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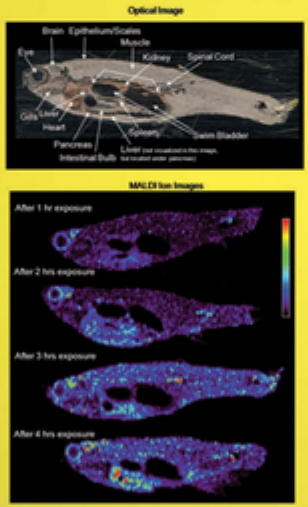
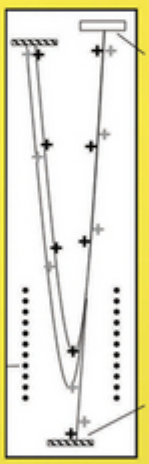
Mass Spectrometry for Drug Discovery and Drug Development





Edited By
WALTER A. KORFMACHER

WILEY

MASS SPECTROMETRY FOR DRUG DISCOVERY AND DRUG DEVELOPMENT

WILEY SERIES ON MASS SPECTROMETRY

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This book is dedicated to the most important people in my life:

Madeleine Korfmacher

Joseph Korfmacher

Mary McCabe

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PREFACE

This book was written as part of a series of books on the utility of mass spectrometry (MS) for various scientific fields. The emphasis for this book is the description of the application of MS to the areas of new drug discovery as well as drug development. MS is now used as the main analytical tool for all the stages of drug discovery and drug development. In many cases, the way MS is applied to these endeavors has changed significantly in recent years, so there is a need for this book in order to provide a reference to the current technology. Thus, the readers of this book would be pharmaceutical scientists including medicinal chemists, analytical chemists, and drug metabolism scientists. This book will also be of interest to any mass spectrometry scientist who wants to learn how MS is being used to support new drug discovery efforts as well as drug development applications.

The book has 15 chapters that are written by experts in the topic that is described in the chapter. The first chapter provides a current overview of the various types of MS systems that are used in new drug discovery and drug development. This chapter will be useful to those still learning about MS as well as experts who want to understand the latest MS technology. One of the major changes in the MS field has been the emergence of high-resolution mass spectrometry (HRMS) as a tool not only for qualitative analyses, but also for quantitative analyses. This change has the potential to produce a true paradigm shift. In the future, it can be predicted that many quantitative bioanalytical assays will shift from using the selected reaction monitoring (SRM) technique with high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) to HPLC-HRMS. Discussions of why and how this will happen can be found in the second, third, and fourth chapters of this book. This shift from HPLC-MS/MS to HPLC-HRMS has the potential to radically change how MS is used in both new drug discovery and drug development. In addition to these three chapters, the final chapter in the book looks at the new topic of quantitative analysis of peptides and asks whether one should use SRM or HRMS for these assays.

Metabolite identification has been a major focus of MS for several decades. Chapter 5 describes the current MS technology that is used for metabolite identification including new software tools that have made this task easier. One of the

newer applications of MS is the quantitative and qualitative analysis of biological drugs; this new topic is described in the sixth chapter along with a discussion of the MS analysis of proteins and peptides. Another important part of drug development is the characterization of impurities and degradation products; the utility of MS for this task is described in the seventh chapter. Medicinal chemists are at the center of all new drug discovery and drug development activities; Chapter 9 describes how MS is used to support the efforts of medicinal chemists in this effort.

An area of continuing interest is the application of MS to surface analysis in order to understand the distribution of drugs and metabolites as well as proteins and peptides on tissue slices from laboratory animal studies and sometimes human clinical tissue samples. Chapter 8 describes the new technique called liquid extraction surface analysis (LESA) that is used for tissue profiling. Chapter 10 discusses MS imaging for proteins and peptides, while Chapter 11 describes the use of MS imaging for drugs and metabolites. Together, these three chapters provide a comprehensive overview of how MS imaging is being used for various drug discovery and drug development applications.

The rest of the book covers various specific topics that are important parts of the drug discovery and drug development process. Chapter 12 deals with the important topic of screening for reactive metabolites. This topic has received increased attention in recent years because of concerns that reactive metabolites may lead to drug safety issues. Two new topics are covered in Chapters 13–14. Chapter 13 describes the use of MS for siRNA applications and Chapter 14 covers the various ways MS is used in the field of metabolomics. The last chapter in the book, Chapter 15, takes a look at the new field of quantitative analysis of peptides using MS techniques.

Overall, this book provides a comprehensive picture of the latest MS technology and how it is being used throughout the various stages of new drug discovery and drug development. I want to thank the authors of each chapter for their efforts and careful attention to detail. I also want to thank Nico Nibbering and Dominic Desiderio, the editors of this MS series, for inviting me to be the editor of this volume. Finally, I want to thank my family for their support of this effort, with special thanks going to Madeleine, my wife.

Walter A. Korfmacher

1

OVERVIEW OF THE VARIOUS TYPES OF MASS SPECTROMETERS THAT ARE USED IN DRUG DISCOVERY AND DRUG DEVELOPMENT

Gérard Hopfgartner

1.1 INTRODUCTION

Since J.J. Dempster published one of the first reports on the detection of volatile organic compounds using electron impact ionization in 1918, significant progress in ion sources and mass analyzers has been achieved. The aim this chapter is to focus on the most commonly used techniques in drug metabolism studies for quantitative or qualitative analysis, and also to discuss some of the "niche" techniques. In terms of the ionization techniques, atmospheric pressure ionization (API) sources including electrospray (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI) have revolutionized the analysis of low molecular weight compounds (LMWCs) by high-performance liquid chromatography-mass spectrometry (HPLC-MS). In addition, matrix-assisted laser desorption/ionization (MALDI) was originally developed for the characterization of biopolymers, but is also attractive for the analysis of LMWCs and for mass spectrometry imaging (MSI) of drugs and their metabolites in tissues. Ambient ionization techniques have also gained interest for the same type of applications. Finally, inductively coupled plasma (ICP) mass spectrometry has also been explored as an alternative detector to ^{14}C-labeled drug for drug metabolism studies.

