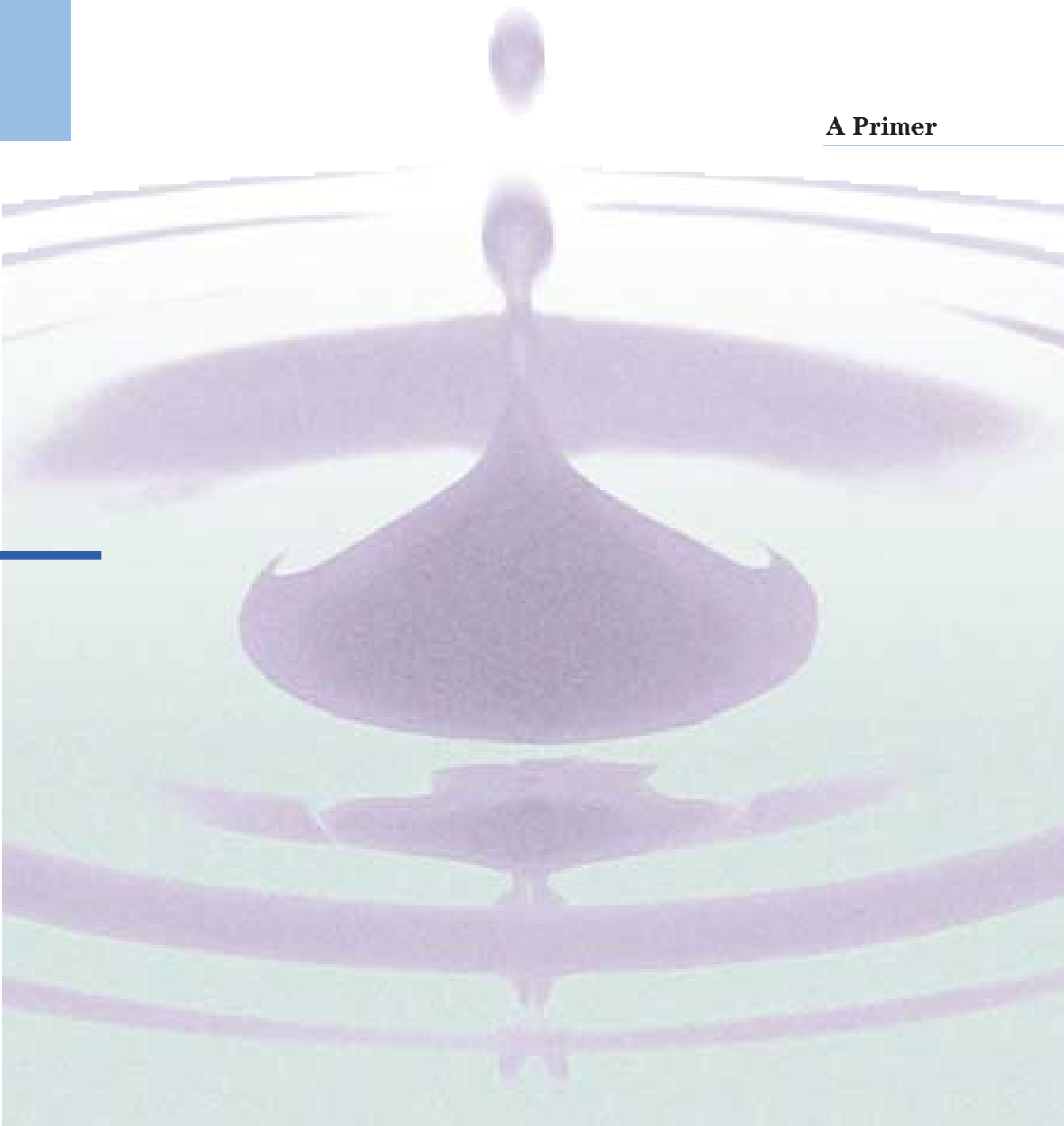
HEWLETT PACKARD
Expanding Possibilities

Basics of LC/MS

A Primer



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Introduction

Liquid Chromatography/Mass Spectrometry (LC/MS) is fast becoming the preferred tool of liquid chromatographers. It is a powerful analytical technique that combines the resolving power of liquid chromatography with the detection specificity of mass spectrometry. Liquid chromatography (LC) separates the sample components and then introduces them to the mass spectrometer (MS). The MS creates and detects charged ions. The LC/MS data may be used to provide information about the molecular weight, structure, identity and quantity of specific sample components.

Sample Types

LC/MS systems facilitate the analysis of samples that traditionally have been difficult to analyze. Despite the power and usefulness of gas chromatography/mass spectrometry (GC/MS), many compounds are impossible to analyze with GC/MS.

LC/MS significantly expands the effective analytical use of mass spectrometry to a much larger number of organic compounds. Gas chromatography and GC/MS can be used to analyze a small percentage of the 9 million registered compounds. Because they impart little or no heat to the analyte molecules, LC and LC/MS-based methods can be applied to most organic compounds. Sample types range from small pharmaceutical compounds to large proteins.

Because it is a much more widely applicable method than GC/MS, LC/MS is suitable for the analysis of large, polar, ionic, thermally unstable and involatile compounds. Some of these compounds can be made amenable to GC/MS by derivatization, but LC/MS eliminates the need for time-consuming chemical modifications. This permits MS analysis of non-volatile, thermally labile, or charged molecules.

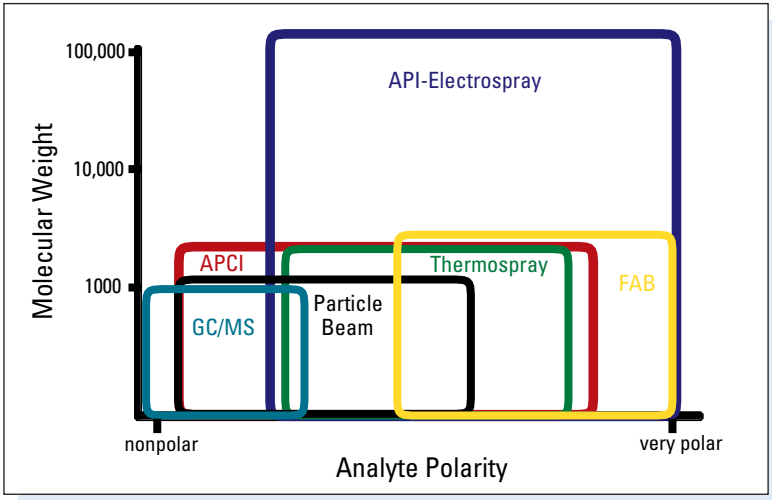


Figure 1. Applications of various LC/MS techniques

Selectivity and Sensitivity

A mass spectrometer combined with a liquid chromatograph can detect masses characteristic of a compound or of a class of compounds. The system can selectively detect compounds of interest in a complex matrix, thus making it easy to find and identify suspected impurities at trace levels.

When configured to simultaneously detect a range of masses (and depending on the compound) LC/MS sensitivity can be comparable to that provided by a diode-array detector (DAD). Far greater sensitivity is possible when the LC/MS is configured to detect only those masses characteristic of the compounds being monitored.

