Making your LC Method Compatible with Mass Spectrometry

Technical Overview

Author

Edgar Naegele

Agilent Technologies, Inc.

Waldbronn, Germany

Introduction

The power of traditional high-performance liquid chromatography with UV detection (HPLC-UV, DAD) can be readily extended by simply coupling a mass spectrometer (MS) to an existing system. In the early years of liquid chromatographic mass spectrometry (LC-MS), this coupling was considered exotic and complex. After more than 15 years of refinement, LC-MS systems are robust and easy to use, and provide specificity unattainable by any other detection scheme. With the increased analytical capability, challenges may be tackled from several different and complementary directions. This Technical Overview provides an overview to the integration of MS into your existing HPLC system, showing the analytical strengths that MS brings and the ease with which it can be added.

Part 1 presents a basic introduction to the technique, applications that highlight its power, and some considerations on sample preparation methods. Part 2 describes the theory of electrospray ionization (ESI), which is the most common technique. Part 3 describes less common alternative techniques like atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) which are quite useful for various applications.

Agilent Technologies

Table of Contents

HPLC/UV/MS: Part 1. Introduction and Applications

HPLC-UV capabilities enhanced by the addition of MS as demonstrated by accelerated stability samples of both a pharmaceutical active and a formulation

Introduction

HPLC is the mainstay analytical technique of today's chemical, pharmaceutical, and bioanalytical industries. MS is a powerful tool for increasing quantitative capability, providing peak identification, and elucidating the structure of unknowns. The addition of MS to HPLC with UV detection to form an LC-UV-MS system (see Figure 1) adds a rich additional data source without compromising existing data collection. This allows the analyst to monitor masses relevant only to the target analytes, and the resulting increased specificity provides multiple advantages:

- Improves sensitivity, resolution, throughput, and productivity

- Integrates easily with HPLC-UV (DAD)

- Enables multiplexed experimental optimization (since the two detection methods are entirely different mechanisms, they combine to yield a single, powerful, orthogonal approach)

- Facilitates problem-solving, as will be described

The Basics of Atmospheric Pressure Ionization (API) Mass Spectrometry

Mass spectrometry detects ionized analytes from the LC eluent after UV detection. The sample is desolvated, ionized, analyzed by mass/charge ratio, and detected. Based on the mass-to-charge ratio (m/z), the mass spectrometer measures unique masses from individual analytes that are used to confirm known compounds and identify unknowns. Analyses are easily performed in both the positive ion mode and the negative ion mode. In positive ion mode, protonation occurs on a basic site of the molecule. In negative ion mode, deprotonation produces a negatively charged molecule.

MS detection for samples in liquid phase (HPLC) is typically accomplished by atmospheric pressure ionization. This methodology is compatible with a broad range of compounds, has femtogram-to-picogram sensitivity, and delivers both qualitative and quantitative information. There are three typical atmospheric pressure ionization methods, with the method deployed largely dependent on the polarity of the analytes: ESI, APCI, and APPI. Of these three methods, ESI is the most common. See following chapters for more detailed discussion on the theory of electrospray as well as additional atmospheric pressure ionization techniques.
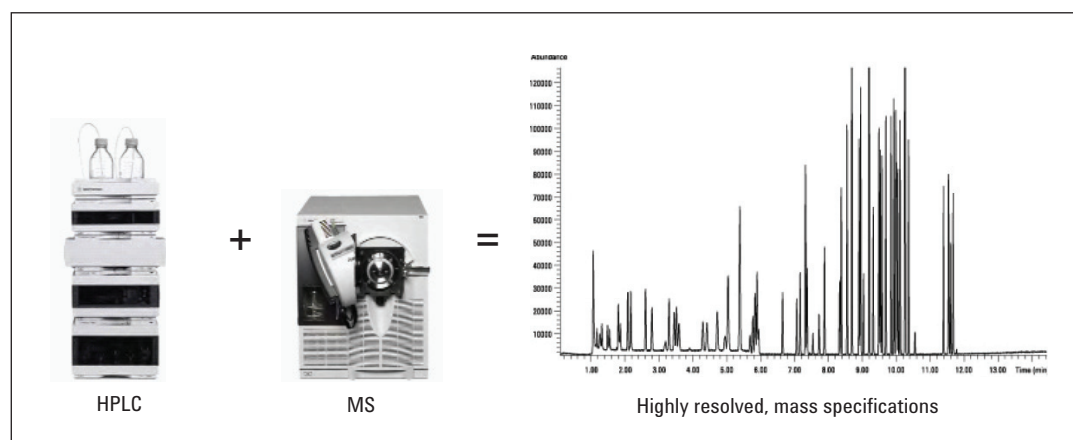

HPLC + MS = Highly resolved, mass specifications

Figure 1
Multiplexing of HPLC with MS yields a powerful tool.

Adapting Existing LC Methods to MS

LC-UV mobile phases typically contain non-volatile buffers which build up in the MS. However, adapting to a volatile buffer at the same pH is generally a simple matter. For example, if working at pH 3 with phosphate, using formate will also bring the pH of the mobile phase to 3 and interface well with the mass spectrometer.

Since MS detection requires the formation of ions, the mobile phase should be used to create charged analytes. This means that mobile phase pH and sample pKa information are critical. Selecting a mobile phase pH that will give positively charged or negatively charged analytes will increase sensitivity. Low pH will generally ionize basic compounds. Acidic compounds require more careful mobile phase pH selection because they are more likely to have pKa values in the pH 2-to-5 region. Neutral compounds can also be analyzed when they associate with ions such as acetate or ammonium that are in the buffer. Applying a voltage to the electrospray probe will induce ion formation, but good mobile-phase selection to provide preionized molecules dramatically increases ion formation, and therefore sensitivity.

Applications Using ESI

The analyses of accelerated stability samples of both a pharmaceutical active and a topical steroid in formulation were chosen to demonstrate the benefits of adding mass

spectrometry to HPLC-UV. Accelerated stability studies are routinely conducted to estimate product shelf-life in the pharmaceutical and biopharmaceutical industries. Impurities or degradates detected by HPLC-UV-MS are quickly and easily identified by molecular weight confirmation. Also, the data show that when an active is tested in formulation, the source of the impurity can be determined as coming from the active or the formulation. These results can then be used to make improvements in both products and processes.

Impurity Identification by LC-MS Coming from a Non-volatile Buffer LC Method and Identification of Unknown Impurities

In the example given in Figure 2, an HPLC-UV method containing phosphoric acid was developed for studying the stability of an active pharmaceutical ingredient. During the analysis of accelerated stability samples, new unknown peaks were observed. An HPLC-UV-MS compatible method with comparable performance to the phosphate method was needed to determine the molecular weights of the impurities.