Triple quadrupole MS systems have become the workhorse for quantitation and, in combination with linear ion traps (LITs), are very attractive for qualitative/quantitative workflows. Ions traps are still used as standalone mass spectrometers

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but more and more in combination with others types of mass analyzers. A new paradigm shift will certainly come from high-resolution, accurate mass systems such as time-of-flight (TOF), ion cyclotron resonance, and Orbitraps, which will allow the application of novel approaches in mass spectrometry for drug metabolism studies. Due to the complexity of the samples, additional orthogonal separation power is always required and ion mobility mass spectrometry could play a more important role in the near future. One of the key problems in HPLC-MS is that the response is compound dependent; accelerator mass spectrometry (AMS) is one option that can be used to overcome this limitation and to provide the ultimate sensitivity in human studies.

1.2 IONIZATION TECHNIQUES

1.2.1 Electrospray

Electrospray is currently one of the most commonly used ionization techniques; in ESI, either singly or multiply charged gas phase ions are generated at atmospheric pressure by electrically charging a liquid flow. It is based on a condensed phase process where preformed solutions ions are transferred to the gas phase. ESI for mass spectrometry was developed by John Fenn and coworkers in an attempt to analyze large biomolecules by mass spectrometry [1]. Charged droplets are generated by applying a strong potential of several kilovolts (2–6 kV) to a liquid stream. An electric field gradient is generated, which induces the deformation of the liquid into a conical shape called the Taylor cone. Then the solution forms a charged aerosol. After size reduction of the droplets by evaporation at atmospheric pressure, ions escape from the droplets and are sampled into the mass analyzer. The concept of applying high potential to a metal capillary to generate ions at atmospheric pressure followed by mass spectrometric detection has also been reported by Alexandrov et al. [2, 3], and they named their method extraction of dissolved ions under atmospheric pressure (EDIAP).

The stability of the aerosol is strongly dependent on the solvent composition, the flow rate, and the applied potential; typically, electrospray works best at the flow rate of a few microliters per minute. To achieve higher flow rates, the spray formation can be assisted by a nebulizing gas (nitrogen), which has been referred to as ionspray [4] or pneumatically assisted electrospray. Most modern instruments can handle flow rates from a few nanoliters per minute to several milliliters per minute. Various atmospheric pressure ion source geometries have been developed, using in most cases some combination of nebulizing gas and heat [5]. Pneumatically assisted electrosprays are well suited as ionization sources for liquid chromatography at various flow rates. It has been stated that ion spray mass spectrometry behaves like a concentration-sensitive detector [6], where the reduction of liquid chromatography column internal diameter should result in an increase of the MS response considering that the same amount of analyte is injected. The actual behavior of ESI sources is very dependent on the ion source geometry and the instrumental settings.

ESI works best with preformed ions in solution and when preformed ions are separated from their counter ions. In 1991, Kebarle et al. [7] reported the electrophoretic nature of ESI, in which the charge balance requires the conversion of ions

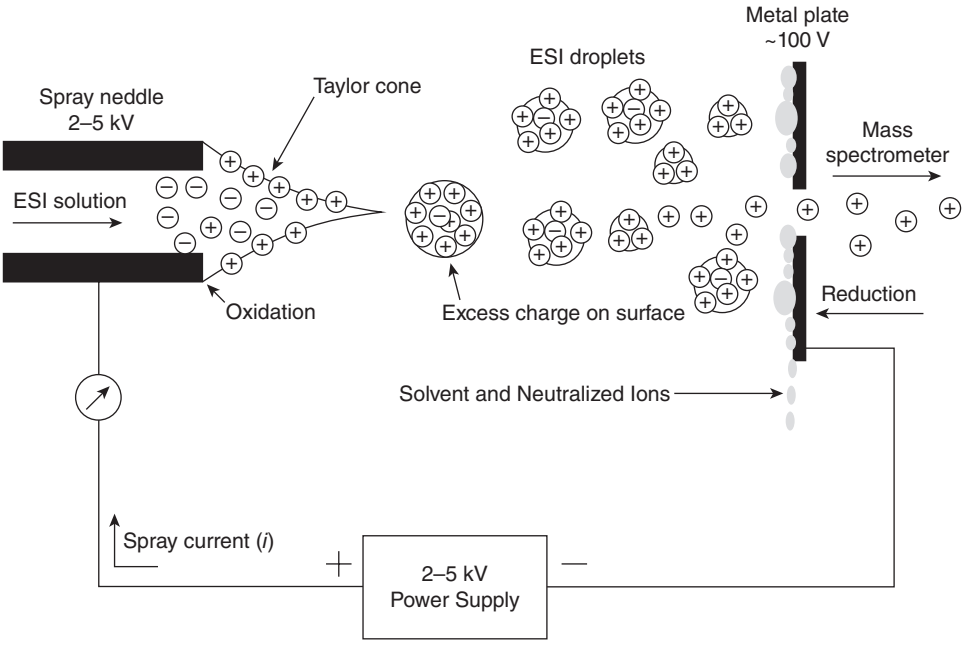


Figure 1.1 Schematic of the electrospray process (adapted with permission from Reference 136).

into electrons. Therefore, oxidation may occur at the needle (Fig. 1.1), and the interface of the mass spectrometer acts as a counter electrode.

Electrospray is particularly suitable for the analysis of inorganic ions and molecules that have acidic or basic functional groups. Organic molecules are generally observed as protonated or deprotonated molecules depending on their pKa. Bases are best detected in the positive mode, while acids give good signals in the negative mode. Therefore, for best signal, the pH of the mobile phase must be adjusted to the acidic or basic nature of the analyte. However, for peptides, it has been shown that intense signals can be observed either in the positive or in the negative mode using strongly acidic or basic solutions, respectively. These observations are reported as "wrong way round" and have been discussed by Zhou and Cook [8]. For many analytes besides the protonated or deprotonated molecules, adduct ions such as sodium or potassium adducts in the positive mode or with formate in the negative mode can be observed. Also, they can also form dimers such as $[2M+H]^+$, which are gas phase reactions [9]. Often it is almost impossible to control the intensity of sodium adducts. The formation of adducts is based on ionization by charge separation which occurs in solution and can be exploited to analyze by ESI polar compounds which are neutral or weakly acidic or basic. In the negative mode, chloride ions adducts can be formed when chlorinated solvents such as chloroform are used [10] or for the analysis of tocopherols and carotenoids where silver ions are added to form $[M+Ag]^+$ ions [11]. Analysis of analytes in highly aqueous solution is more challenging in the negative mode than in the positive mode. This is mainly due to an electrical discharge occurring at the tip of the sprayer (corona discharge)

resulting in the chemical ionization of the analyte and the solvent [12, 13]. Generally, negative ESI operated at lower potential and compressed air is preferred to nitrogen as nebulizing gas.