Complementary Information

Using MS in combination with other LC detectors gives richer information. For example, a DAD acquires data on selected ultraviolet (UV) and visible (Vis) wavelengths and spectra. This information is useful for identifying unknown peaks and for determining peak purity or for both. An MS acquires mass information by detecting ions; it offers molecular-weight and structural information. The LC/MS can be used with analytes that do not have chromophores. The two orthogonal sets of data can be used to confidently identify, confirm, and quantitate compounds. In addition, an LC/MS can be used as a highly selective and sensitive tunable detector. An MS chromatogram for a single mass often produces an interference-free signal that offers high precision and low minimum detection limits.

Using both a UV detector and a mass selective detector is more effective than using either one alone. There are compounds (such as metabolites or degradents) for which the UV-Vis spectra of two analytes will be very similar and it may be difficult to detect an impurity based on UV spectra alone. It is also possible to have impurities that have the same mass, especially at lower molecular weights. It is rare, however, for two components to have identical UV-Vis spectra and mass. Figure 2 shows the ability to separate polymer components from an unresolved peak using the information available in a mass spectrum. This separation would not be possible using a conventional UV detector.

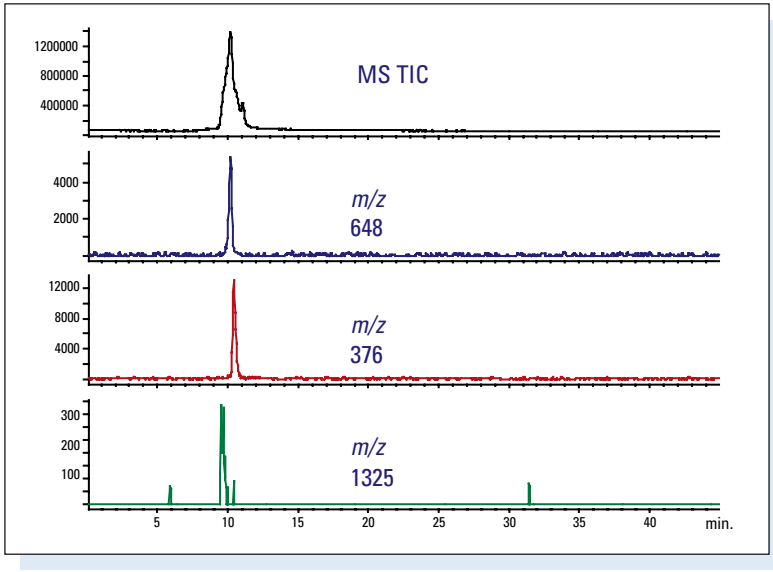


Figure 2. Separation of isomers in a chromatographically unresolved peak

Instrumentation

LC/MS systems have improved dramatically over the last 20 years. Instruments have been transformed from complex, high-cost, highly advanced research tools to low-cost, robust, easy-to-use routine detectors. And, as the instruments have been refined, more applications have been developed.

Interfacing LC and MS

There has been a major focus on improving the interface between the LC and the MS. Liquid chromatography uses high pressure to separate a liquid phase and produces a high gas load. Mass spectrometry requires a vacuum and a limited gas load. For example, common flow from an LC is 1 ml/min of liquid which, when converted to the gas phase, is 1 l/min. However, a typical mass spectrometer can accept only about 1 ml/min of gas. Furthermore, an LC operates at near ambient temperature where as an MS requires an elevated temperature. There is no mass range limitation for samples analyzed by the LC but there are limitations for an MS analyzer. Finally, LC can use inorganic buffers and MS prefers volatile buffers.

Recent developments in atmospheric pressure ionization sources have expanded the molecular weight, sample polarity, and flow-rate limitation of older LC/MS techniques. In many cases, analysts are able to use unmodified high-pressure LC methods.

Atmospheric Pressure Ionization

Atmospheric pressure ionization (API) techniques are soft ionization processes well suited for the analysis of large and small, polar and nonpolar, labile compounds. These techniques can be used to rapidly confirm the identity of a wide range of volatile and nonvolatile compounds by providing sensitive and accurate molecular-weight and fragmentation information. API techniques can be used in metabolite confirmation analysis of most pharmaceutical compounds, and other applications.

API-Electrospray

Application

API-electrospray (API-ES) is useful in analyzing samples that become multiply charged such as proteins, peptides, and oligonucleotides, as well as in analyzing samples that are singly charged, such as benzodiazepines and sulfated conjugates.

API-ES can be used to measure the molecular weights of most polymers, peptides, proteins, and oligonucleotides up to 150,000 Daltons quickly and with high mass accuracy. In biopharmaceutical applications, chemists use API-ES to speed protein characterization, to accurately identify and characterize post-translational modifications, and to quickly confirm the molecular weight of synthetic peptides.

Process

API-ES is a process of ionization followed by evaporation. It occurs in three basic steps: (1) nebulization and charging; (2) desolvation and; (3) ion evaporation.

Nebulization

The HPLC effluent is pumped through a nebulizing needle which is at ground potential. The spray goes through a semi-cylindrical electrode which is at a high potential. The potential difference between the needle and the electrode produces a strong electrical field. This field charges the surface of the liquid and forms a spray of charged droplets. There is a concentric flow of gas which assists in the nebulization process.

Desolvation

The charged droplets are attracted toward the capillary sampling orifice. There is a counterflow of heated nitrogen drying gas which shrinks the droplets and carries away the uncharged material.

Ionization

As the droplets shrink, they approach a point where the electrostatic (coulombic) forces exceed the cohesive forces. This process continues until the analyte ions are ultimately desorbed into the gas phase. These gas-phase ions pass through the capillary sampling orifice into the low-pressure region of the ion source and the mass analyzer, see Figure 3.

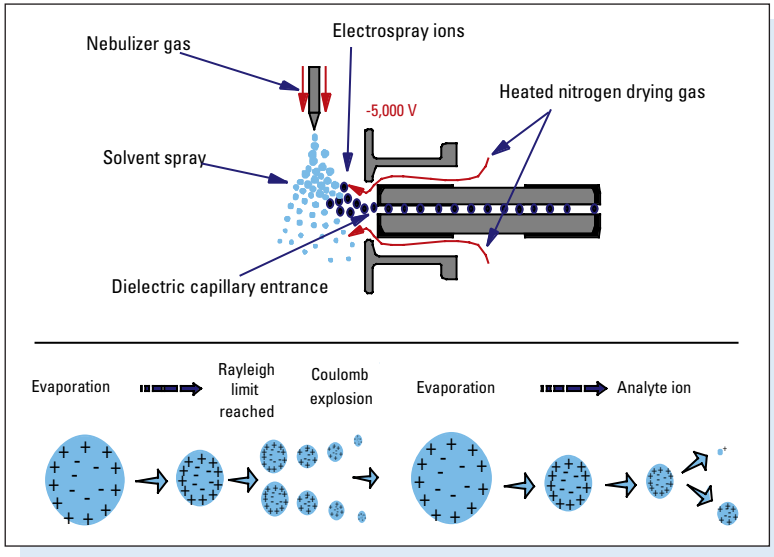


Figure 3. API-electrospray ionization

Atmospheric Pressure Chemical Ionization

Application

Atmospheric pressure chemical ionization (APCI) is an ionization technique that is applicable to a wide range of polar and nonpolar analytes that have moderate molecular weights.