Volatile formic acid replaced the phosphoric acid/triethylamine components used in the original LC method. The resulting LC-MS analysis showed a retention-time shift, which was acceptable for this particular study, but the chromatography was improved with less fronting, yielding excellent detection of the active pharmaceutical compound (APC).
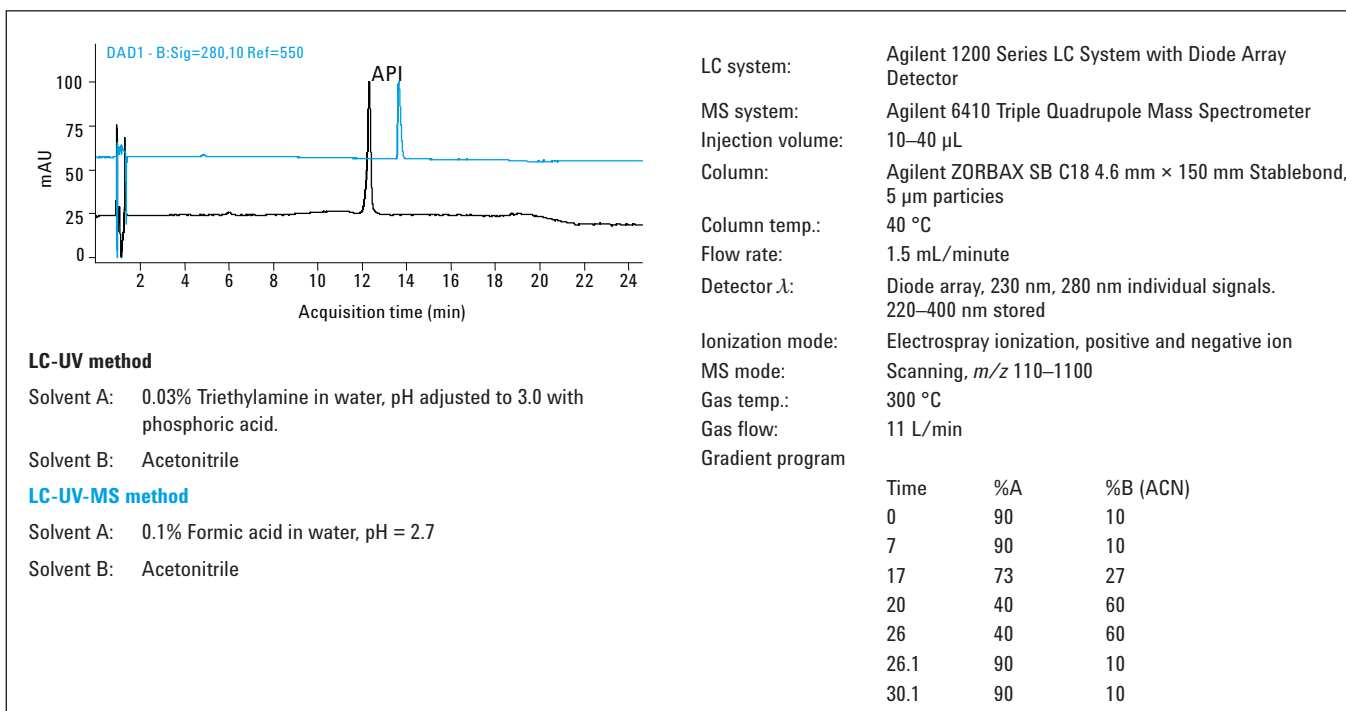

LC-UV method

Solvent A: 0.03% Triethylamine in water, pH adjusted to 3.0 with phosphoric acid.

Solvent B: Acetonitrile

LC-UV-MS method

Solvent A: 0.1% Formic acid in water, pH = 2.7

Solvent B: Acetonitrile

LC system:	Agilent 1200 Series LC System with Diode Array Detector
MS system:	Agilent 6410 Triple Quadrupole Mass Spectrometer
Injection volume:	10–40 µL
Column:	Agilent ZORBAX SB C18 4.6 mm × 150 mm Stablebond, 5 µm particies
Column temp.:	40 °C
Flow rate:	1.5 mL/minute
Detector λ:	Diode array, 230 nm, 280 nm individual signals. 220–400 nm stored
Ionization mode:	Electrospray ionization, positive and negative ion
MS mode:	Scanning, m/z 110–1100
Gas temp.:	300 °C
Gas flow:	11 L/min
Gradient program	

Time	%A	%B (ACN)
0	90	10
7	90	10
17	73	27
20	40	60
26	40	60
26.1	90	10
30.1	90	10

Figure 2
Method comparison for Active Pharmaceutical Ingredient analysis (API).

The LC-UV-MS analysis of an actual accelerated stability sample shown in Figure 3 indicates a match of the five impurities between the MS total ion chromatogram (TIC) and the UV trace with adequate resolution of known impurities.

After a degradation period of two months, four known impurities were identified in the degraded samples. More importantly, two unknown impurities were also present above the 0.1% threshold. These unknown impurities labeled by the observed molecular weights 358 and 497 are shown in Figure 4.

Note that impurity B is not seen in the TIC from the MS data. The absence of the peak in the TIC does not mean impurity B was not detected, although differences in intensities are to be expected when comparing the UV and MS data. The TIC scans the entire range of molecular weights selected by the user and within the instrument's range. Knowing that the impurity is present from the UV (DAD) data and knowing the identity, one can readily find the impurity in the mass spectrometry data by searching for impurity B's molecular weight. This is easily done by extracting the molecular weight in an extracted ion chromatogram (chromatogram of only specific molecular weight ions).

This demonstrates how easy it is to confirm impurities when mass spectrometry and UV data are complementary, as in Figure 4, and reference material is available, as in Figure 5. In Figure 5, the mass spectral data of impurity B match that of the reference standard in both the positive ion mode and the negative ion mode. The molecular weight, 165, is confirmed by the protonated molecular ion in the positive ion mode (M+H m/z 166) and the deprotonated ion in the negative ion mode (M-H m/z 164). Also, ion fragments from the molecular ion produce a spectral match between the sample and the reference standard (m/z 138 and m/z 136).
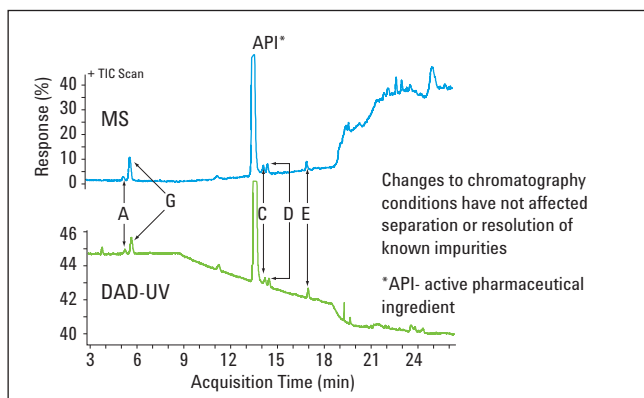