Typical flow rates for electrospray and pneumatically assisted electrospray range from μL/mL to mL/min. Electrospray can also be operated at very low flow rates; indeed, nanoelectrospray (flow rates <500 nL/min) was developed with the intention to minimize sample consumption and maximize sensitivity [14]. The infusion of a few microliters will result in a stable signal for more than 30 min using pulled capillaries [5] or chip-based emitters [15, 16]. With the infusion signal, averaging allows one to improve the limit of detection in tandem mass spectrometry. The uniqueness of nanoelectrospray is that at nL/min flow rates the droplet sizes are in the submicron range and that the complete spray is sampled into the mass spectrometer. Nanoelectrospray has become particularly important in combination with nanoflow liquid chromatography or chip-based infusion [17]. The ionization efficiency is strongly analyte dependent. Thus, in drug metabolism studies, the relative signal intensities from the sample cannot be correlated directly to the relative abundance of the metabolites. Hop et al. [18] reported that the uniformity of the ionization response could be improved, compared with ESI, by using a chip-based nanoelectrospray source. They argue that the generation of a high electric field around the nozzles produces a large excess of protons and smaller droplets, which minimizes the differences in the ionization efficiency for the analytes.

Hirabayashi et al. [19] described an alternative to ESI called sonic spray. In their device the liquid is sprayed using a high-velocity nebulizing gas. Ions are produced without the application of heat or an electric potential typically at sonic gas velocity. For the analysis of labile compounds and noncovalent complexes the use of a cold spray ionization source was also described [20]. The solution is sprayed into a liquid nitrogen cooled electrospray source. The operating temperature is in the range (ca. –80 to 10°C) that minimizes fragmentation of the analytes compared with conventional electrospray.

The qualitative or quantitative outcome of an electrospray analysis may be strongly dependent on the settings of the experimental parameters such as solvents, flow rate, electrode, electric field, and additives, as well as the nature of the analytes (metal ions, LMWCs, polymers, oligonucleotides, peptides, or proteins). Therefore, understanding the mechanisms of how gas phase ions are formed from ions in solution is important, and reviews have been carried out by Kebarle and Verkcerk in this regard [21]. Two major mechanisms have been proposed—(1) the ion evaporation model (IEM) proposed by Iribarne and Thomson [22] and (2) the charge residue model (CRM) described by Dole et al. [23]—and have been a subject of extensive discussion [4, 24, 25].

In 1968, Dole et al.[23] reported the electrospray analysis of diluted solutions of synthetic polymers, in the negative mode, into air at atmospheric pressure, where the macroion current was detected by a Faraday cage after the light ions have been repelled from the beam by negative voltages on a repeller grid. At that time, there was no evidence of any possible solution to the "vaporization problem," for large polyatomic molecules such as proteins without extensive fragmentation and decomposition [26]. Regarding the formation of gas phase ions, Dole's proposition was that evaporation of solvent would increase the surface-charge density until it reached the Rayleigh limit at which the forces due to Coulombic repulsion and surface tension become comparable. The hydrodynamic instability results in the formation

of a jet of smaller droplets repeated until complete dispersion of the liquid. Ultimately, the droplets become so small that they contain one single solute molecule, and this molecule becomes an ion, thus a "charge residue," when it retains some of the droplet charge as the last of the solvent is vaporized.

Iribarne and Thomson [22], interested in the study of small ions, proposed in 1976 the atmospheric pressure IEM consistent with the scenario described by Dole et al. [23] in that a sequence of evaporation and Coulombic explosions leads to droplets of 10^{-6} cm where charge densities are so high that the resulting electrostatic field at their surface is high enough ($>10^9$ V/m) to push solute ions into the gas phase. The high electric field responsible for the ion evaporation generated by the size reduction of the droplets by heat becomes competitive with further solvent evaporation. In the experiments conducted by Iribarne and Thomson, charged droplets where generated by pneumatic nebulization and the electric field was applied across the plume of evaporating spray to extract small ions and is sometimes referred to as aerospray [27]. Most published work suggests that that the two models strongly depend on the nature of the analyte and that most molecules follow the IEM proposed by Iribarne and Thomson, while large macromecules undergo mostly the charge residue mechanism. A recent study by Nguyen and Fenn [28], where the authors demonstrated the benefit of adding solvent vapor to bath gas, showed that in electrospray—at least for singly and doubly charged peptides—most gas phase ions are likely produced by the IEM rather than by the CRM. Further work indicates that CRM is preceded by IEM, in particular when buffers such as ammonium acetate or triethyl acetate are used [29], or that more nuanced emission mechanisms appear, ranging from pure ion evaporation (PIE) for small ions to pseudo-Rayleigh ion release (PRIR), a mechanism that yields charge states that are nearly indistinguishable from the CRM, for large ions [30].

Blades et al. [7, 12] showed in the early 1990s, when using stainless steel capillaries, the presence of nickel Ni(II) and iron Fe(II) ions in the electrospray solution. They compared the electrospray process with that of an electrolysis cell where an electrochemical (EC) oxidation reaction occurs at the tip of ESI capillary. In general, most of the analytes investigated are not, under standard conditions, affected by the electrochemical process that occurs in electrospray and is most pronounced at very low flow rates. Electrochemistry can be applied ion purpose for chemical derivatization, for signal enhancement, or for the oxidation and cleavage of peptides and proteins [31, 32]. EC oxidation is also of interest in the field of drug metabolism because it can mimic oxidation reactions catalyzed by the enzymes from the cytochrome P450 family [33]. EC cells (couloumetric, amperometric, or in-source) can be easily implemented online prior to MS detection with or without chromatography. In general, the conversion rate is high, but EC does not fully reflect the *in vivo* situation. However, there are two major advantages of EC over the *in vivo* technique: (1) no endogenous cofactors are needed that can affect the MS response, and (2) the direct detection of reactive metabolites becomes possible.

1.2.2 Atmospheric Pressure Chemical Ionization (APCI) and Atmospheric Pressure Photoionization (APPI)

APCI had been commercially available long before ESI but remained a niche technique for HPLC-MS [34] before its use in the quantitative analysis of pharmaceuticals in biological fluids, which started in the early 1990s [35] (Fig. 1.2).