Process

APCI, a process of evaporation followed by ionization, is complementary to API-ES.

Nebulization and Desolvation

APCI nebulization is similar to that in API-ES. However, APCI nebulization occurs in a hot (typically 250°C–400°C) vaporizer chamber. The heat rapidly evaporates the spray droplets, resulting in gas-phase HPLC solvent and analyte molecules, see Figure 4.

Ionization

The gas-phase solvent molecules are ionized by the discharge from a corona needle. In APCI there is a charge transfer from the ionized solvent reagent ions to the analyte molecules in a way that is similar to chemical ionization in GC/MS. These analyte ions then are transported through the ion optics to the filter and detector.

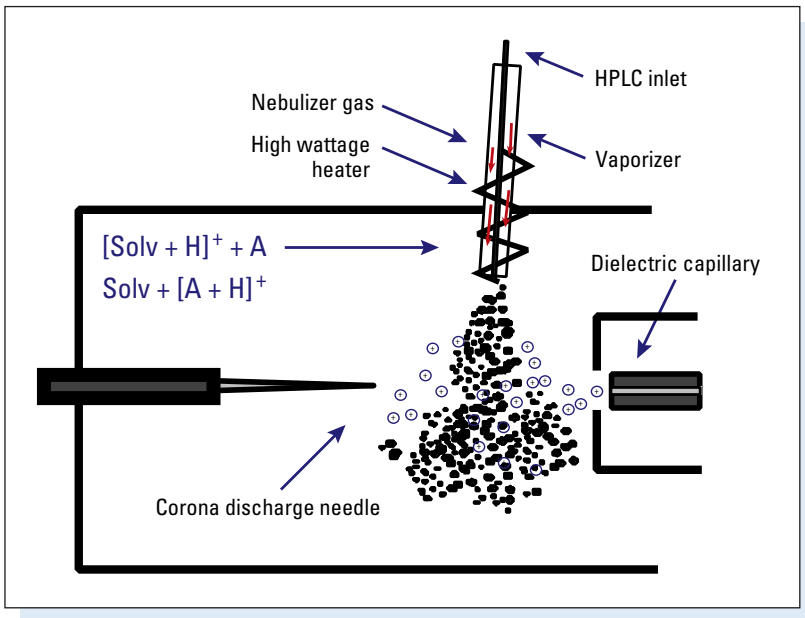


Figure 4. Atmospheric Pressure Chemical Ionization (APCI)

Scan and SIM

Mass spectrometers can be operated in either a scan mode or a selected ion monitoring (SIM) mode (Figure 5).

Scan Mode

In the scan mode, the instrument detects signals over a mass range (e.g. from 50–2000 m/z) during a short period of time (e.g. 2 sec). During this scan period, the MS electronics sequentially read the signals detected within narrower mass intervals until the full mass range is covered. The spectra that are stored represent the detected signal for the full mass range. Since full mass spectra are recorded, this mode of operation is typically selected for qualitative analysis, or for quantitation when all analyte masses are not known in advance.

Samples may be introduced into a mass spectrometer by infusion or through an HPLC. In the latter, it is important to match the peak width and the scan range. The narrower the peaks, the shorter the total scan time must be in order to get proper peak definition. In order to get a short total scan time, it may be necessary to reduce the scan range.

SIM Mode

Mass spectrometers can also operate in the selected ion monitoring (SIM) mode. Rather than scanning continuously, they can be set to only monitor a few mass-to-charge ratios (m/z). As a result the quadrupole is able to spend significantly more time sampling each of the m/z values, with a concomitant and large increase in sensitivity. Moreover, because the cycle-time between data points is often shorter than it is in scan mode, quantitative precision and accuracy are improved through optimal peak-shape profiling.

Since the m/z values to be sampled must be set in advance, SIM is most often used for target compound analysis. For analyses consisting of multiple target compounds, SIM ion sampling choices can be time-programmed to match compound elution time windows. No data is collected at m/z values other than those specifically sampled, so SIM is rarely used in qualitative analysis.

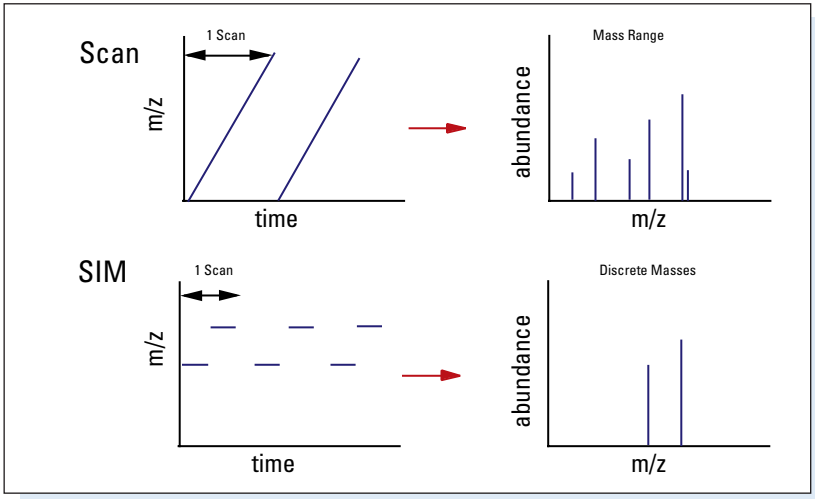


Figure 5. Scan and SIM data acquisition

Collision-Induced Dissociation

MS/MS is accomplished by a process called collision-induced dissociation (CID) in which ions break apart as a result of collisions with other molecules. Electrospray ionization can also be used to produce CID spectra even with a single quadrupole system. In many instances, a single quadrupole system can be used for work that has traditionally required triple quadrupoles or ion traps.

Electrospray is a soft ionization technique that produces a large number of molecular adduct ions. Adduct ions are typically protonated parent ions $[M+H]^+$. These ions are guided into the vacuum region by applied voltages on lenses. By changing the voltage, various degrees of fragmentation may be achieved. With a low voltage, there is little fragmentation; with higher voltages, the parent ion is fragmented to a larger degree. Electrospray CID has the advantage of more efficient transfer than in a triple quadrupole.

There are some limitations to using CID in a single quadrupole mass spectrometer. In a triple quadrupole or ion trap, a single ion can be selected and fragmented. In CID with a single quadrupole instrument, there will be multiple ions in the source that are fragmented. These other ions may interfere with the analyte of interest by generating additional fragments. In many cases, this problem can be solved by improving the chromatography to isolate the analyte (Figure 6).