Figure 3
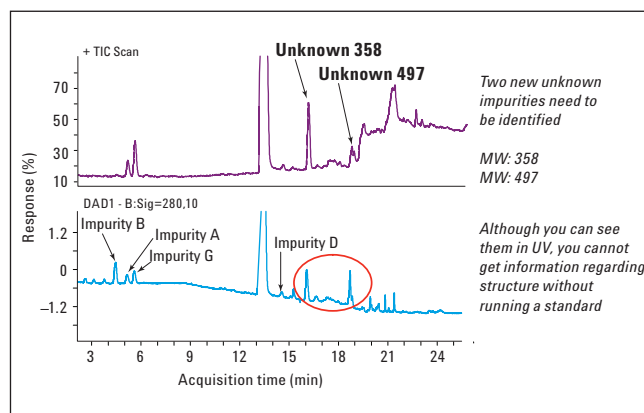**Comparing LC-UV and LC-MS data.**

Figure 4
Acquiring more information with mass spectrometry: Analyzing impurities in two-month stability samples.
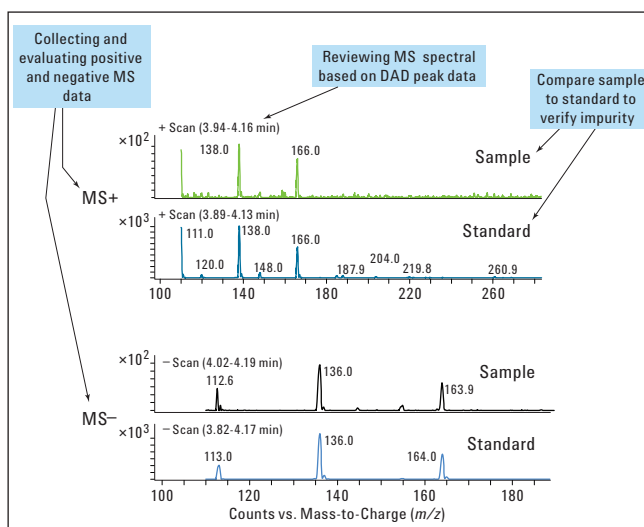

Figure 5
The power of tandem UV and MS: Matching of known impurities.

Another difference between the UV and MS scans is the presence of new and unknown impurities detected at 18.8 min. An expanded view of the TIC, the UV (DAD) scan, and two extracted ion chromatograms (EIC) at *m/z* 653 and 498 are compared in Figure 6. The TIC shows two co-eluting peaks while in the UV (DAD) chromatogram, we see one main, unidentified peak of interest. By extracting specific ions on EIC, compounds of molecular weights 653 and 498 are shown to elute at similar retention times. The mass peak at *m/z* 498 corresponds to the peak of interest in the UV trace.

The positive and negative mass spectra for the peak of interest at 18.8 min are shown in Figure 7. The molecular weight is confirmed to be 497 from both the positive and negative ion MS traces.

Identification of Impurities of a Topical Steroid Formulated in Oil at Low Levels

New challenges arise from accelerated stability samples of active ingredients in formulation. The source of new impurity peaks becomes ambiguous as it is not always clear if the impurities come from the formulation matrix or the API. For the following example, a MS compatible HPLC-UV method was developed for formulation analysis. Many new peaks were observed when analyzing the accelerated stability sample. Although some peaks were identified based on retention times, others remained unknown. The steroid, formulated at 0.1%, required identification of impurities at low ppm levels (~0.1% of the API). When MS detection is added to the LC-UV method, impurity identification becomes more complete and accurate.

A six-month accelerated stability sample of a topical steroid in oil at low levels showed significant impurities by LC-UV as seen in Figure 8. Three impurities had similar retention times to peaks in the placebo, but two were present in much greater amounts and had retention times similar to known API impurities. Initially, the peak at 21.1 min was assigned to the formulation matrix, the peaks at 27.6 and 39.3 were assigned to known API impurities, and the peak at 48.7 was unknown. These identifications were made by LC-UV, but were they correct?

Based on the UV response, it is very difficult to determine if the larger impurity peaks in the six-month sample are due to matrix or the API. When MS detection was added, unequivocal determination of the source of the impurities was possible.

Figure 6
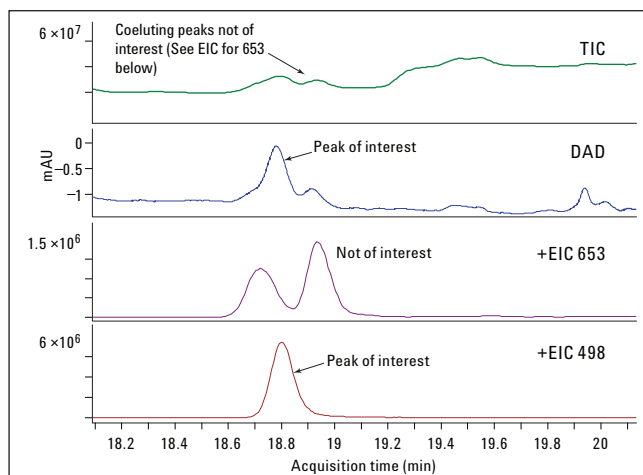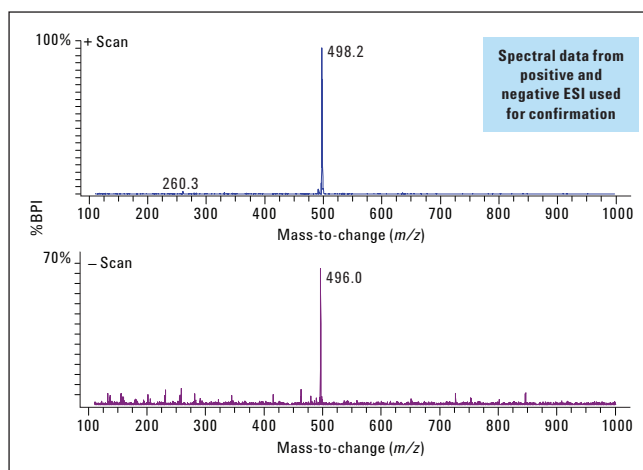**Finding the unknown impurities using UV and MS.**

Figure 7
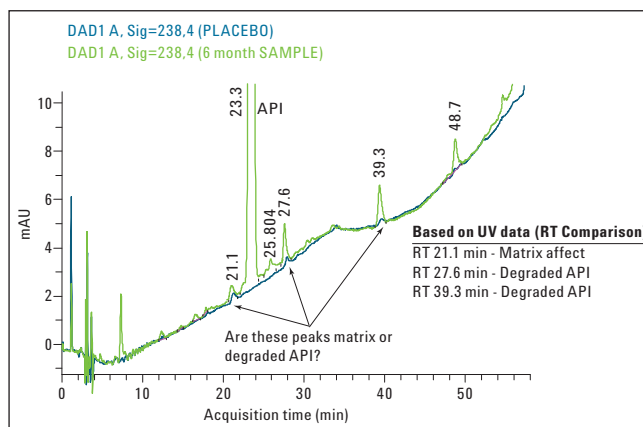**Mass spectral data identifies the peak of interest: Molecular weight of 497 confirmed.**

Figure 8
Comparing samples: Changes in chromatography with six-month sample.