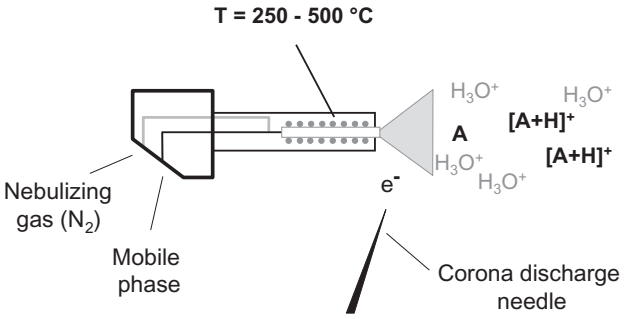


Figure 1.2 Schematic of the heated nebulizer probe.

APCI has an ionization mechanism similar to chemical ionization where gas-phase ion-molecule reactions occur between a neutral molecule and reactant ions [36]. It is a gas-phase ionization process that occurs at atmospheric pressure where the analyte is dissolved in a liquid and introduced through a heated quartz or ceramic tube (heated nebulizer probe) into the source. With the help of heat (T = 200–600°C) and a nebulizing gas (N_2) the liquid and the analyte are completely vaporized prior to being bombarded by electrons generated from a needle by a corona discharge. The discharge current is typically in the range of 1 to 5 μA. Early APCI-MS utilized a ^{63}Ni β-emitter to generate the electrons. APCI is not suited for macromolecules and forms mostly singly charged ions for molecules with a molecular weight of less than 2000 Da and which have some thermal stability. Despite the relatively high temperature of the tube, the temperature of the spray remains in the range of 120–200°C. While the typical liquid flow rates for APCI are in the range of 50–1000 μL/min for most APCI HPLC-MS applications, APCI on a microchip has also been described [37]. The reactants ions are formed through several steps. Initially, the electrons ionize the nitrogen or oxygen to form $O_2^{+\bullet}$, $N_2^{+\bullet}$ ions, which through charge-transfer mechanism form in several steps $H_3O^+(H_2O)_n$ cluster ions. In the presence of more basic molecules such as ammonia, methanol, or acetonitrile reactant ions such as $NH_4^+(H_2O)_n$ or $CH_3OH_2^+(H_2O)_n(CH_3OH)_m$ or $CH_3CNH^+(H_2O)_n(CH_3CN)_m$ will react with the neutral analytes in positive ion mode to form $[M+H]^+$ ions by proton transfer. In some cases, ammonium adduct ions $[M+NH_4]^+$ are formed. The MS response is mainly dependent on the proton affinity of the analyte gas phase acid–base chemistry. In the negative mode, ions are formed either by (1) resonance capture (AB–>AB–), (2) dissociative capture (AB–>B–), or (3) ion-molecule reaction (BH–>B–). Electron capture ionization can be exploited in APCI by derivatizing the analyte to form pentafluorobenzyl derivatives. An increase in sensitivity of 2 orders of magnitude has been demonstrated when compared with conventional APCI methodology for the analysis of steroid, steroid metabolite, prostaglandin, thromboxane, amino acid, and DNA adducts [38].

APPI was developed in an attempt to directly ionize molecules which could not ionize properly with other common API techniques. The setup for APPI [39–43] is very similar to that of APCI. The liquid phase is also vaporized by a heated pneumatic nebulizer and only the corona discharge is replaced by a gas discharge lamp

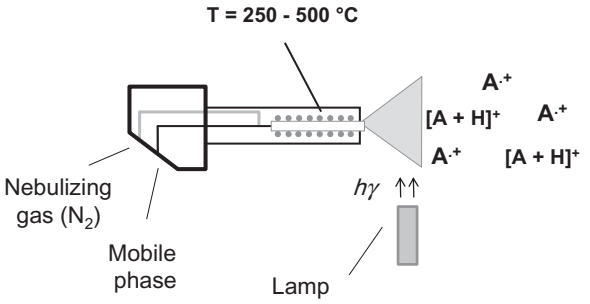


Figure 1.3 Schematic of the photoionization probe.

(Krypton, 10.0 eV) that generates ultraviolet (UV) photons in vacuum. Most analytes have ionization potentials below 10 eV, while typical HPLC solvents have higher ionization potentials (methanol 10.8 eV, acetonitrile 12.2 eV, water 12.6 eV) (Fig. 1.3).

The energy of the photon absorbed by the molecule causes the ejection of an electron and generates a radical cation. While mainly formation of even electron ions has been reported by direct photoionization, the addition of dopants such as toluene or acetone will generate protonated ions with better sensitivities. The ionization process is not fully understood, but two different mechanisms can occur: (1) dopant radical cations react with the analyte by charge transfer, or (2) the dopant radical cations can ionize the solvent molecules by proton transfer, which can then ionize the analyte. APPI can also be performed in the negative mode. As with APCI, APPI can handle a large range of analytes. Cai et al. [44] compared the APPI, APCI, and ESI performance of 106 standard compounds and 241 proprietary drug candidates that represented a wide range of chemical space. They found that APPI is an excellent complementary tool for ionizing compounds that are not ionized by ESI or APCI, and suggested that the technique may be more universal than was previously believed. The performance of APPI is dependent on the flow rate, and better sensitivities than APCI have been reported at lower flow rates. Because APCI and APPI are gas-phase ionization processes, it appears that, compared with ESI, they are less vulnerable to matrix effects [42]. APPI is a useful tool for a large variety of neutral analytes such as steroids [44].

