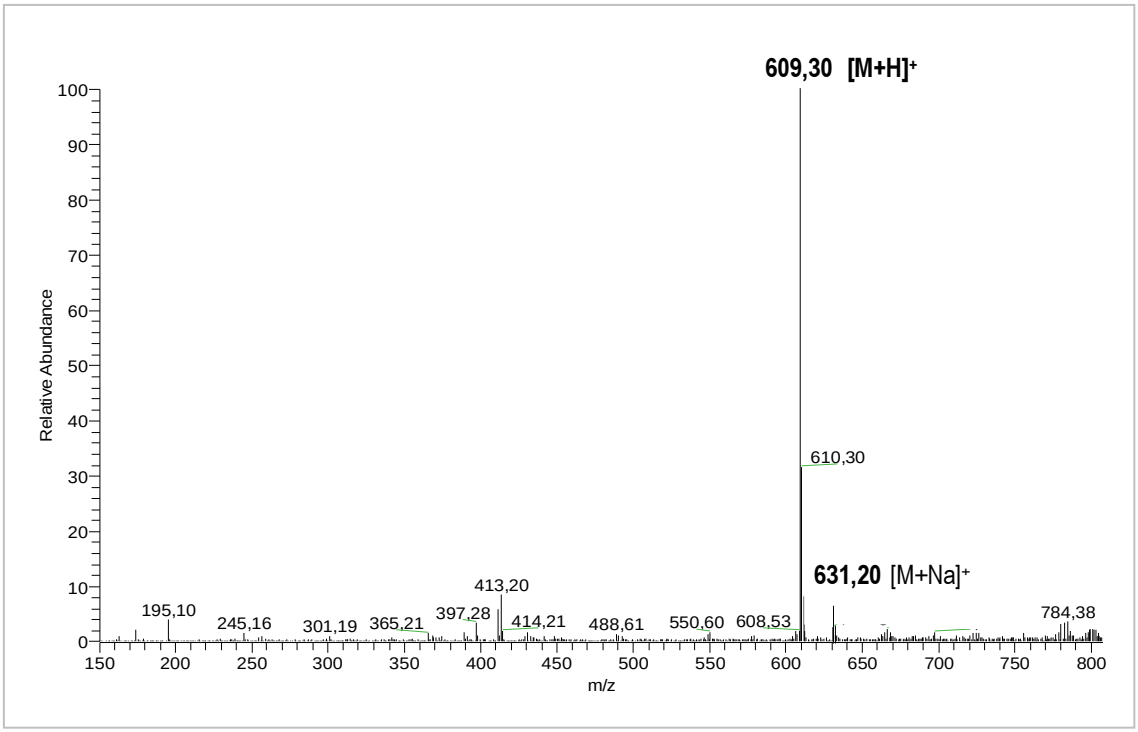


Figure 75. MS positive spectra of reserpine, molecular ion at m/z 609

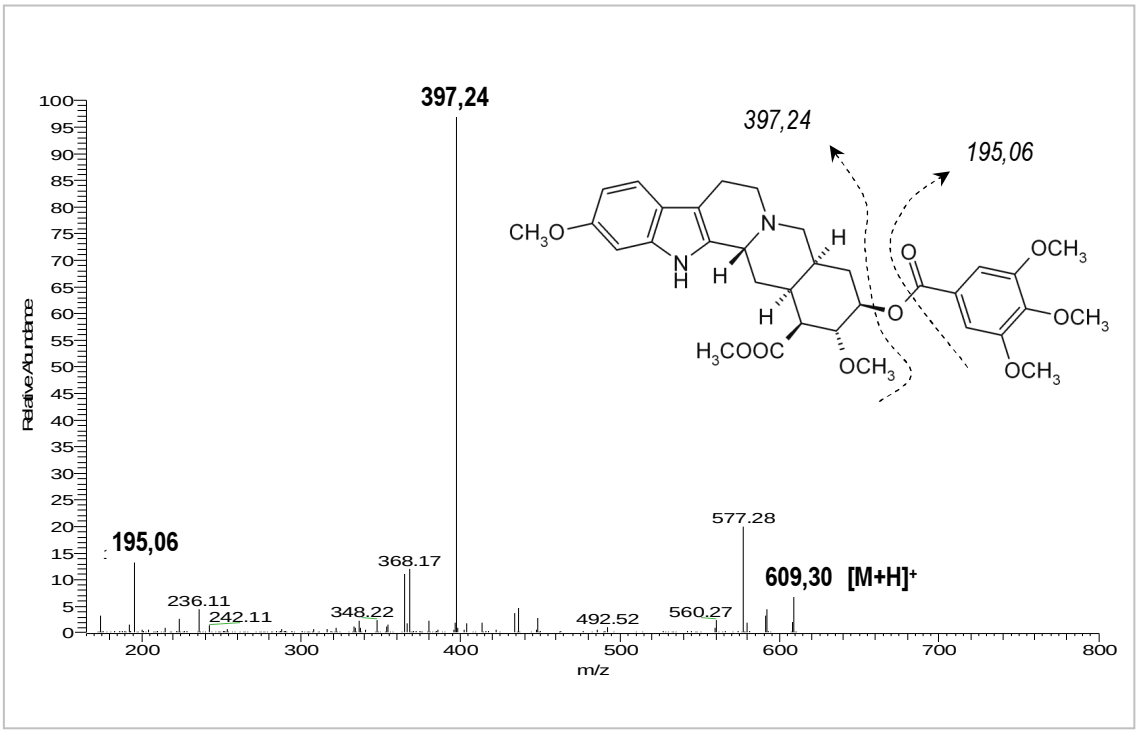


Figure 76. MS/MS spectra of molecular ion of reserpine and related specific fragments

3.3 PLANT TISSUE ANALYSIS

In-vivo analysis of plant tissues was an early demonstrations of the capabilities of PADI. Plant tissues can be interrogated by simply directing the spray onto native or cut tissue surfaces. Several plants are known to contain a wide variety of alkaloids which show different distributions in the different types of plant tissues were investigated. [70]

PADI spectra of plants often showed protonated alkaloids as abundant ions. Besides alkaloids, ordinary metabolic products such as amino acids, polar lipids, or carbohydrates were also detected. Although the qualitative determination of constituents in natural samples is troublesome, the reproducibility of spectra obtained from the same type of tissue provides information on the alkaloid content of plant parts. Possible application areas of plant analysis by PADI include pharmacognosy.

Cortex analysis of *Cinnamomum zeylanicum* species (Cinnamon).

Figure 77. Cinnamon foliage and flowers