The peak at 27.6 minutes was initially assigned as an API impurity by LC-UV. Figure 9 shows the mass spectra associated with the impurity found in the sample. The positive ion spectrum (top left) shows the protonated molecular ion at M+H m/z 427 for the sample impurity. The negative ion spectrum (bottom left) does not show the deprotonated molecular ion m/z 425, only the trifluoroacetic acid (TFA) adduct m/z 539. As such, the positive ion mode was chosen to determine the source of the impurity. The positive ion mass chromatograms (top and bottom right) show a strong molecular ion peak for the sample, but no peak in the formulation placebo. The MS results definitely show that the impurity is not a matrix product of the formulation; thus it is a degradation product of the API. With the inclusion of mass spectrometry, the impurity peak at 27.6 is confirmed to be due to API degradation.

A second impurity peak (at 39.3 minutes) was originally identified as another API degradation peak by LC UV. The reference standard for the tentatively identified impurity, impurity number 12, shows a protonated molecular ion at M+H m/z 495 (Figure 10, top left) in the positive ion mode and a M+TFA m/z 607 adduct ion in the negative mode. Mass spectra of the sample peak do not show either of these ions. Instead a m/z 227 ion was observed for the 39.3 minute peak (top center and top right) in the positive ion mode and a m/z 339 ion (bottom center and bottom right) in the negative mode. These
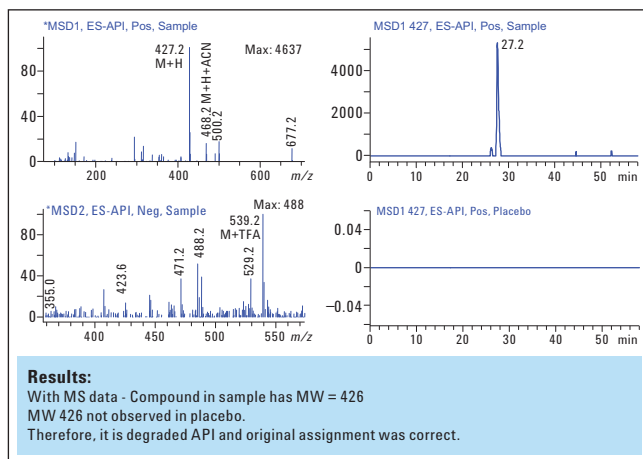
Figure 9
Identifying peak at 27.2 min with mass spectrometry.

data conclusively show that the impurity peak was incorrectly identified as the degraded impurity no. 12 by LC-UV and might be an increased amount of a matrix peak.

The peak at 39.3 minutes was incorrectly assigned based on LC-UV retention time alone. Based on MS, the impurity peaks at 21.1, 39.3, and 48.7 minutes were all due to matrix degradation.
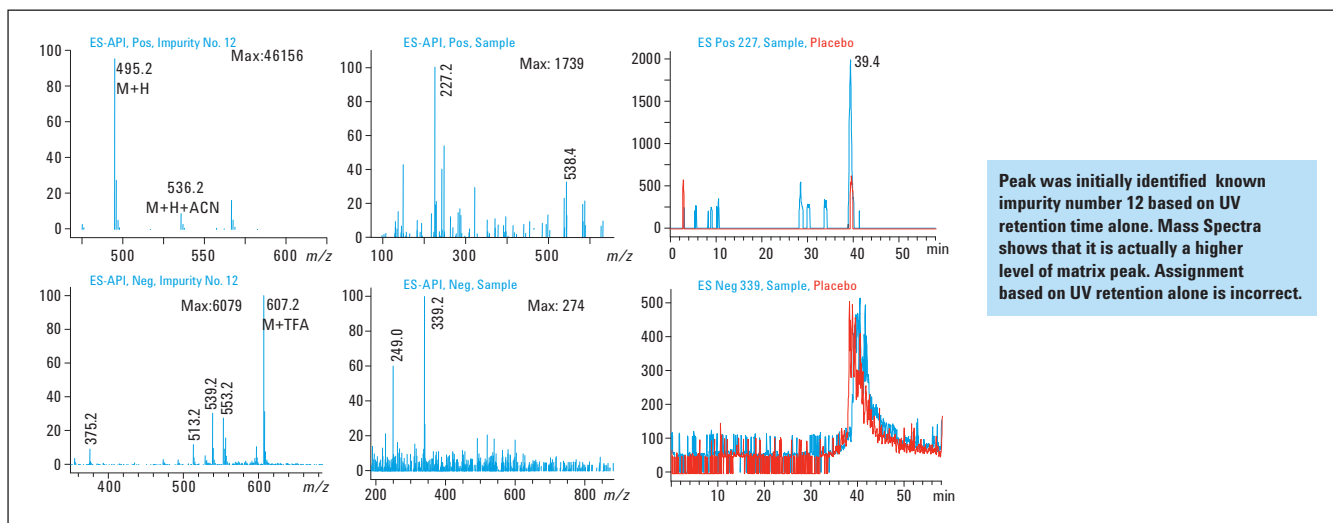
Figure 10
Identifying peak at 39.3 min with mass spectrometry.

7

Considerations for LC Performance with MS

As previously discussed, using the appropriate mobile phase buffers and pH is critical to robust and sensitive MS performance. Sample preparation is also important to the success of HPLC-UV-MS analysis. Inadequate sample preparation can result in ion signal suppression or interferences. The main items to consider prior to ESI-MS are:

- Matrix components: Salt suppresses the ionization process, detergents interfere with the evaporation process, and high analyte concentrations can saturate the detector.

- Eliminate matrix/salt/detergent effects. Some typical techniques are ultrafiltration, solvent extraction/desalting, liquid-liquid extraction, solid-phase extraction (SPE), immunoaffinity, on column concentration, and column switching (LC/LC).

- If salts and detergents cannot be avoided, remove them using chromatography (a short column is sufficient) or a cut-off filter.