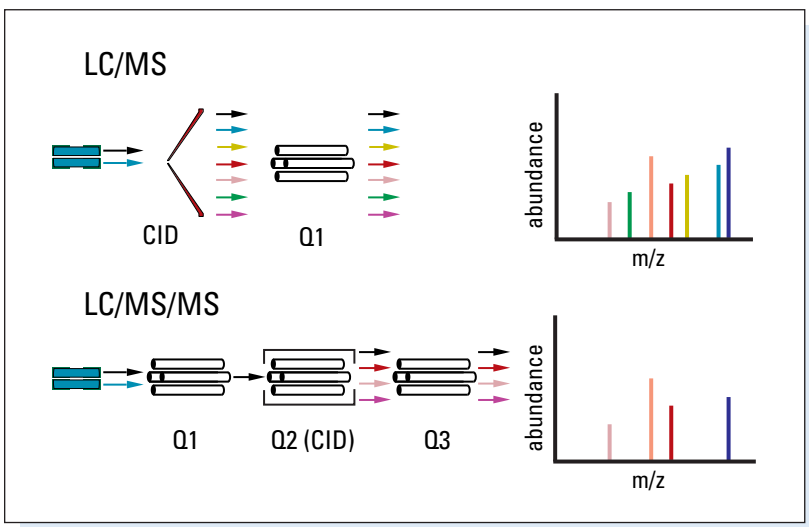


Figure 6. CID with single quadrupole and triple quadrupole mass spectrometers

Adapting LC Methods

Compared to previous LC/MS interfaces, the API-ES and APCI interfaces are relatively rugged. In many cases, existing methods may be used with little or no adaptation. Some instruments allow flow rates of 1 to 2 ml/min without splitting.

One of the most critical factors in adapting LC methods is the choice of buffer. Involatile buffers interfere with good MS performance. For the best long-term perfomance, it is highly recommended that the method be modified to use a volatile buffer.

Modern mass spectrometer designs incorporate a number of features to increase their tolerance for involatile buffers. In the first-generation systems, the spray from the LC was directed into the lens axis for quadrupole systems. In newer designs, the flow is directed orthogonal to the lens axis and ions are focused into the mass filter (Figure 7). The extraneous material is pumped away.

Figure 8 shows a source that had been subjected to 600 injections with a complex involatile salt solution (approximately 9 g/l). Even after this abuse, the performance was only slightly degraded. This ruggedness makes this technique attractive for laboratories in which there are many users of the system and in which large varieties of sample are commonly handled. The instrument does not need to be finely adjusted for every sample.

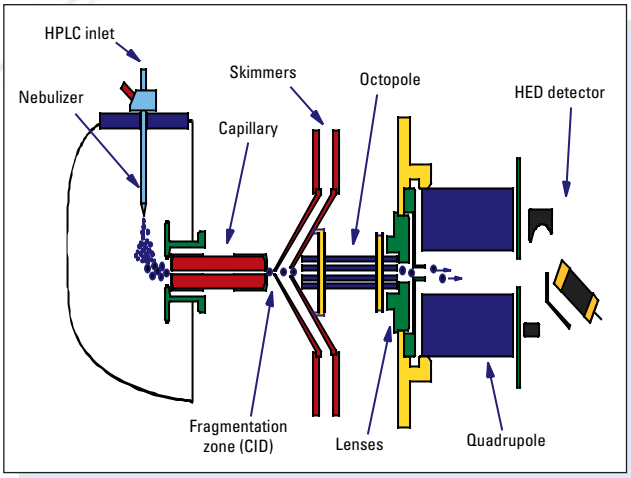


Figure 7. Orthogonal spray of API-electrospray source

Figure 8. Salt deposits on an HP 1100 Series LC/MSD System API interface

Applications

LC/MS is suitable for many applications, from pharmaceutical development to environmental analysis. The ability to detect a wide range of compounds has made API techniques popular with scientists in a variety of fields.

Molecular Weight Determination

One of the key applications of API-electrospray and APCI LC/MS systems is in determining molecular weights.

Differentiation of Similar Octapeptides

Figure 9 shows the spectra of two peptides whose mass-to-charge ratio differ by only 1 amu. The only difference in the sequence is at the C-terminus where one peptide has threonine and the other has threonine amide. The smaller fragements are identical in the two spectra, indicating that large portions of the two peptides are similar. The larger fragments contain the differentiating peptides.

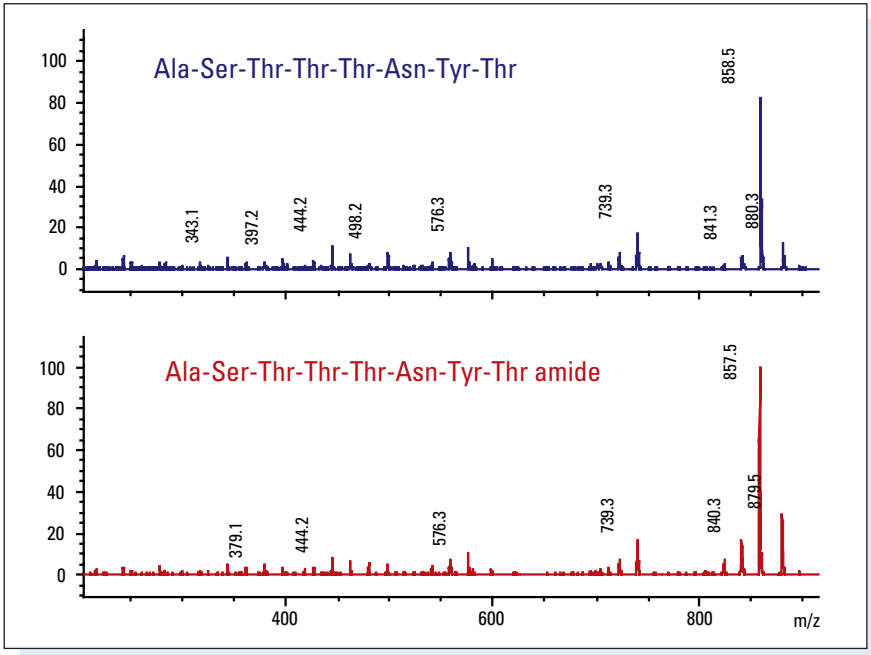


Figure 9. Octapeptides with 1 amu difference

Determining the Molecular Weight of Green Fluorescent Protein

API-electrospray LC/MS can be used to rapidly determine the molecular weight of a protein. Although pure proteins can be done by infusion, this recombinant protein required chromatography. Green flourescent protein (GFP) is a 27,000 Dalton polymer with 238 amino acids. It emits a green light when excited by UV.

Even though GFP is a very large molecule, a mass spectrometer with a smaller mass range can be used to determine its molecular weight. Since the mass spectrometer measures the mass-to-charge ratio, the molecular weight of a large molecule such as GFP can be determined if it is multiply charged.

The upper part of the display in Figure 10 shows the mass spectrum of the chromatographic peak. There is a regular pattern to the spectral peaks, each one of which represents the molecule with a different number of charges. The lower display is a deconvoluted spectrum generated by the data system for the singly charged molecule.