1.2.3 Ambient Desorption Techniques

Direct analysis of solid sample or analytes present on solid surfaces without any sample preparation has always been a topic of interest but somewhat neglected with the success of HPLC-MS. Ambient ionization mass spectrometry allows the direct analysis of samples in their native state. Its major advantage over MALDI is that no matrix is needed to be deposited on the sample. Since the introduction in 2004 of desorption electrospray ionization (DESI) [45] and direct analysis in real time (DART) [46], there has been a continuous development in the field and more than 30 new techniques have been described [47, 48]. They can be classified in three

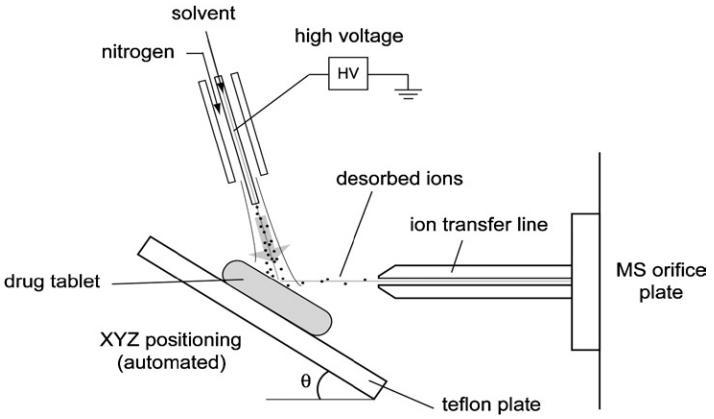


Figure 1.4 Desorption electrospray ionization interface. The sample is placed in front of the orifice and is hit by nebulized droplets. Desorbed ions are then sampled into the mass spectrometer.

groups: (1) ESI or spray-related techniques, (2) spray-based photon/energy, and (3) APCI-related techniques. For drug metabolism studies, ambient ionization mass spectrometry can be applied to analysis of pharmaceuticals and metabolites in tissues [49, 50].

1.2.3.1 DESI Desorption electrospray ionization is an atmospheric pressure ionization method based on charged liquid droplets that are directed by a high-velocity gas jet (in the order of 300 m/s) to the surface to be analyzed. Ions directly produced from the surface to be analyzed are then sampled into the mass spectrometer [51]. The incident angle of the spray plume (relative to the sample) has been extensively investigated and was found to be optimal at around 45–55° (Fig. 1.4).

Various reports on the mechanism of DESI have been published [52, 53] suggesting that both a heterogeneous charge-transfer mechanism and a droplet pick-up mechanism of ionization is occurring. Bereman and Muddiman [54] reported that the spray generates an initial wetting of the surface to analyze, allowing an extraction of the analyte into the film before a delayed formation of the droplets containing the analytes, which subsequently form ions and are sampled into the mass spectrometer. Therefore, the extraction recoveries are certainly dependent on the film properties and the impact of the spray on the sample. DESI has been demonstrated as able to analyze a large variety of analytes from pharmaceuticals to proteins, which are present in many different types of samples. Particularly attractive is the possibility of determining the active components of pharmaceutical tablets [50]. DESI has also been used to make tissue images for the detection of drugs and their metabolites [49] in tissue samples. It has also been applied to dried blood spot analysis [55].

1.2.3.2 DART Direct analysis in real time [46] is based on the reaction of corona discharge-generated metastable helium atoms with oxygen/water (negative mode)

or with water clusters (positive mode). The analytes are ionized by the reactant ions either by cluster-assisted desorption or by proton exchange. Both methods generate mostly protonated or deprotonated molecular ions. Various applications of both techniques in the analysis of mass spectrometric profiling of intact biological tissue, the characterization of the active ingredients in pharmaceutical samples formulated as tablets and ointments, and the sampling of plant material have been reported. Although DART is a new development, simpler devices have been described for the analysis of volatile or semi-volatile liquid or solid materials. Atmospheric pressure solid probe analysis (ASAP) is based on APCI with corona discharge while the analytes are desorbed by a hot nitrogen gas stream (350–400°C) [56].

With all the new development in ionization techniques, it becomes quite difficult to compare them. A couple of reports stated that because negative ion (NI) APPI and DART can produce negative ions by EC, dissociative EC, proton abstraction, and halide attachment, these methods clearly ionize a wider array of compounds than NI APCI, ESI, or even chemical ionization (CI). McEwen et al. [57] investigated the API mechanism of APPI, APCI, and DART, and found that irrespective of the initial method of ionization, similar ion/electron molecule chemistries dominates. They suggest that it is not the initial method of producing the primary ions that defines the ionization efficiency, but it is the ion/electron molecule reactions. Furthermore, the sensitivity difference between the techniques should be more related to instrumental settings and configuration than to the primary ionization method.

1.2.3.3 Liquid Microjunction Surface Sampling Probe The group of van Berkel further developed a concept presented by Wachs and Henion [58] referred to as liquid microjunction surface sampling probe (LMJ-SSP) [59, 60]. Basically it is a liquid extraction probe that allows the extraction of analytes from a surface followed by electrospray analysis. Another type of liquid extraction probe, referred to as "sealing" surface sampling probe (SSSP), was introduced by Luftmann et al. [61] for the analysis of thin layer chromatography (TLC) spots. In LMJ-SSP the analyte is reconstituted from the surface by connecting the probe composed of two channels with a wall-less liquid microjunction. The liquid flow rate is regulated by the aspirating rate from the nebulizing gas of the pneumatically assisted electrospray or APCI probe. Basically any solvent combination can be applied that is compatible with the ionization method. The probe can operate in the discrete mode or in the rastering mode. The concept was also implemented to a commercially available system, the NanoMate (Advion). The chip-based infusion nano-ESI system (Advion, Ithaca, NY) allows the analysis of LMWCs from three types of surfaces: (1) stainless steel plates, (2) paper, or (3) tissues [60]. The principle of the microliquid junction remains, but the operation of the probe is somewhat different and is depicted in Figure 1.5.

A robotic arm picks up a conductive tip that is filled (typically 5 μL) with a solvent mixture and move above the specific sample to analyze. The tip is lowered to the sample and the liquid creates a junction with the target to extract the analyte. After a certain time the liquid is withdrawn and the tip is moved to the back of an ESI chip composed of 100 to 400 microfabricated nozzles. By applying a suitable potential and pressure to the tip a nanoelectrospray is generated operating at flow rates of between 20 and 500 nL/min. For each analysis a new nozzle and tip are used, allowing for carryover-free analysis.