- Concentration issues: Dilute the sample in the solvent composition that exists at the start of the LC method.

Conclusion

The addition of mass spectrometry to HPLC-UV provides capabilities to solve problems and allow for definitive identifications not possible with HPLC-UV analyses alone. In the examples given, the addition of MS identified the molecular weights of unknowns and differentiated the source of degradation as being from either the active pharmaceutical ingredient or the matrix of the formulation. All this was obtained while performing the conventional HPLC-UV analysis typically used for monitoring and quantitation.

Chromatographic performance was maintained when switching the solvent system to MS-compatible solvents. The addition of a single quadrupole mass spectrometer used in these examples significantly enhanced the problem-solving capabilities of HPLC. The addition of a mass spectrometer to HPLC-UV can also accelerate method development by facilitating peak tracking.

The ease of implementing MS along with the industry-proven robustness of the technique has made HPLC-UV-MS an indispensible tool for rapid and definitive analyte analysis and characterization.

HPLC/UV/MS: Part 2.
Theory of Electrospray Ionization Mass Spectrometry and its Coupling to HPLC-UV

Introduction

As described in Part 1, the analytical capability of high-performance liquid chromatography is greatly enhanced by the addition of MS. MS provides analyte specificity that is structurally based, providing unparalleled analyte identification and confirmation. The structure of unknown components can often be rapidly elucidated, providing answers to problems during routine analyses.

The most common approach to MS detection for liquid samples is atmospheric pressure ionization. For atmospheric pressure ionization, the sample is desolvated, ionized, analyzed by mass/charge ratio, and detected. The three typical atmospheric pressure ionization methods are ESI, APCI, and APPI. The appropriate method largely depends on the polarity of the analytes; however, ESI is the most common and will be described below. APCI and APPI will be described in Part 3.

Electrospray Ionization

ESI can be used for both high and low molecular weight compounds, making it suitable for a great diversity of samples. Almost anything that can form an ion in solution and can be separated in a volatile mobile phase can be analyzed by ESI. ESI has femtogram-to-picogram sensitivity, and can deliver both qualitative and quantitative information.

Several factors relating to ionization are obvious from the molecular structure of the analyte. Samples that contain heteroatoms typically accept a charge on N, S, O atoms (for example, carbamates, benzodiazepines, acids, or bases), and hence analyze well by ESI. Along the same line, compounds that can accept a charge by induction can also be analyzed by ESI. With electrospray ionization, strongly non-polar samples should be avoided as charge induction will be inefficient and not much signal will be produced. These compounds are better tackled by APCI or APPI (see Figure 11).

ESI uses an electric field to generate a fine spray of droplets. The charged droplets are attracted toward the MS inlet, passing through a counter-flow of heated nitrogen drying gas. This desolvation process shrinks the droplets and carries away uncharged material. The droplets continue to shrink until the repulsive electrostatic (coulombic) forces exceed the droplet cohesive forces leading to droplet explosions. This process is repeated until analyte ions are ultimately desorbed or ejected into the gas phase. By applying another model, the electrospray process, may be thought of as ionization followed by ion evaporation driven by strong electric fields on the surface of the microdroplets (see Figure 12).

API-ESI = Atmospheric pressure electrospray ionization
APCI = Atmospheric presure chemical ionization
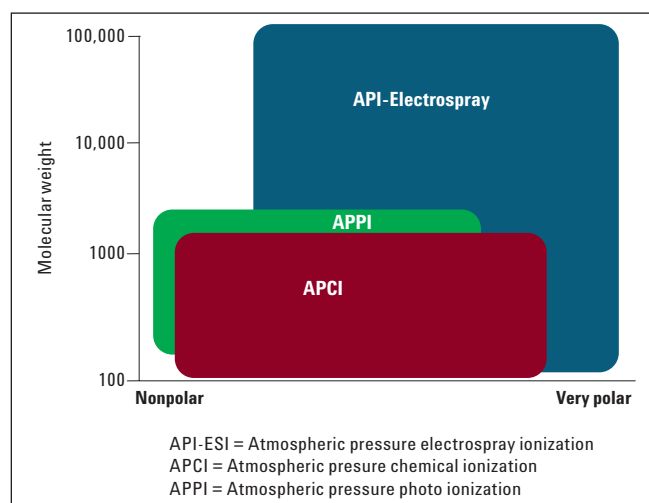APPI = Atmospheric pressure photo ionization

Figure 11
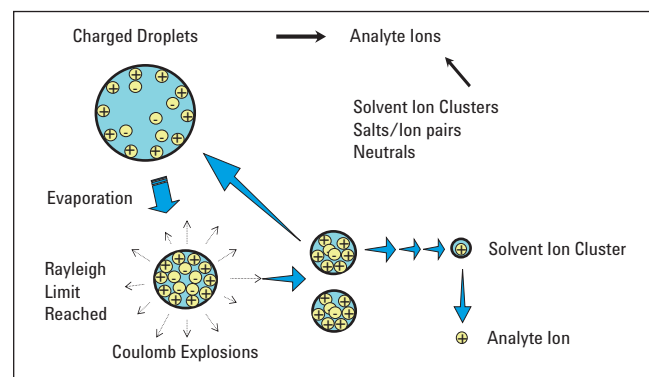**Applicability of LC/MS ionization techniques.**

Figure 12
Theory of electrospray ionization.

The formation of ions in ESI is highly dependent on the pH value of the mobile phase and the pKa value of the analyte, as electrospray requires preformed ions in solution. This follows the well-known principles of acid-base theory (shown in Figure 13) to produce either positive or negative ions which are detected in the positive or negative mode respectively.

Typical examples of data obtained as an ESI-MS spectrum of a small molecule and large molecule are shown in Figure 14. ESI-MS tends to be a soft ionization process leading to limited analyte breakdown or fragmentation (Figure 14a). In this figure, the amine, phenylbutazone, picked up a hydrogen atom (H) under ESI conditions and provided a very simple M+H ion as the base peak.

Most mass spectrometers have acceptable ranges for mass detection of compounds typical of the pharmaceutical and bio-pharmaceutical industries. With large molecules such as proteins and peptides, typically carrying multiple ionizable sides, multiply-charged ions are produced. Since the detector monitors the mass/charge, multiple charges allow these large molecules to be detected in a MS such as a single quadrupole LC/MS system with a mass range of (for example) 2000 Daltons even if their mass is in the range of 10,000 Daltons.

An example of the ESI spectra of the protein myoglobin is shown below in Figure 14b. The spectrum is spread out between 600 and 1200 amu, depicting the typical pattern of a mass spectral envelope of multiply-charged ions. With simple algebra, one can extract the molecular ion from the location and spacing of these multiply-charged peaks. The process of de-convoluting the molecular ion is generally performed by software and can also easily be performed manually. The bottom panel shows the de-convoluted spectrum, with a molecular ion at 16,959 Daltons.