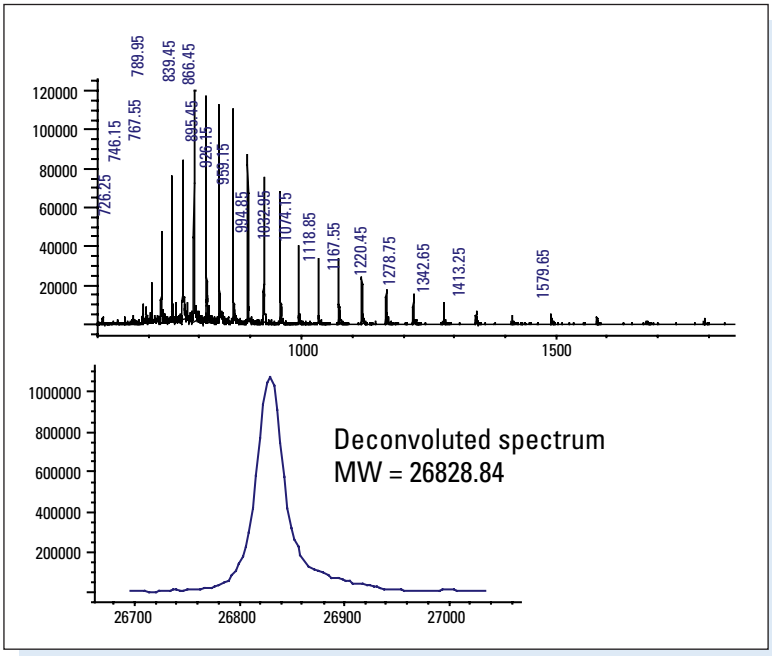


Figure 10. Molecular weight determination of Green Fluorescent Protein by API-electrospray

Combinatorial Chemistry

Combinatorial chemistry has transformed the drug discovery process. Rather than creating compounds through manual manipulations, robotics is applied to generate whole classes of compounds from sets of reagents. Reactions are typically done using 96-well capacity (or greater) plates. These plates are loaded into an autosampler that injects samples into the LC/MS. The instrument acquires the data and produces a report showing whether the compounds detected are of the expected molecular weight.

Recent advances in software allow many chemists to use the high sample capacity of automated LC/MS systems. Trays are loaded on the autosampler; the method is specified; the instrument analyzes each well in the plate; and the data system prints a report. Figure 11 shows a typical display of a sample tray. Green dots show the locations where the reaction product is of the expected molecular weight. Red dots show where the reaction product did not have the expected molecular weight. A more detailed display and report of each position is possible.

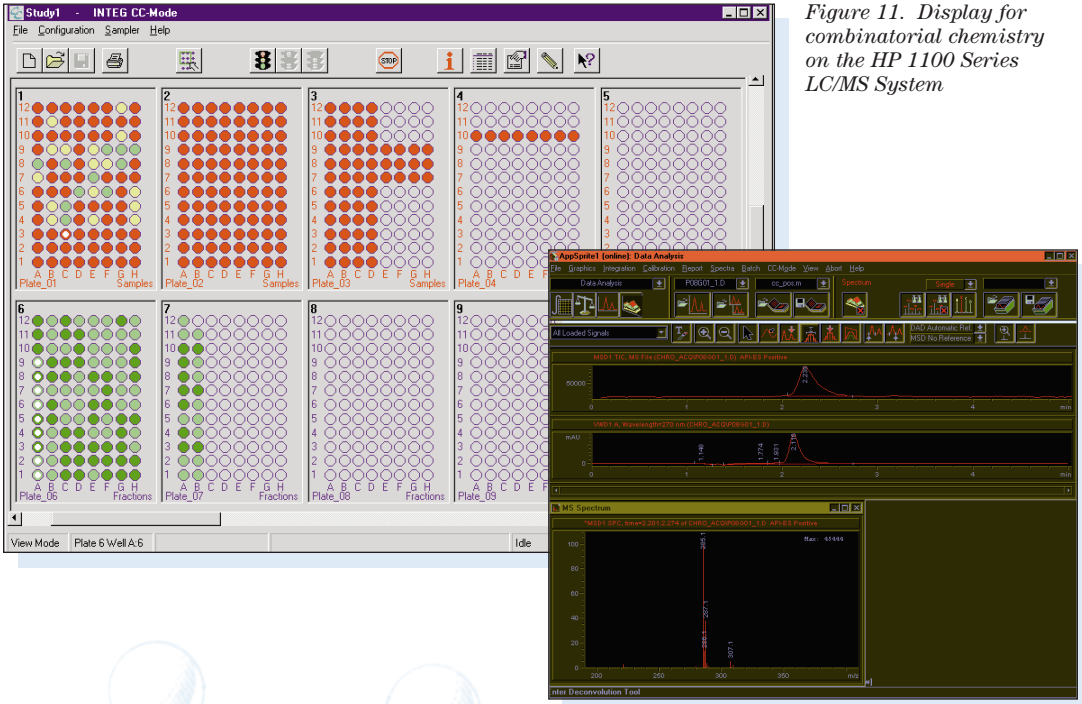


Figure 11. Display for combinatorial chemistry on the HP 1100 Series LC/MS System

Pharmaceutical Applications

Rapid Chromatography of Benzodiazepines

The information available in a mass spectrum allows some compounds to be separated even though they are chromatographically unresolved. In this example, a series of benzodiazepines was run using both UV and MS detectors. The UV trace could not be used for quantitation, but the extracted ion chromatograms for the MS could be used.

The mass spectral information provides additional confirmation of the identity. Chlorine has a characteristic pattern because of the relative abundance of the two most common isotopes. In Figure 12, the triazolam spectrum shows that the molecule has two chlorines, and the diazepam spectrum shows that it has only one.