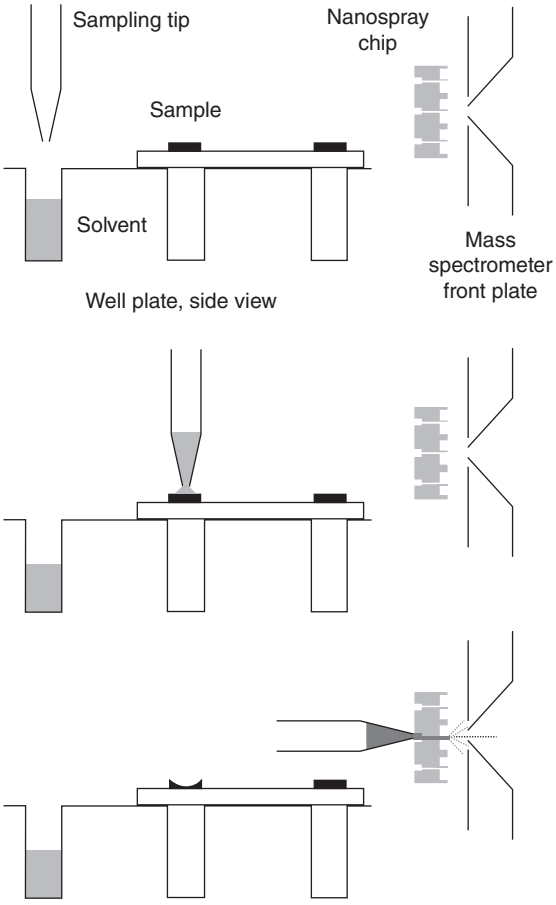


Figure 1.5 NanoMate chip-based infusion nano-ESI system.

1.2.3.4 LAESI Another ambient ionization technique strategy is based on electrospray-assisted laser desorption/ionization. The laser desorption brings the analyte in the gas phase, which is then ionized by electrospray. Basically the sample is deposited orthogonally to an electrospray source and irradiated by laser operating at a defined wavelength. Several variations of this approach, include (1) matrix-assisted laser desorption/ionization ESI (MALDIESI) with an UV laser [62], (2) the use of a nitrogen laser to desorb neutral analytes by a thermal process without matrix (ELDI) [63], and (3) the use a mid-infrared (IR) laser for water-rich samples, which has been described by Nemes and Vertes [64] as consisting of a combination of mid-IR and LAESI. The interaction with the charged droplets generated by the electrospray and neutrals emerging from the laser ablation produces some fused particles, which are the basis of the LAESI signal. The main aim of LAESI is the analysis of untreated water-rich biological samples under ambient conditions, and it was successfully applied on a variety of analytes, including pharmaceuticals, small dye molecules, peptides, proteins, explosives, synthetic polymers, and animal and

plant tissues, in both positive and negative ion modes. Urine and blood samples have also been analyzed directly without any pretreatment.

1.2.4 MALDI

In the late 1960s laser ionization was mostly investigated for the analysis of organic solids and inorganic samples. In the late 1980s MALDI emerged from the efforts to analyze macromolecules by mass spectrometry. Two groups have been able to demonstrate the use of laser ionization to obtain singly charged mass spectra of proteins larger than 100 kDa [65, 66]. The group led by T. Tanaka [65] mixed the analyte in a matrix of glycerol and cobalt and used a nitrogen laser operating at 337 nm for sample ionization. The other group, formed by M. Karas and F. Hillenkampf [67], developed MALDI where originally the dissolved analyte is mixed with a matrix solution containing UV-absorbing molecules such as nicotinic acid and subjected to an Nd:YAG laser operating at 266 nm for ion generation.

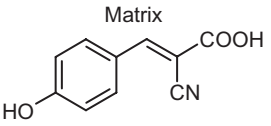
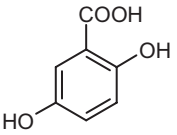
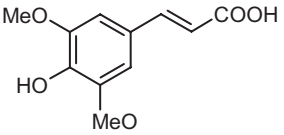
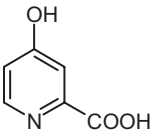
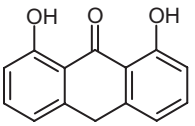
In both methods, singly charged ions are formed and the laser ionization technique was coupled with a TOF mass analyzer. In MALDI, the analyte is typically diluted in a large excess of matrix. A few microliters of the solution is spotted onto a MALDI target where the sample crystallizes. After introduction of the target into the vacuum, a short pulse of a few nanoseconds from a UV laser is used to desorb and ionize the sample. The nitrogen laser emitting at 337 nm and the Nd:YAG laser emitting at 355 nm or 266 nm are the most widely used, with an operating frequency range of 20–200 Hz. More recently lasers with frequencies as high as 1000 Hz are used, in particular in MSI applications. MALDI has largely replaced former techniques such as fast-atom bombardment (FAB) for the analysis of high molecular weight compounds such as peptides and proteins [68], synthetic polymers [69], DNA [70], and lipids [71]. In most cases intact singly charged ions of the analytes or their dimers and more rarely multiply charged ions are observed. As potential matrices, many compounds have been tested randomly [72] or by design [73, 74], but only a few are now used routinely, and the most common matrix compounds are listed in Table 1.1.

The ionization mechanisms involved in MALDI are still under investigation and several of them have been proposed [75]. Unfortunately, the current understanding is not sufficient to allow the selection of the best matrix compound based only on the physicochemical properties of the analytes. In MALDI the matrix serves several functions. First, the matrix must have a strong UV chromophore to absorb the incident laser light and to allow an efficient energy transfer to the matrix causing the sample to disintegrate. Second, after ablation the matrix is vaporized together with ions and neutrals and prevents the formation of aggregates (Fig. 1.6).

Ionic compounds, which are liquids at room temperature, characterized by a very low vapor pressure, have been reported as interesting MALDI matrices because for the analysis of LMWCs the intensity of matrix-derived peaks was found to be lower than conventional matrices. These matrices are generally formed by mixing 2,5-DHB, CHCA, or sinnapinic acid (SA) with an equimolar amount of organic base such as tributylamine, pyridine, or 1-methylimidazole [76].