Neutral molecules, which have a propensity for hydrogen bonding, can form adduct ions with ammonium or alkali metal ions (add a buffer of ammonium acetate or sodium acetate to facilitate ionization); typical examples are carbohydrates.

ESI Related to Chromatography Conditions

Mass spectrometry has certain operating parameters that need to be considered when coupling with HPLC. Lower LC flow rates are generally preferred since the MS operates under vacuum and can only handle a limited gas load generated from the LC eluent. MS works at elevated temperatures and requires volatile mobile phase buffers as the mass analyzer separates and detects gas phase analyte ions based on a mass to charge ratio (m/z).

As described above, ionization factors must be considered. The consistent and stable ion formation is the foundation for reproducible measurements and is driven by well-understood chemistry. The ion formation in the liquid to gas interface (the MS source) is the key to success; thus, different types of ion
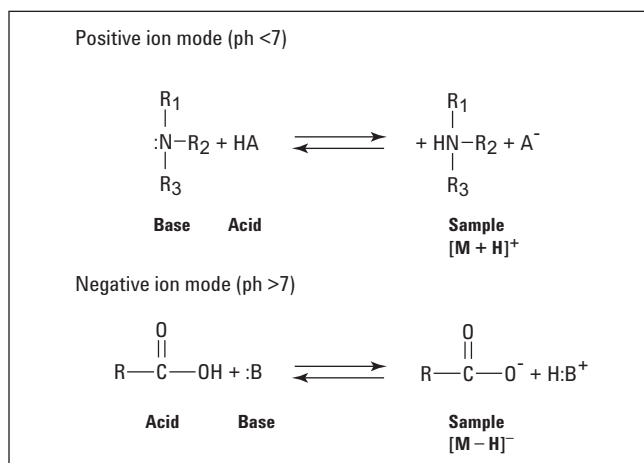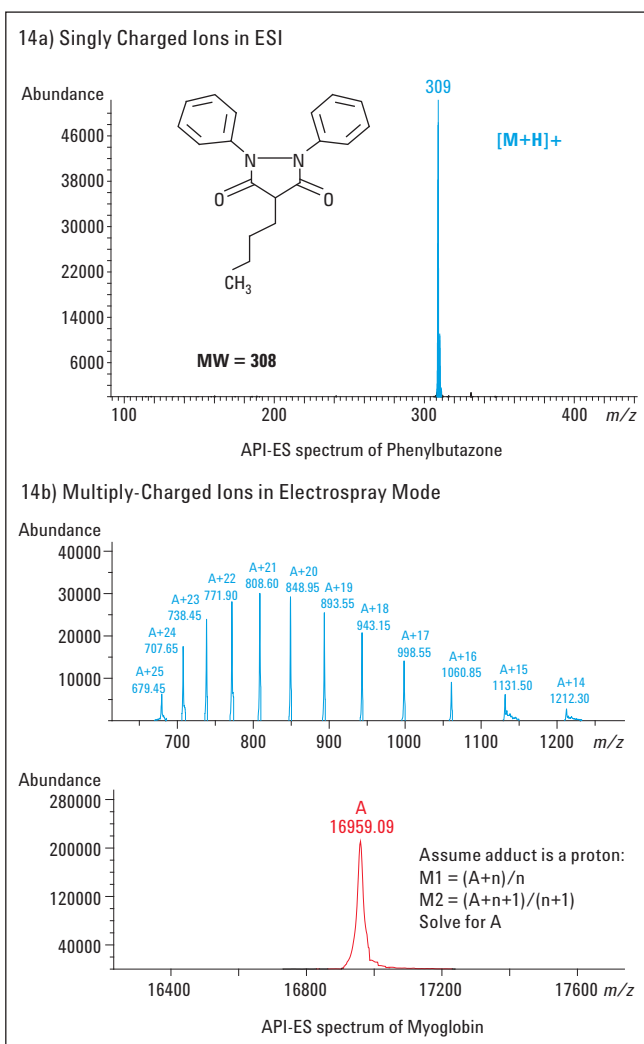

Figure 13
Solution chemistry.

Figure 14
ESI-MS of singly-charged and multiply-charged compounds.

sources are available for interfacing HPLC to a mass spectrometer. The rest of the MS instrument is robust, requiring little attention for routine analyses.

When choosing chromatographic conditions with an electrospray ion source, one should use the HPLC mobile phase to create charged analytes. This means that mobile phase pH and sample pKa information is critical. One can quickly select a mobile phase pH that will give positively charged or negatively charged analytes and thereby increase sensitivity. Low pH will generally ionize basic compounds. Acidic compounds require more careful mobile phase pH selection because they are more likely to have pKa values in the pH 2-to-5 region; finding a good buffer is therefore more difficult. Neutral compounds can also be analyzed when they associate with ions such as acetate or ammonium that are in the buffer. Applying a voltage to the electrospray probe will induce ion formation, but good mobile phase selection to provide ionized molecules dramatically increases ion formation and therefore sensitivity.

ESI-MS interfaces operate over a wide flow rate range, from 5 µL/min up to 2.0 mL/min, for ESI with thermal gradient focusing (Agilent Jet Stream). Most often they are operated at or below 0.5 mL/min, with optimum sensitivity achieved at lower flow rates. Standard 2.1 mm id HPLC columns are compatible with most HPLC instruments and are ideally operated around 0.25 mL/min. This mobile-phase flow rate leads to good sensitivity with ESI.

ESI is readily compatible with reversed-phase and normal phase solvents. Since heating is not required there is little chance for flammability problems with normal-phase solvents.

LC/ESI-MS has some limitations from a chromatographic perspective. ESI is particularly well suited for the analysis of small, polar analytes which might be difficult with the reversed phase retention (when selecting a mobile phase to provide ionized molecules). In addition, adduct ions are possible with some analytes (in addition to or in place of the protonated molecular ion, M+H). LC/ESI-MS also has poor compatibility with non-volatile modifiers and ion-pairing agents.

Mobile Phase Buffer Selection

Volatile buffers are used to modify mobile-phase pH in mass spectrometry not only because of deposit build-up from non-volatile buffers but also because metal ion buffers interfere with ionization and surfactants interfere with droplet evaporation. The volatile buffers may also be added to the mobile-phase eluent as a post-column addition. This technique preserves chromatographic separation while optimizing analyte ionization.