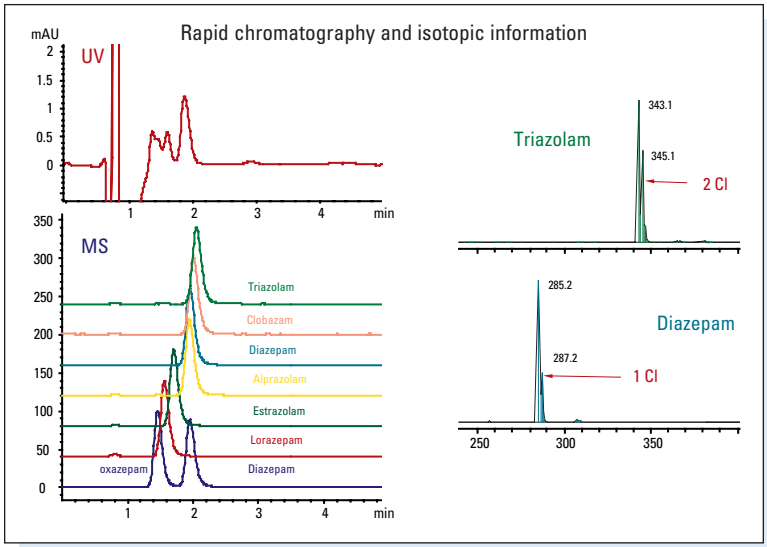


Figure 12. Benzodiazepines by API-ES

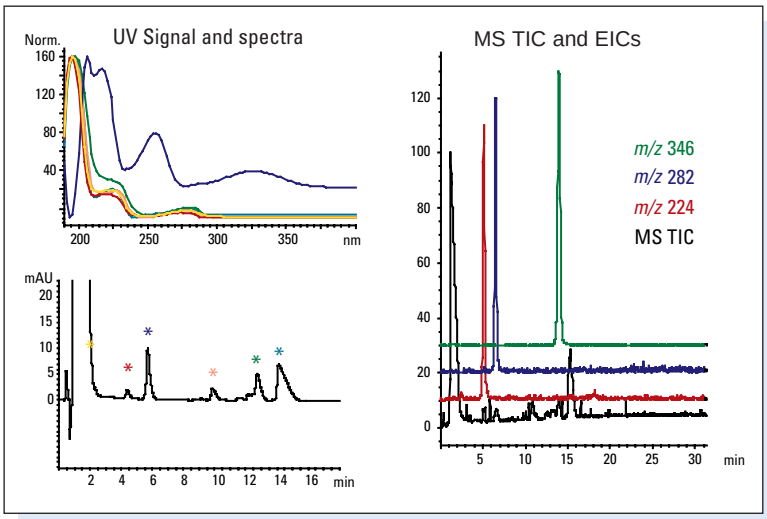


Figure 13. Salbutamol degradation products

Detection of Degradation Products for Salbutamol

Detecting degradation products can often be difficult because they can be structurally very similar to the original product. If the chromophoric region is intact, the two compounds cannot be distinguished with a UV detector.

The UV spectra for the salbutamol and its degradation products are very similar. The unique mass spectral fragments can be used to identify the compounds. Figure 13 shows the extracted ion chromatograms for various masses.

Biochemical Applications

Detection of Glycosylation in a Tryptic Map of a 60 kD Protein

Proteins are sometimes chemically or enzymatically digested to identify the components. The digestion mixture is then chromatographed to produce a peptide map. Figure 14 shows two chromatograms for a 60,000 Dalton glycoprotein from a mass spectrometer and a UV detector.

Although the chromatographic patterns with the two detectors are very similar, the mass spectrometer can provide additional data. Glycosylation is the covalent modification of certain amino acids which result in the attachment of complex carbohydrates. The sugars can be fragmented from the peptide fragments using in-source collision-induced dissociation (CID). The code at the right in Figure 15 is used to designate various markers. Since glactose and mannose have the same molecular weight, they cannot be distinguished from each other. Therefore, they are designated simply as Hex.

Figure 16 shows the extracted ion chromatogram (EIC) for three of the glycosylation markers. The fragments with the glycosylation markers can be easily identified.

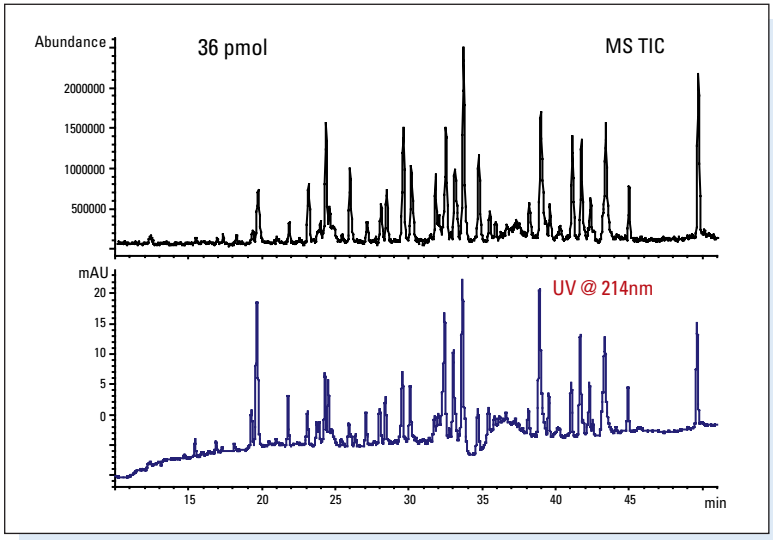


Figure 14. Tryptic map of a 60 kD protein

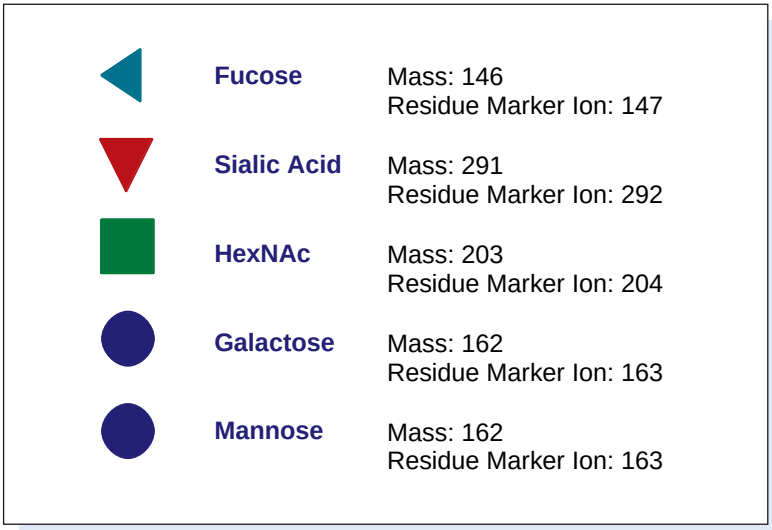


Figure 15. Typical glycosylation markers

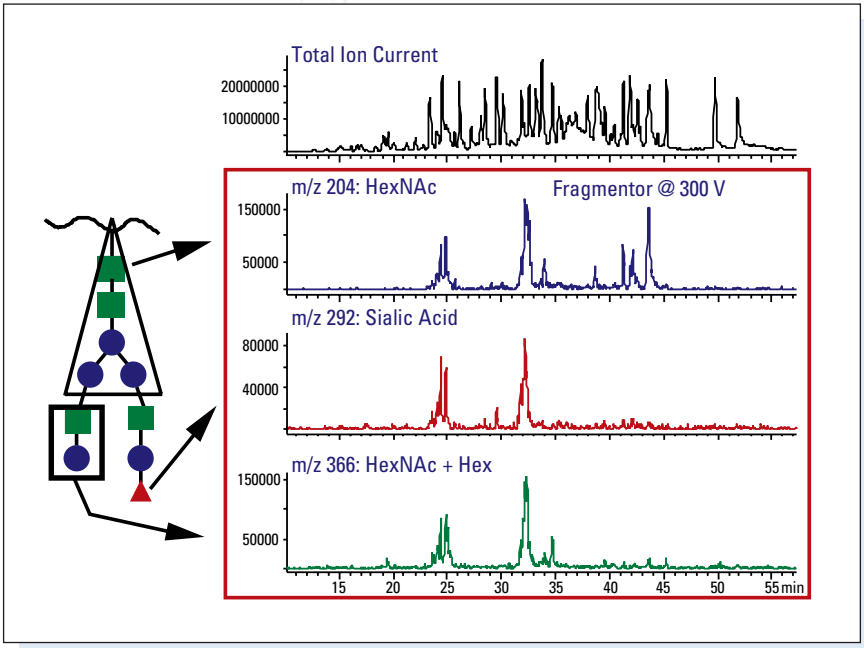


Figure 16. Detection of glycosylation

Clinical Applications

High Sensitivity Detection of Trimipramine and Thioridazine

For some compounds, MS provides more sensitive detection. Trimipramine is a tricyclic antidepressant with sedative properties. Thioridazine is a tranquilizer. Figure 17 shows these compounds in a urine extract at a level that could not be detected by UV. To get the maximum sensitivity, the analysis was done by selected ion monitoring.