Cinnamon (Cinnamomum verum, synonym C. zeylanicum) is a small evergreen tree belonging to the family Lauraceae, native to Bangladesh, Sri Lanka (Ceylon), the neighboring Malabar Coast of India, and Myanmar (Burma), and also cultivated in South America and the West Indies. [71] It is often confused with other similar species and the similar spices derived from them, such as Cassia and Cinnamomum burmannii, which are often called cinnamon too.

The name cinnamon comes from Greek *kinnámōmon*, but in many other languages, particularly Europeans, it has a name akin to French *cannelle*, diminutive of *canne* (reed, cane) from its tube-like shape (figure 78).

Figure 78. Quills of true cinnamon bark and ground cinnamon

We performed direct analysis of Cortex and identify Cinnamic Aldehyde, or Cinnamaldehyde (figure 79); it is the organic compound that gives cinnamon its flavor and odor. This pale yellow viscous liquid occurs naturally in the bark of cinnamon trees and other species of the genus Cinnamomum. The essential oil of cinnamon bark is about 90% cinnamaldehyde. The natural product is trans-cinnamaldehyde. The molecule consists of a phenyl group attached to an unsaturated aldehyde. As such, the molecule can be viewed as a derivative of acrolein. Its color is due to the π → π* transition: increased conjugation in comparison with acrolein shifts this band towards the visible. [72]