In general acidic solutions favor positive ion mode analysis (formic acid, 0.1–1.0%; acetic acid, 0.1–1.0%; or TFA [trifluoroacetic acid], 0.05–0.2%). Ammonium salts favor production of single ammonium adducts (ammonium acetate or ammonium formate). Basic solutions favor negative ion mode

analysis (triethylamine or ammonium hydroxide [pH 10–11]). Ion pairing reagents can ionize and create high MS background and strong ion pairing with an analyte can prevent ionization of the analyte. Also, some mobile phase additives will cause persistent background problems (TEA interferes in the positive ion mode [m/z 102] and TFA interferes in the negative ion mode [m/z 113]).

A summary of the volatile mobile phase choices that will work for LC/MS are shown in Figure 15. For positive ion analysis of basic analytes, the buffer choices will be acetate, propionic acid, formate, and TFA. These buffers provide the most reliable and consistent chromatography.
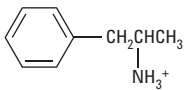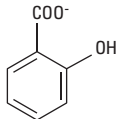

Positive ion detection of basic analytes	Negative ion detection of acidic analytes
Buffer choices (10 mM or less)	Buffer choices (10 mM or less)
• Acetate pKa 4.8	• Ammonia pKa 9.2
• Propionic acid pKa 4.9	• Diethylamine pKa 10.5
• Formate pKa 3.8	• Triethylamine pKa 10.7
• TFA highly acidic	• Piperidine pKa 11.1
Typical analytes — amines, amides, antibiotics	Typical analytes — acids, hydroxyls, phosphates, sulfates

Figure 15
Mobile phase polarity and buffer selection for ESI.

Compounds that protonate to form a positive charge tend to be basic; for example, amphetamine, where the amino functional group can be ionized. Acidic compounds deprotonate and are negatively charged, for example salicylic acid where the carboxylic acid group can be ionized. Analysis can be performed by positive and negative ion ESI and one wants to choose buffers that create these charged analytes based on the sample pKa.

For optimum MS performance, buffer concentration should be less than 10 mM for better droplet evaporation. For negative ion detection of acidic analytes at high pH, some good buffer choices are ammonia, diethylamine, TEA and piperidine. Compromises are possible to work closer to pH 7 where compound classes like carboxylic acids will have a negative charge, but the mobile phase will not compromise column life. In general, to optimize the mobile phase:

• adjust the pH to be 1 to 2 units away from the pKa of the analytes,

• avoid using salts and detergents, and

• use solvents that enhance ion desorption (for example, solvents with low surface tension and low heat of vaporization).

Considerations for LC Performance with MS

Evaluating Robustness

It is important to distinguish the mass spectrometer source from the analyzer. The source is where the liquid-to-gas phase transition and analyte ionization occurs. The operator has significant control over reactions taking place in this region. A correct setup (mobile phases, flow rates, source parameters) leads to robustness, while inferior setup can lead to problems. The analyzer region is considered everything behind the first vacuum region; this region is very robust and can be treated as a black box for normal applications.

The chromatography must resolve interfering compounds and the analyte must ionize in the source. These two criteria are essential; all other criteria are secondary.

When adapting a method from HPLC-UV to HPLC-UV-MS, one should consider fitness for purpose. In general there are no hard-and-fast rules about buffers and modifiers, but simply guidelines. Choices outside those guidelines will not damage the instrument, although frequent source cleaning may be necessary. If the sample set is small, method development time is limited. The use of an established method with a semi-volatile or non-volatile buffer might be the most pragmatic choice. A simple cleaning of the source when the analysis is complete will prevent problems with subsequent analyses.

In short, the standard guidelines given above can be modified, but one must be mindful of the price one has to pay (loss of sensitivity, more cleaning).

Adapting Existing LC Methods to LC/ESI-MS

If an existing method does need to be adapted to LC/MS, some slight changes in the separation may occur, but that can be optimized. First, replace non-volatile buffers with volatile buffers at a concentration of < 10 mM for electrospray. For example, replace phosphate buffers with acetate or formate buffers. TFA may be used as well. If the chromatography changes too dramatically and a non-volatile buffer must be used, then select a buffer where only the anionic or cationic part is non-volatile—for example, ammonium phosphate instead of potassium phosphate. The column id and flow rate should be kept low to increase sensitivity as much as possible and minimize the build up of non-volatiles. Maintain the pH as in the original separation if possible. If working at pH 3 with phosphate, replacement with formate is recommended first since formate will also buffer at pH 3. If any type of ion-pair reagent must be used to increase retention, then the use of a volatile ion pair reagent like heptaflurobutyric acid (HFBA) and tributylamine (TBA) is recommended. In general it is possible to adapt an existing method using the same basic principles and paying attention to the original pH of the separation.

Electrospray Source Settings

To achieve optimum sensitivity, the ESI source requires specific temperatures, flow rates, and voltages for good droplet desolvation and ion evaporation. Settings for a typical electrospray ionization source are shown in Figure 16.

Nebulizer Pressure
 • Higher liquid flow requires higher nebulizer pressure for efficient spray
 • 100 to 1000 µL/min = 15 to 60 psi

Vcap
 • Just need enough to perform the electrospray. (set and forget)

Drying Gas Flow
 • High water needs higher flow
 • High gas flow keeps the transfer capillary clean when analyzing dirty samples or using semi-volatile modifiers.
 • When in doubt, use excess (set high and forget unless sensitivity is an issue)

Drying Gas Temperature
 • Higher for low vapor pressure solvents
 • Start with 300-350 °C

Fragmentor
 • Low value breaks up solvent clusters but transmits molecular ions (typical)
 • Normally set and forget

Figure 16
Typical electrospray source settings.

Conclusion

The placement of a MS detector in tandem after an LC-UV (DAD) provides increased performance and capability. Such enhancements include increased specificity, resolution, sensitivity, productivity, and problem solving capabilities. MS offers, in addition to UV detection, a sensitive detection of both polar and non-polar compounds. A few considerations such as flow rate, mobile phase buffer selection, and MS source parameters are important for optimum HPLC-UV-MS performance. Chromatographic conditions have a significant impact on mass spectrometric analysis and can be optimized and adjusted easily. Whether analyzing small molecules or large peptides and proteins, even a single quadrupole mass analyzer has sufficient mass-resolving power for excellent, highly sensitive quantitative and qualitative mass spectral analysis.

HPLC/UV/MS: Part 3.
APCI and APPI as Alternative Ionization Techniques for MS

Introduction

As described in Parts 1 and 2, the coupling of MS to HPLC-UV provides a robust analytical technique that provides a dimension of specificity with problem solving capabilities not possible with HPLC-UV alone. In Part 2, ESI was presented as the most common approach to atmospheric pressure ionization. In this section, two other approaches, APCI (Atmospheric Pressure Chemical Ionization) and APPI (Atmospheric pressure photoionization), are presented.