Matrix background can be fully eliminated by applying matrix-free laser desorption ionization where the sample is placed on a photoactive but nondesorbable support [77]. Desorption/ionization on porous silicon (DIOS) [78] without any

TABLE 1.1 Various Matrices Used for Matrix-Assisted Laser Desorption/Ionization

Matrix	Name	Comments
	α-cyano-4-hydroxy-cinnamic acid (4-HCCA)	Peptides, low molecular weight compounds
	2,5-dihydroxy benzoic acid (DHB)	Proteins
	Sinnapinic acid (SA)	Proteins
	4-hydroxypicolinic acid (HPA)	Oligonucleotides
	1,8,9 antrancetriol (dithranol)	Polar and apolar polymers

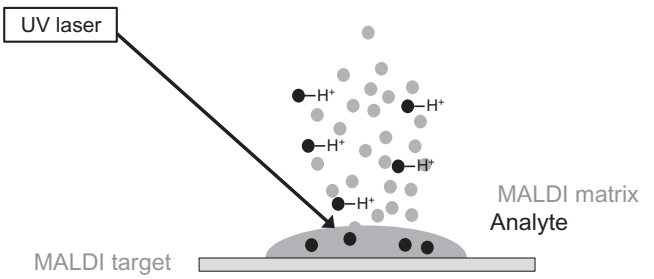


Figure 1.6 Matrix-assisted laser desorption/ionization (MALDI).

matrix has been described for the analysis of LMWCs with no chemical background [79, 80].

Compared with electrospray HPLC-MS, MALDI-MS has an intrinsic advantage in that it can achieve a higher sample throughput because the analyte separation can be decoupled from the mass spectrometric analysis and even multiplexed. The MALDI target plate can be easily archived, which allows simply the re-analysis of

selected samples. One important difference between ESI and MALDI is that in ESI multiply charged ions are formed, allowing the analysis of proteins on almost any type of mass analyzer, while in MALDI a TOF mass analyzer is required in the linear mode to cover the high mass range typically needed for proteins.

The higher throughput capability of MALDI and the different ionization mechanisms make this technique also an attractive alternative to ESI for the analysis of LMWCs [81]. However, interferences of matrix ions with the protonated molecules of the LMWCs may jeopardize somewhat the larger application of MALDI-TOF despite significant improvements in the mass resolution of TOF instruments [82]. Due to the high analysis speed in the selected reaction monitoring mode (SRM), the coupling of a MALDI source with a triple quadrupole mass analyzer for quantitative analysis or for tissue imaging is particularly attractive. Combined with a triple quadrupole LIT MALDI offers interesting perspectives in the analysis of pharmaceuticals, drugs of abuse, or peptides [83, 84]. Because MALDI is a desorption technique, it is particularly suited for the analysis of surfaces such as biological tissues [85] and for the generation of mass spectrometric images of endogenous or exogenous analytes. For mass spectrometric imaging (MSI) applications, the matrix is applied on the complete surface of the tissue generally by spraying. The laser resolution is about 50–200 μm and is operated either in rastering or in discrete mode and complete analyte distribution (LMWCs, peptides, proteins) images can be recorded [86–88].

While MALDI has been widely used in the vacuum with TOF, TOF/TOF or triple quadrupole instrument its application at atmospheric pressure (API-MALDI) has also been described [89]. API-MALDI sources are commercially available and can be mounted on any type of electrospray-based instrument. Schneider et al. [90] performed a comparative study of vacuum-MALDI and API-MALDI on a triple quadrupole LIT instrument. They concluded based on the signal/background ratio that for peptides analysis both techniques provided similar results, while API-MALDI was more difficult to optimize and that thermal degradation of the analytes were observed.

Surface enhanced laser desorption/ionization (SELDI) is a distinctive form of laser desorption/ionization where the target is used in the sample preparation procedure and the ionization process [91]. The SELDI target surface can act as solid phase extraction or an affinity probe depending on the chemical or biochemical treatment of the chip surface. Chromatographic surface is used in sample fractionation and purification of biological samples prior to direct analysis by laser desorption/ionization. SELDI is mainly applied in biological fluids for protein profiling and in biomarker discovery by comparing protein profiles from control and patient groups.

1.2.5 ICP

Inductively coupled plasma mass spectrometry (ICP-MS) has gained significant interest over the last years as a sensitive technique for absolute quantitation of elements, in particular, metals. Detectable drugs or analytes are limited to halogen-, sulfur-, metal-, and metalloid-containing molecules [92]. In (ICP) ionization, the sample must be in the liquid form pumped with a peristaltic pump into a nebulizer, where it is nebulized with the help of argon gas. The fine droplets are separated from the larger ones in the spray chamber, and emerge into the ICP plasma torch.

An ionized gas is formed by the interaction of a high magnetic field, produced by a radio frequency (RF), and argon and, when bombarded with electron forms a high temperature plasma discharge. Despite the limited use of ICP-MS, it is an alternative technique for drug metabolism studies in particular since it can be combined with liquid chromatography and the response is independent from the analyte structure.

1.3 MASS ANALYZERS

1.3.1 Triple Quadrupole Systems

A two-dimensional (2D) quadrupole field is generated when applying an electric potential ($\Phi(x,y)$ within four hyperbolic or circular rods placed in parallel with identical diagonal distances from each other. The rods are electrically connected in pairs. An alternating RF potential (V) and a positive direct current (DC) potential (U) is applied on one pair of rods $\Phi(x)$ while a negative potential is applied on the other pair $\Phi(y)$. The ion trajectory is affected in the x and y directions by the total electric field composed of a quadrupolar alternating field and a constant field. When accelerated ions enter the quadrupole they maintain their velocity along the z-axis (Fig. 1.7).

The motion of ions in the quadrupole (x and y) is quite complex and its stability is defined by the solution of the Matthieu equations. The Matthieu equation contains two parameters (terms a and q), which are proportional to the RF and DC potentials, respectively. The solutions of the Matthieu equations are classified stable and unstable. An ion motion is stable when the amplitude of its oscillations never reaches the rods of the quadrupole. For a detailed description of Matthieu equations, please see the book by March and Todd [93]. To be detectable, an ion must have a stable trajectory in the x and y directions. Although there are an infinite number of

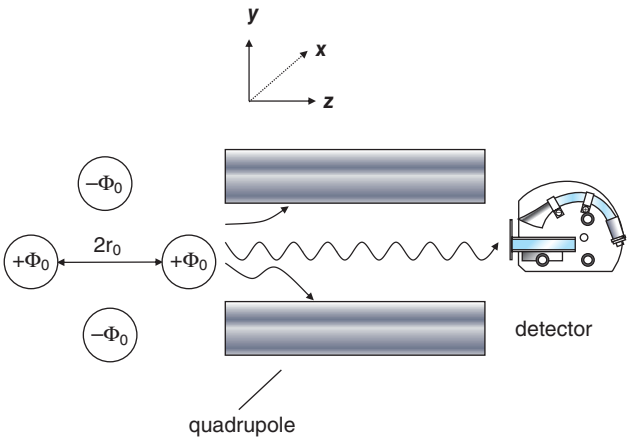


Figure 1.7 The quadrupole mass analyzer is formed by four circular or hyperbolic rods placed in parallel, Φ quadrupolar potential.