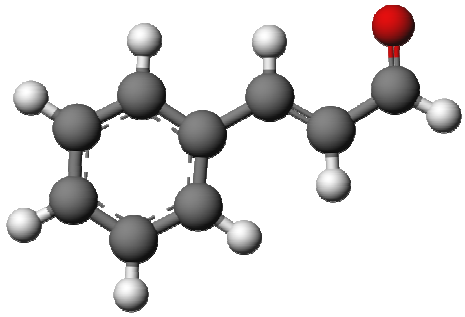


Figure 79. 3D molecular formula of Cinnamic Aldehyde

Active Principle: Cinnamic Aldehyde

Systematic IUPAC name: (2E)-3-phenylprop-2-enal

Molecular Formula: C_9H_8O

Exact Mass: 132.057515

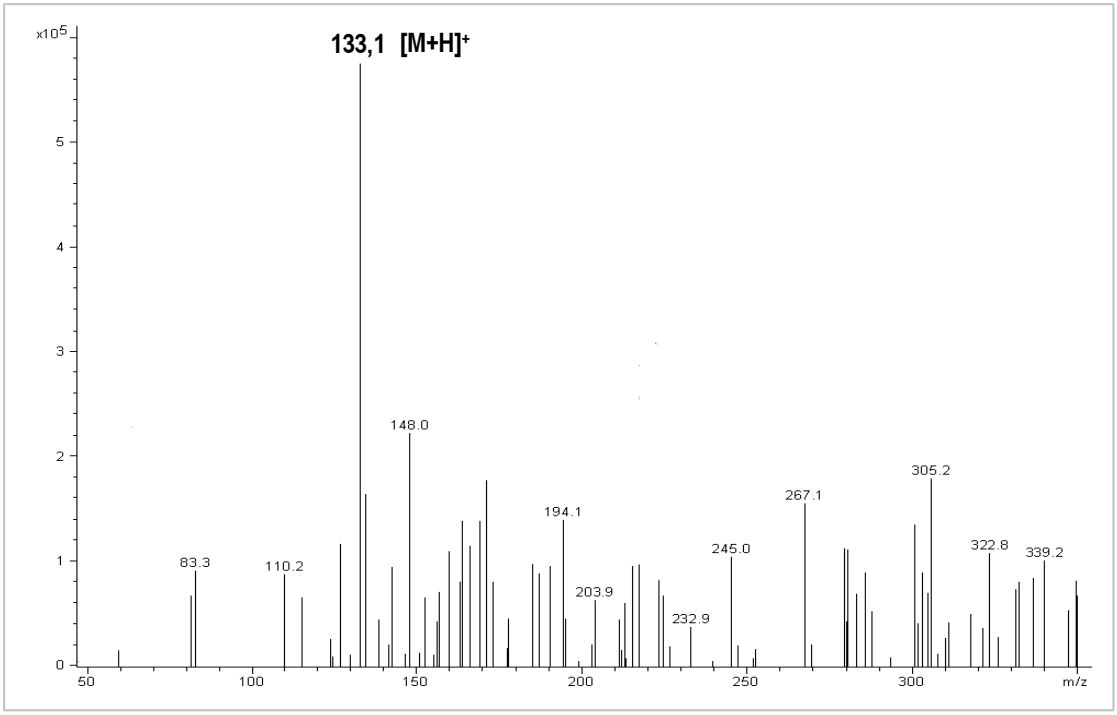


Figure 80. MS spectra, in positive mode, of cortex of *Cinnamomum zeylanicum* specie

Direct analysis of cortex was performed by setting the sample on the surface of sample holder by cyanoacrylic resin. This simple approach permitted to identify, in positive ion mode, the molecular ion of Cinnamic Aldehyde, at m/z 133, as most abundant signal in MS spectra (figure 80).

Seeds analysis of *Pimpinella anisum* fruits (Anise).

Figure 81. Pimpinella *species*

Figure 82. Anise seeds analysed

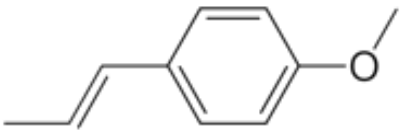


Figure 83. Anethol molecular formula

Anise (*Pimpinella anisum*) is a flowering plant in the family Apiaceae native to the eastern Mediterranean region and Southwest Asia (figure 81). It is known for its flavor, which resembles liquorice, fennel and tarragon. Anise is sweet and very aromatic, distinguished by its licorice-like flavor. The seeds, whole or ground, are used in a wide variety of regional and ethnic confectioneries. [73]

We direct analysed seeds of Anise (figure 82) and identified the aromatic compound of Anise, Anethole (also para methoxy phenyl propene, p-propenylanisole, and isoestragole; figure 83). It contributes a large component of the distinctive flavors of anise. Chemically, anethole is an aromatic, unsaturated ether. It has two cis-trans isomers, involving the double bond outside the ring. The more abundant isomer, and the one preferred for use, is the trans or E isomer (trans-anethole).