Food Applications

Aflatoxins are toxic metabolites produced by certain fungi in foods. Figure 18 shows the total ion chromatogram for four aflatoxins; each could be uniquely identified by their mass spectra (Figure 19).

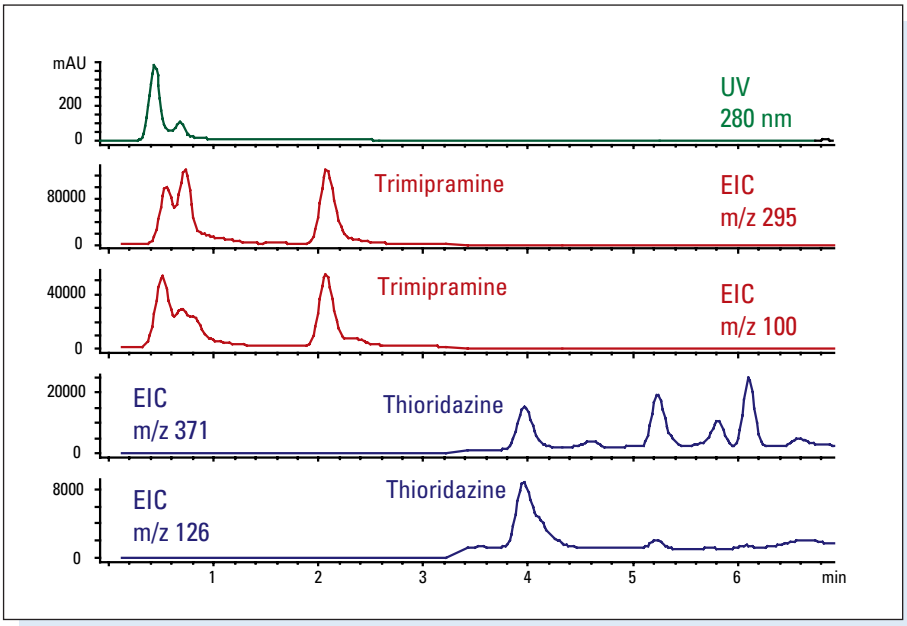


Figure 17. Trimipramine and thioridazine in urine extract

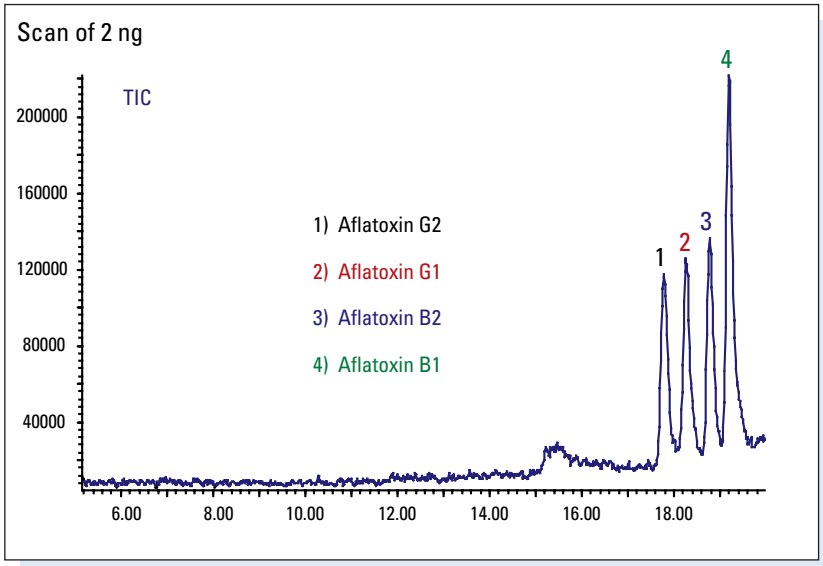


Figure 18. Afltoxins by API-ES

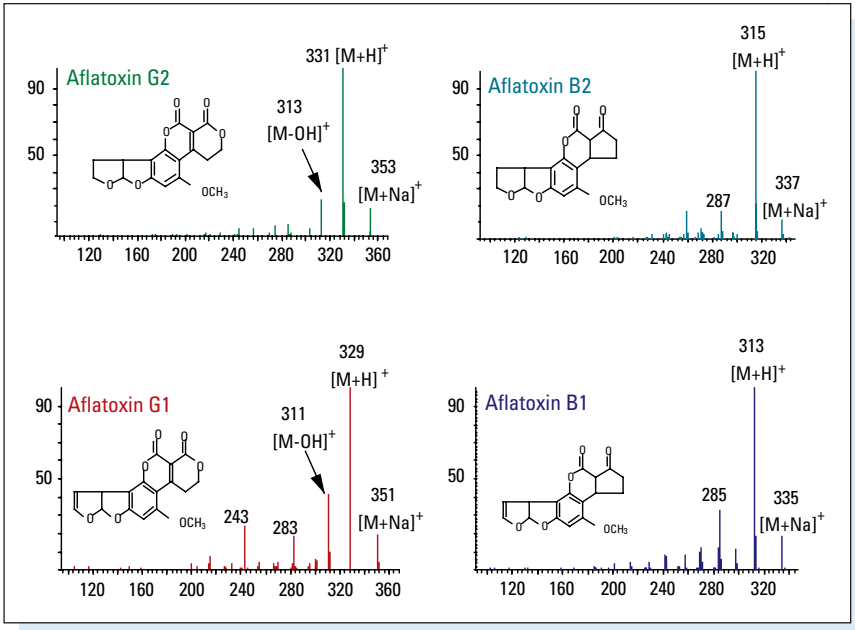


Figure 19. Mass spectra of aflatoxins

Environmental Applications

Carbamates by APCI-LC/MS

Carbamates are a class of pesticides usually analyzed by post-column derivatization and fluorescence detection. The chromatogram in Figure 20 is that of a tomato extract that has been spiked with 11 carbamates. Using APCI, these compounds are detected without derivitization. The extracted ion chromatogram displays the peaks for the individual compounds.