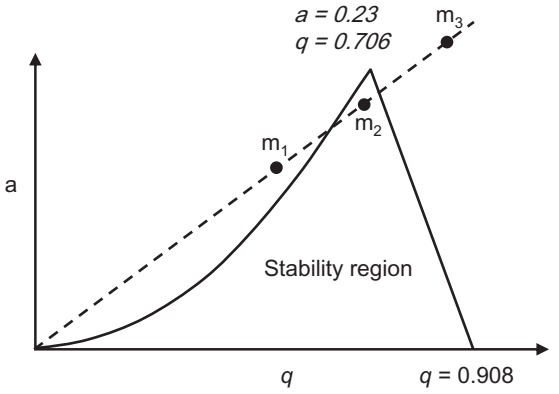


Figure 1.8 First stability region with scan line.

stability regions, most commercial quadrupoles operate in the "first" stability region, which is illustrated in Figure 1.8.

To detect an ion m_2 (Fig. 1.8) the RF and DC voltage are to be set in such a manner that m_2 is almost at the apex of the stability diagram. A lighter ion m_1 and a heavier ion m_3 with larger and smaller a and q values are outside the stability diagram. To obtain a mass spectrum, the RF and DC voltages are cramped together at a fixed ratio while their respective amplitudes are increased. In a quadrupole mass analyzer, when the DC voltage of the quadrupole is set to 0 and the RF voltage is maintained ions remain focused with no mass selectivity. Therefore, RF quadrupoles are ideal as ion guides or as collision cells. Typically, quadrupole mass analyzers operate at a unit mass resolution corresponding to a peak full width at half maximum (FWHM) of 0.6–0.7 *m/z* units. The resolution can be tuned by changing the a/q or U/V ratio. Resolution corresponding to a peak width of 0.1 *m/z* units without significant loss in sensitivity has also been reported [94, 95]. The mass range of quadrupole is typically between *m/z* 5 to *m/z* 4000 and dependent on the operating frequency (in the KHz–MHz range). The higher the frequency the lower the mass range. Often quadrupole mass analyzers are wrongly considered as slow scanning instruments. In general, most quadrupoles used for LC-MS are operated with scanning speeds of 500 to 2000 units/s, and a triple quadrupole with a scan speed of more 10,000 units/s have also been commercialized. Most common ionization sources are available on quadrupole instruments including electron ionization (EI), ESI, APCI, APPI, and MALDI.

A triple quadrupole instrument (QqQ) is a combination of two mass resolving quadrupole (tandem mass spectrometry) separated by a collision cell, which is also a quadrupole operating in the RF-only mode (Fig. 1.8). A common nomenclature is to use (Q) to describe a quadrupole that is operated in the RF/DC mode and (q) for a quadrupole that is operated in the RF-only mode. Tandem mass spectrometry or MS/MS is required to obtain structural information, in particular for soft ionization techniques such as electrospray. In a first step, a specific *m/z* ion (precursor ion) is selected in the first mass analyzer (Q1). Collision-induced dissociation (CID)

occurs in the collision cell (q2) where precursor ions collided with a neutral gas such as argon or nitrogen. The fragment ions (product ions) are then sorted according to their mass-to-charge ratio in the second mass analyzer (Q3) and recorded by the detector. Because the generation of the product ions is performed sequentially, this type of CID experiment is called MS/MS in space; this is in contrast to quadrupole ion traps (QITs) where MS/MS experiments are performed in time. On triple quadrupole mass spectrometers, the potentials used to perform CID are in the range of 0–250 V. As the collision energy is defined in electrons volts (eV) it is therefore dependent on the charge of the ions. For a potential difference of 30 volts, the collision energy for a singly charged precursor ion would be 30 eV, and for a doubly charged precursor ion, 60 eV. The nature of the collision gas (N_2 or Ar) does not affect the product ion spectrum, except the energy needed to achieve similar fragment ratios. The gas pressure in the collision cell mainly influences the sensitivity, while collision energy changes the nature and intensity of the fragments (Fig. 1.9).

Quadrupole mass analyzers can be operated in transmission, scan, or fixed mode. Various types of MS and MS/MS experiments can be performed on a QqQ and are summarized in Table 1.2. A symbolism to describe various MS/MS or multistage MS^n experiments have also been proposed [96, 97].

The product ion mode is generally used to record the collision-induced fragments of a precursor ion to allow structural elucidation. The precursor ion mode is used

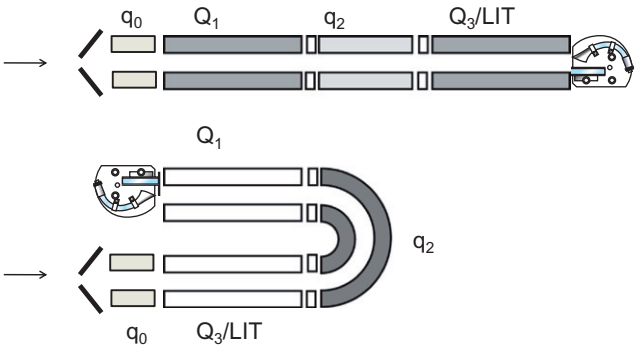


Figure 1.9 Schematic of a triple quadrupole instrument, q0 focusing quadrupole, Q1 and Q3 mass analyzing quadrupoles, q2 collision cell. In the present configuration, the collision energy (CE) is determined by the potential difference between q0 and q2.

TABLE 1.2 Settings of the Q1 and Q3 Quadrupoles for the Various Scan Modes of a Triple Quadrupole

Mode	Q1	Q3
Full Scan Q1/Single ion monitoring (SIM) Q1	Scan/fixed	RF mode
Full Scan Q3/Single ion monitoring (SIM) Q3	RF mode	Scan/fixed
Product ion scan (PIS)	Fixed	Scan
Precursor ion scan (PC)	Scan	Fixed
Neutral loss (NL)	Scan	Scan-neutral loss offset
Selected reaction monitoring (SRM)	Fixed	Fixed