Active Principle: Anethole

Systematic IUPAC name: 1-methoxy-4-(1-propenyl)benzene

Molecular Formula: $C_{10}H_{12}O$

Exact Mass: 148.088815

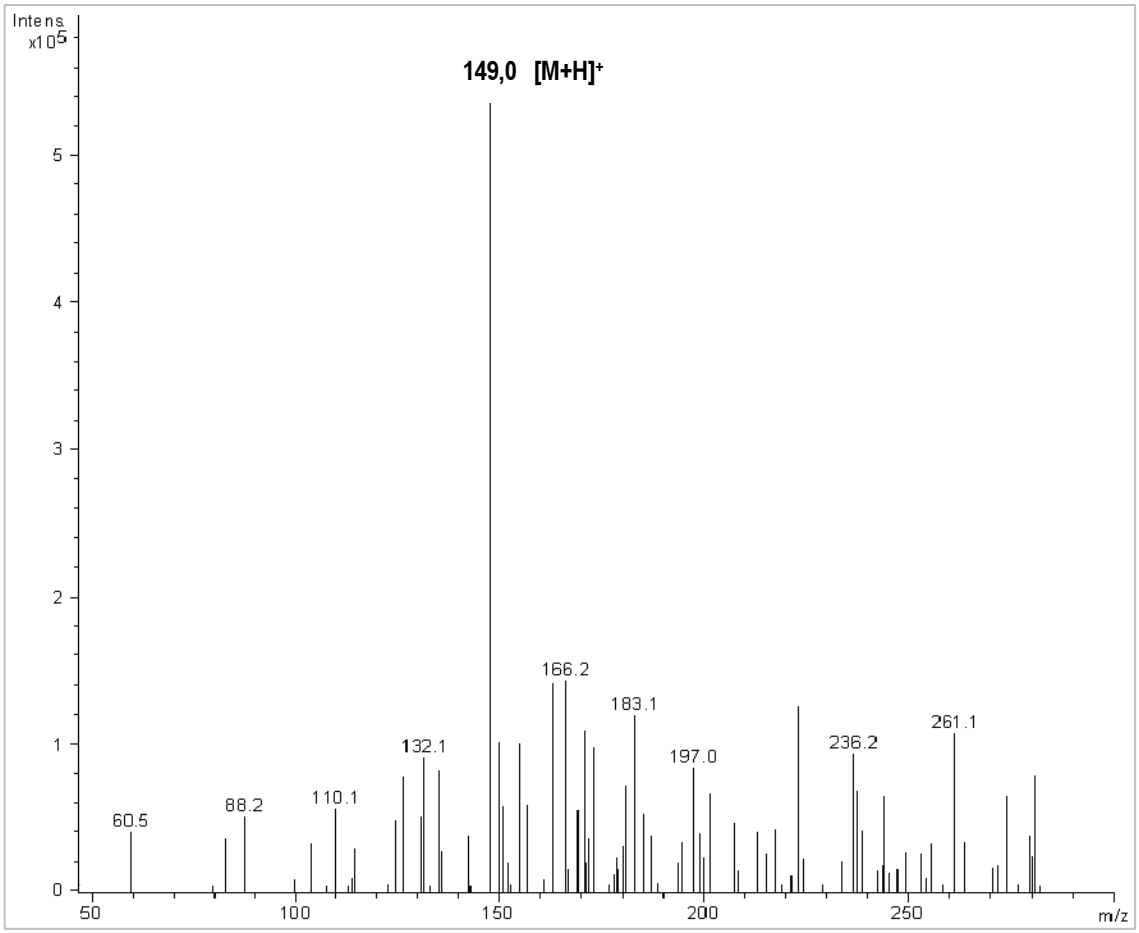


Figure 84. MS spectra, in positive mode, of seeds of *Pimpinella anisum* specie

Also in this case, direct analysis was performed by setting the seeds on sample holder by cyanoacrylic resin.

In figure 84 is reported MS spectra obtained by this direct analysis: we are be able to identify, in positive ion mode, the molecular ion of Anethole at m/z 149, as most abundant signal.

Roots analysis of fresh and dried of *Echinacea pallida* e *Echinacea angustifolia ibrida* species

Finally we studied the roots of both fresh and dried of *Echinacea pallida* e *Echinacea angustifolia ibrida*. Echinacea is a genus of nine species of herbaceous plants in the family Asteraceae which are commonly called purple coneflowers (Echinacea angustifolia, Echinacea atrorubens, Echinacea levigata, Echinacea pallida, Echinacea paradoxa, Echinacea purpurea, Echinacea sanguinea, Echinacea simulata, Echinacea tennesseensis) All are endemic to eastern and central North America. Some species are used in herbal medicines and some are cultivated in gardens for their showy flowers. (figure 85) A few species are of conservation concern.[74]

Figure 85. The spiny center of *Echinacea*

Like most crude drugs from plant or animal origin, the constituent base for echinacea is complex.

To perform direct analysis both fresh and dry roots of *Echinacea pallida* e *Echinacea angustifolia ibrida* were fixed on sample holder by cyanoacrylic resin, as shown in figure 86 and 87.

Figure 86. *Echinacea pallida*, fresh and dried

Figure 87. *Echinacea angustifolia*, fresh and dried

Two main groups of interest compounds in roots of *Echinacea* are phenolic compounds, show in figure 88 and derived primarily from caffeic acid, and lipophilic compounds show in figure 89. The echinacoside (figure 88A) is the most important compound of this family.