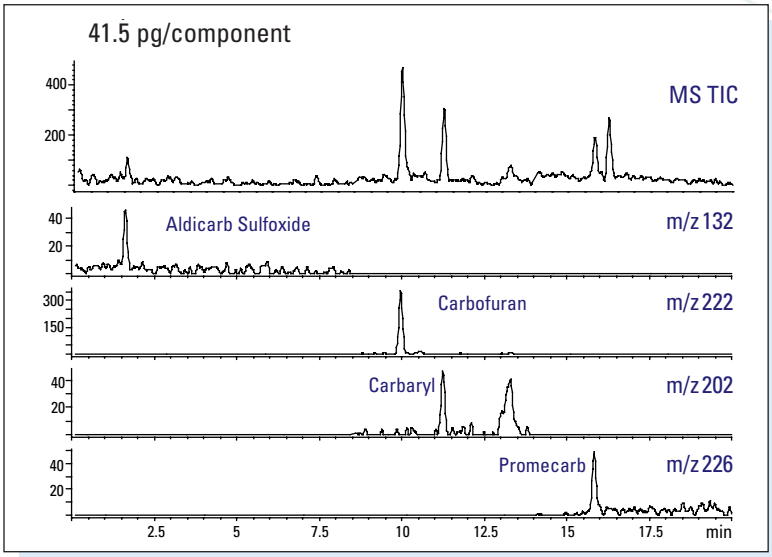


Figure 20. Carbamates by APCI

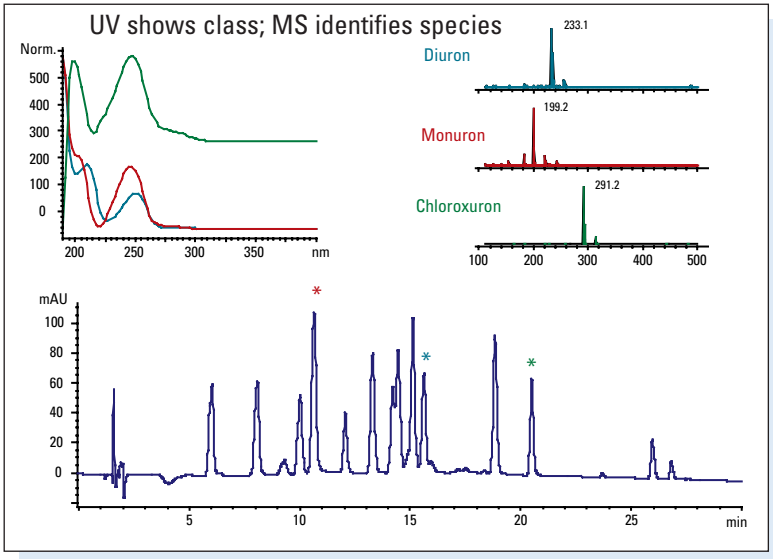
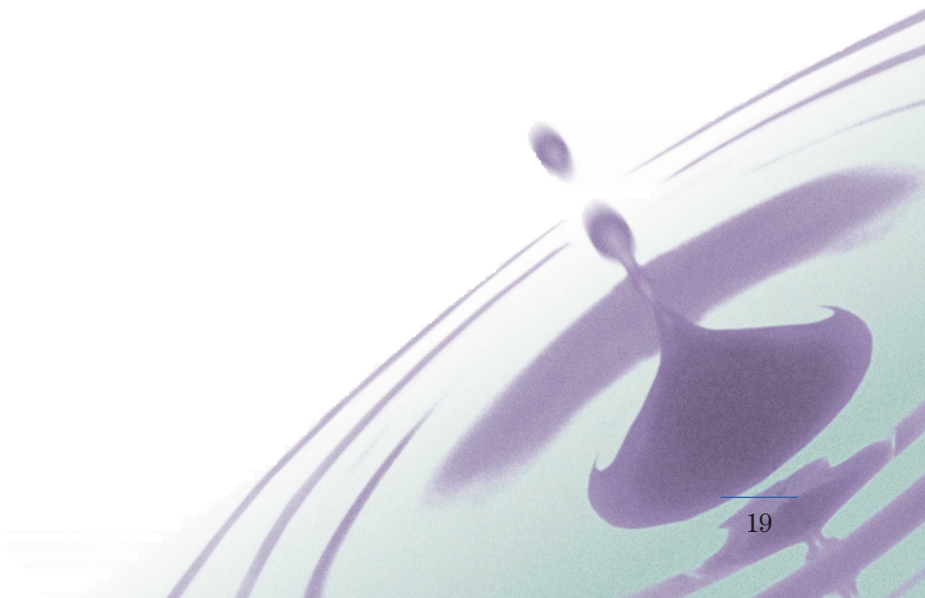


Figure 21. Phenylurea herbicides by API-ES

Detection of Phenylurea Herbicides

Many of the phenylurea herbicides are very similar and difficult to distinguish with a UV detector (Figure 21). Monuron and diuron have one ring and differ by a single chlorine. Chloroxuron has two chlorines and a second benzene ring attached to the first by an oxygen. The UV-Vis spectra are similar for diuron and monuron, but different for chloroxuron. Each of these compounds has a distinct mass spectrum. The standards were run with an API-electrospray LC/MS system.



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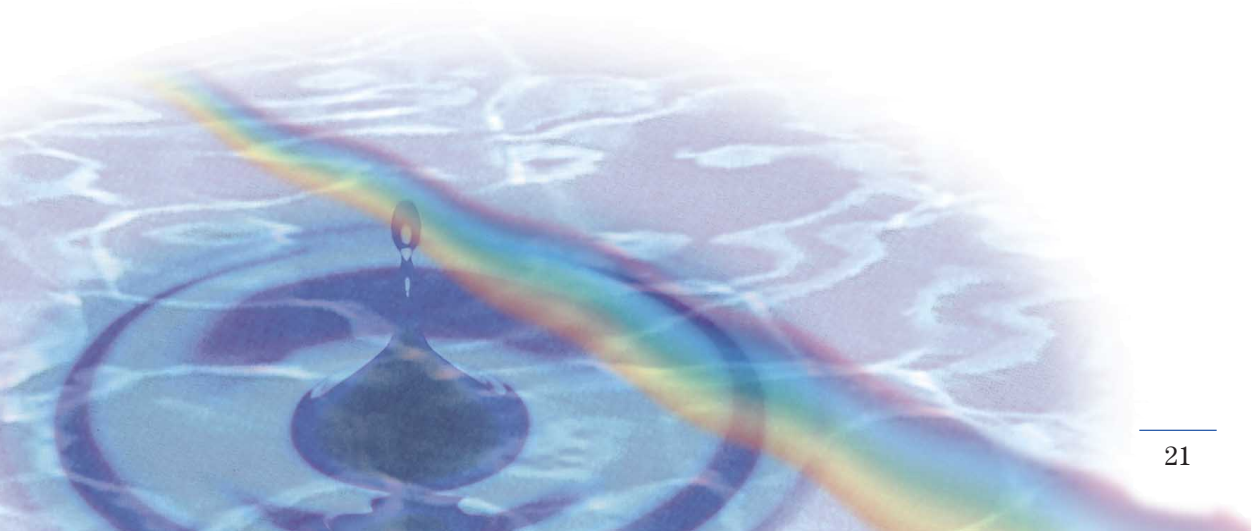

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