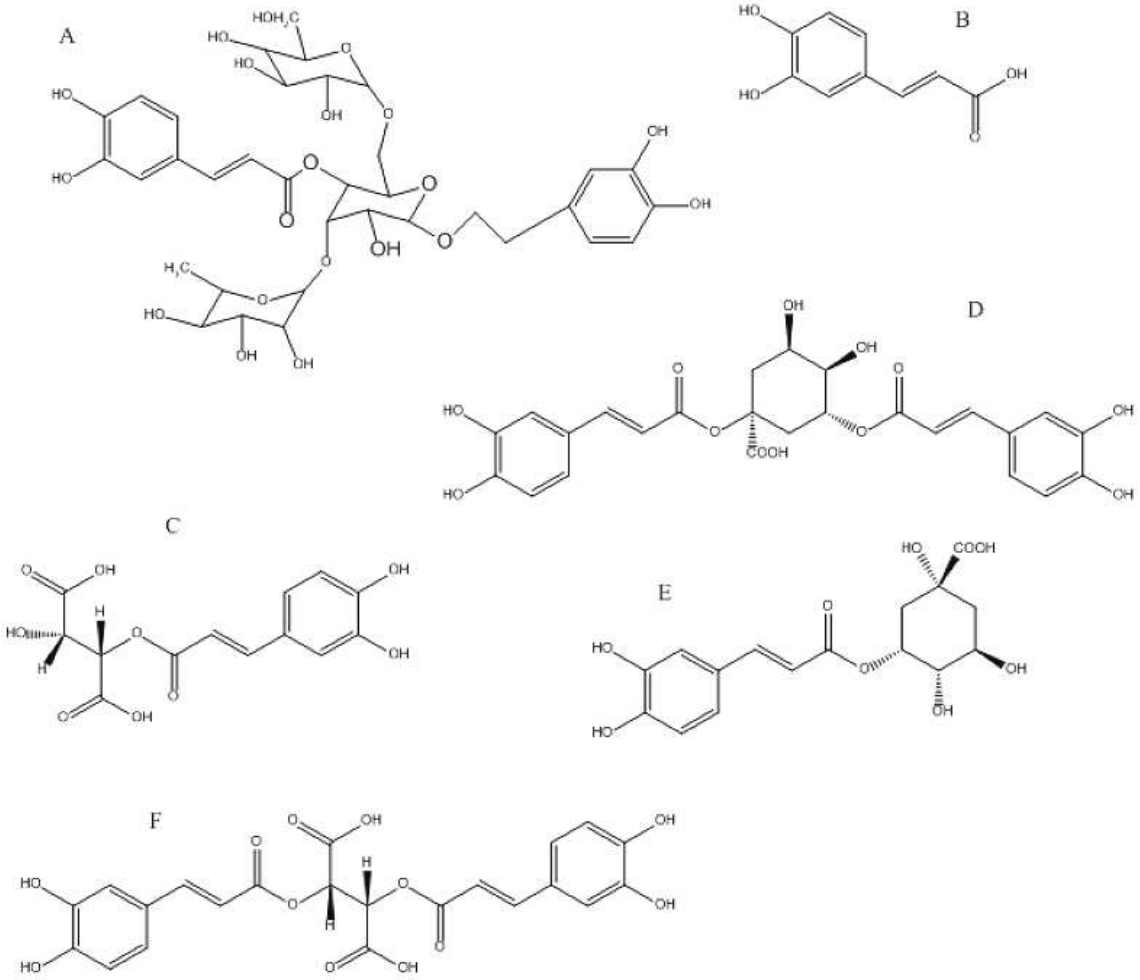


Figure 88. Phenolic compounds in roots of *Echinacea*

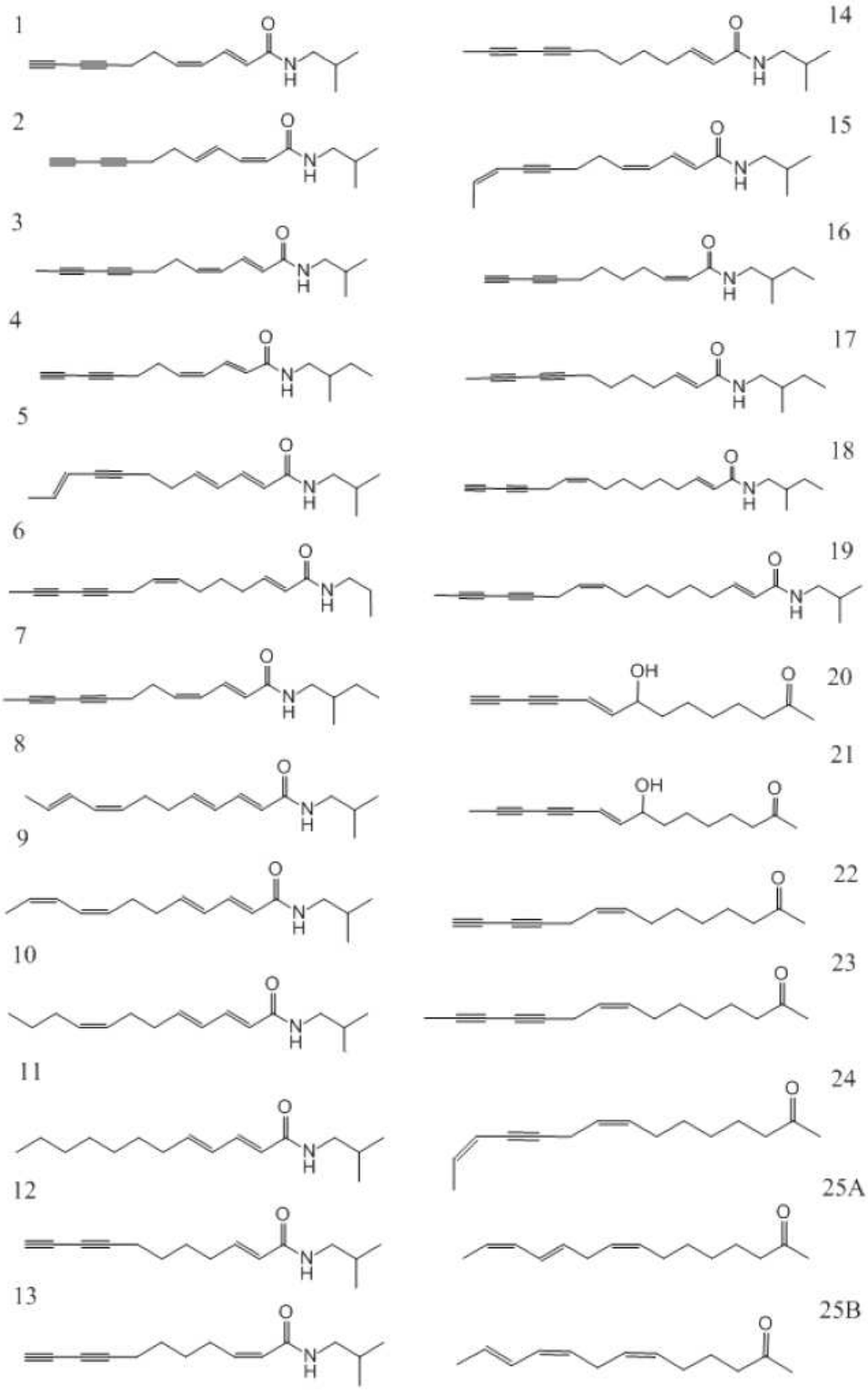


Figure 89. Lipophilic compounds in roots of *Echinacea*

The spectra obtained in positive and negative mode shows that there are no significant differences between the inner and outer, probably for the solubilization of the same metabolic.

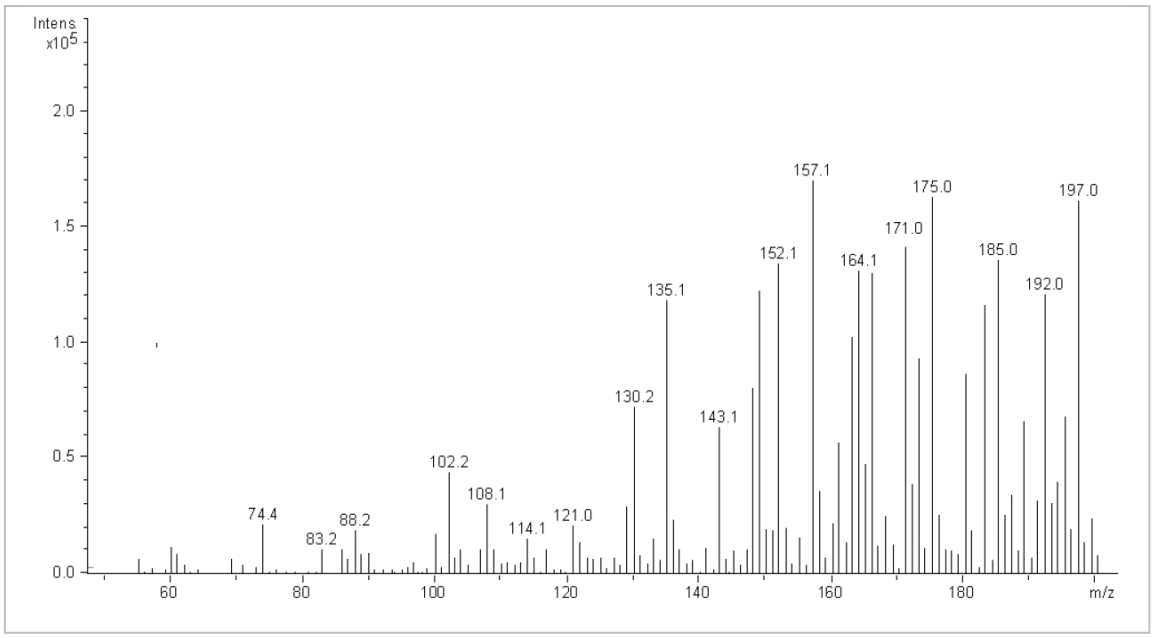


Figure 90. MS spectra, in positive ion mode, of dried root of *Echinacea pallida*

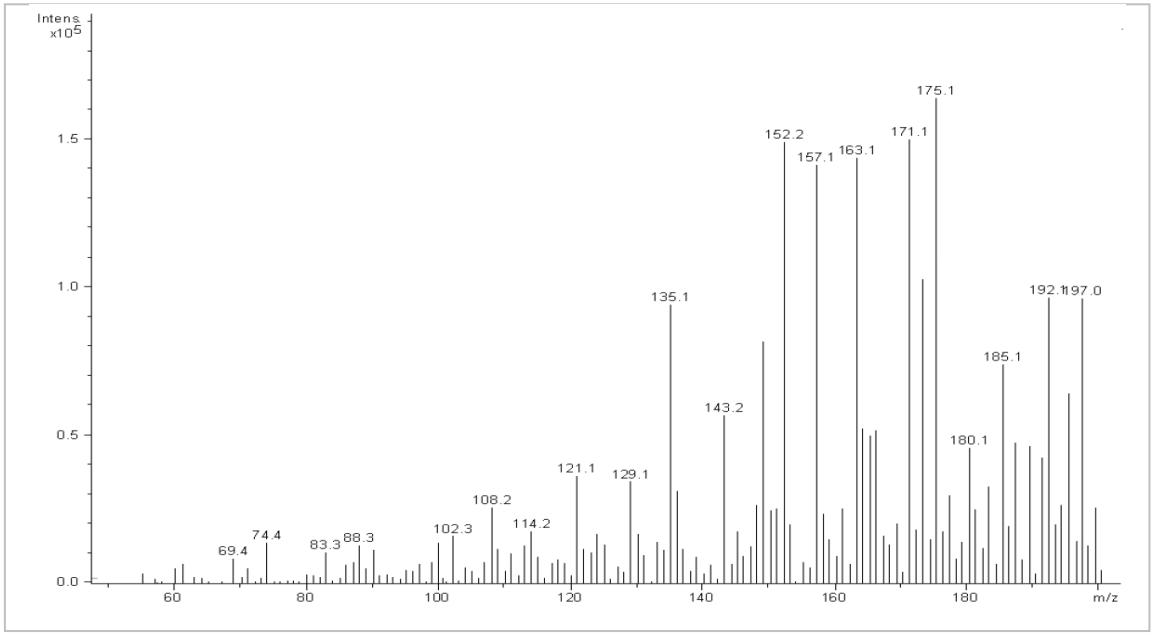


Figure 91. MS spectra, in positive ion mode, of dried root of *Echinacea angustifolia ibrida*

Analyzing the spectra obtaining, we identify the ions related to analyte n. 9 and isomers (figure 89), while the parent compound, the echinacoside (figure 88A) was not identified. This result could be related to the nature of the glucosidic compound, its thermal instability within the capillary heater or its low solubility in the solvent used. The presence of compound n. 9 isomers (figure 89) does not allow a definitive structural assignment, also on the basis of MS/MS spectra, but the PADI system has resulted in a "finger printing" of a large number of metabolites (figure 90 and 91).

From a theoretical point of view the analysis of the fresh root should have led to more significant results, but it did not happen. Indeed, the spectra (in ways both positive and negative ions) from both outside surface and inner part of fresh root is not allowed to identify with sufficient reliability the metabolites identified in the analysis of dried root. Probably because the solubilization of metabolites of the solvent is sprayed on fresh tissue with a yield less, in relation to the increased presence of liquid.

4 CONCLUSIONS

It is to be expected that the diffusion of MS applications will grow in the future, due to relevance of the information that quantitative MS can provide, in particular in the field of public health. For this reason, some basic information of the phenomena which form the basis of this instrumental approach, and some theoretical aspects, have been investigated to understand the real mechanism involved in ions formation.

The data obtained by the present investigation can be summarized as follows:

1. the presence of strong electrical fields leads to an increase of ion signal, but in their absence the production of MH^+ species of analytes is still observed in high yield as already described by Haddad et al. [11]; keeping sprayer, sample holder and entrance capillary at ground potential and without any addition of organic acid to the sprayer solvent, MH^+ ions are still detectable, proving that their production is due to pneumatic effects and not by electrospray-related mechanism;
2. the impact of the sprayed droplets leads to the formation of a solvent thin layer in which the analytes are solubilized. The presence of this thin layer is essential for ion detection: its stability will critically depend on the sprayed solvent and auxiliary N_2 flow;
3. the interaction of further solvent sprayed droplets with the solvent thin layer on the surface leads on one hand to a local heating of the solution, with subsequent decrease of the solvent pk_a, on the other to the emission of secondary droplets of the analyte solution (as described by Costa and Cooks [50]). In the case of solvent with low pk_a values, this could lead to the analyte protonation. However the comparison of the results obtained with different solvents on zopiclone tablets seems to indicate that this phenomenon has only a minor value. The best results have been obtained with acetonitrile

and not with more protonating solvents as water and methanol. Furthermore the results obtained cannot be ascribed to a more effective solubilization of zopiclone in specific solvents: in fact by using chloroform as sprayed solvent [which is the best solvent for zopiclone and exhibits a pk_a value of 25, very close to that of acetonitrile (pk_a = 24)] no signal corresponding to protonated molecules was obtained;

4. the valid results obtained by acetonitrile could be rationalized invoking the occurrence of thermally activated isomerization of acetonitrile, leading to a highly reactive species able to protonate the analyte. However the direct role of acetonitrile as protonating medium has been excluded by spraying CD_3CN: also in this case the production of MH^+ (and not MD^+) is observed, proving that the proton does not originate from the solvent;
5. considering the high N_2 flows (and the consequent high kinetic energy) employed for the experiments, the ionization of N_2 by collisional phenomena has been considered. $N_2^{+\cdot}$ could lead, through a charge-exchange reaction, to a short-lived odd electron ion of the analyte, which behaves as protonating media for neutral molecules.

The results obtained indicate that the formation of MH^+ analyte ions in pneumatically solvent spraying on a surface can originate by many different factors, either chemical or physical, and that further investigations are needed to evaluate their roles in the analysis of different classes of compounds.

In synthesis, PADI is a convenient means of direct transporting analyte in droplets from surface for ionization at ambient conditions.

The promise of no sample preparation is one advantage that PADI and other techniques, DESI and DeSSI offers, however it is acknowledged that some sample preparation may be required under certain circumstances. Another advantage is the fact that the sample is fully accessible to manipulation during the experiment and that direct analysis of living systems is at least conceivable.

Compared with DESI and DeSSI, this simpler technique may be advantageous in several cases as it avoids the use of high voltages or acidic solvents, thus providing a more friendly (high) voltage and acidic-free environment in which to perform ambient ionization. This feature may be important, for instance, when using for in vivo applications of ambient MS.

The ambient MS method described here should help drive the next revolution in MS, making the uniquely sensitive and specific general-purpose technique of MS accessible to a wider range of scientists studying additional types of problems. The development of ambient MS, especially if successfully implemented on miniature mass spectrometers, could be an almost perfect tool for in-situ applications.

Application areas of PADI analysis could include environmental applications, high-throughput analysis, clinical diagnostics, food analysis, forensics and many others. A potentially important and broad area of application of PADI is forensics, in which on-location analysis, speed, specificity, and wide applicability are all important. Applications include monitoring for public safety (for example, explosives, chemical agents, biological pathogens, etc.) as well as monitoring for drugs of abuse, poisons, and in overdose situations.

Understand the mechanism underlying the process of desorption/ionization is a fundamental step to choose carefully the solvent used in relation to each analyte to be identified. This will allow to monitor selected compounds in complex matrices: this capability is a significant advantage to its applicability to both small and large molecules and to perform spatial imaging for particular molecules.

In conclusion, PADI technique can now be applied to many types of samples. Will require studies involving samples of biological origin, carefully considering the specific features of each matrix of interest.

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