Atmospheric pressure ionization is the most common sample introduction for MS detectors in liquid phase separations (HPLC). For API, the sample is desolvated, ionized, analyzed by mass/charge ratio, and detected. The three typical API methods are ESI, APCI, and APPI. The appropriate method largely depends on the polarity of the analytes.

After the eluent passes through the UV detector, the mass spectrometer detects ionized analytes. Based on the mass to charge ratio (m/z), the mass spectrometer measures unique masses from individual analytes that are used to confirm known compounds as well as identify unknowns. Analyses are easily performed in both the positive ion mode and negative ion mode. In the positive ion mode, protonation or the addition of hydrogen occurs on a basic site of the molecule. In negative ion, deprotonation produces a negatively charged molecule.

As discussed in Parts 1 and 2 of this technical overview, ESI is the most common method used for LC/MS. ESI can be used for both high and low molecular weight compounds, making it suitable for a large number of diverse samples—almost anything that can form an ion in solution and can be separated in a volatile mobile phase.

Not all samples or chromatography techniques are best analyzed by ESI. While electrospray may be thought of as ionization followed by ion evaporation, not all analytes can be ionized in solution or use chromatography conditions that are suitable for ESI. In these cases, the alternative ionization methods of APCI or APPI may provide a solution (Figure 11).

Atmospheric Pressure Chemical Ionization (APCI)

APCI is distinguished from ESI as it uses a corona discharge to ionize molecules. Unlike ESI, no preformed charged analytes are needed because of a charge transfer process that occurs in APCI. APCI generates ions by first nebulizing the liquid analyte into small droplets. This is followed by evaporating the droplets to produce gas phase solvent and analyte molecules, the solvent molecules being ionized by the corona discharge. A corona discharge needle serves as a charge source. The gas phase analyte is then ionized by gas phase chemical ionization (CI) via proton addition, proton abstraction, or by electron capture processes. CI is a process where the solvent acts as a CI reagent gas to ionize the sample. The ionization process is protonation (for example, H_3O^+) for bases and a charge exchange deprotonation for acidic compounds. APCI is also an electron capture mechanism for halogens and aromatics. The evaporation and ionization processes are shown in more detail in Figure 17.

$$[Solv+H] + A \rightarrow Solv + [A+H]$$

Analyte containing aerosol

Evaporation

Vapor

Corona needle

Charged reagent gas formed

Charge transferred to analyte

Analyte ions
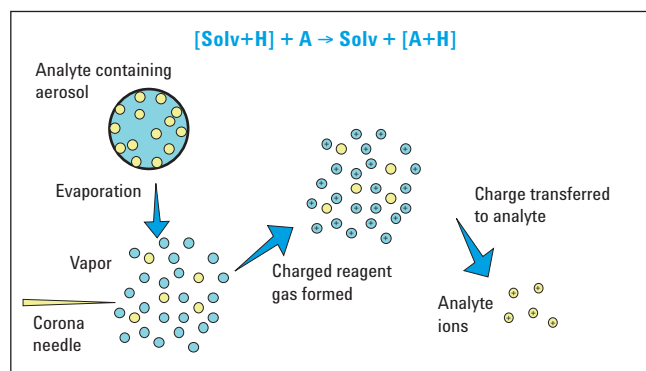
Figure 17
Theory of atmospheric pressure chemical ionization (APCI).

Just as in the API-electrospray design, the APCI inlet is positioned orthogonally to the inlet of the capillary, uses the same nebulizer design, and takes advantage of the drying gas heater design. All of this results in low noise, high signal, and maximum system uptime at high HPLC flow rates. A high probe temperature is typically used to desolvate and vaporize the

sample, although temperatures that are too high can lead to sample decomposition. The externally removable corona discharge needle can be easily replaced without venting the vacuum system or opening the spray chamber (Figure 18).

APCI is a good technique for small molecules with molecular weights of less than 1500 Daltons. These small molecules can be polar or somewhat non-polar (substituted PAHs and PCBs, fatty acids, phthalates). APCI is not a good technique for biomolecules (peptides and proteins) because this form of ionization rarely results in multiply-charged species. Therefore, for large molecules that ionize, the mass-to-charge ratio would remain high and would generally not be in the range of the MS instrument. In general, APCI should be thought of as a complementary technique to ESI, because in APCI, samples targeted are generally those not charged in solution. Samples that charge in solution will typically be detected with greater sensitivity using ESI. APCI is also not the method of choice for thermally unstable or photosensitive samples. These tend to fragment completely, thus not producing the parent ion which is desirable in LC/MS for molecular weight and compound identification. These types of molecules are better attempted by atmospheric pressure photoionization (APPI) discussed later in this technical overview.

A general comparison of ESI and APCI is presented in Table 1. For sensitivity, if a sample can be ionized by both techniques, electrospray is generally more sensitive and has less background noise. However, ESI is more adversely affected by sample and solvent matrix than APCI (for example, signal suppression). Electrospray also requires a lower concentration of volatile buffers relative to APCI. The choice of organic solvent strongly affects ionization in APCI while ionization in ESI is largely dependent on the choice of mobile phase buffers. Another significant difference is that ESI works best at low flow rates (< 500 µL/min) while APCI has a broad flow rate range up to 1.5 mL/min. APCI is more sensitive and has less noise than ESI at high flow rates (> 750 µL/min). ESI, like a UV detector, is a concentration sensitive isolation technique while APCI is mass sensitive. For APCI, sample dilution is not a factor for sensitivity.

There are advantages of APCI over ESI. APCI is less sensitive to solution chemistry effects than ESI. APCI tolerates higher flow rates than ESI and accommodates some solvents that are not compatible with ESI. Most importantly, APCI may ionize neutral or more non-polar compounds that cannot be ionized by ESI.
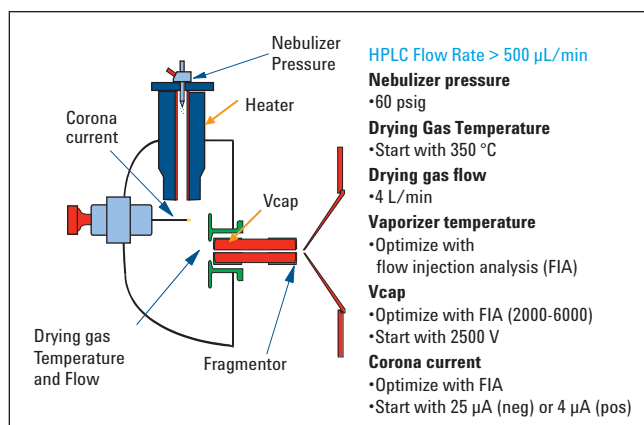
HPLC Flow Rate > 500 µL/min
Nebulizer pressure
•60 psig
Drying Gas Temperature
•Start with 350 °C
Drying gas flow
•4 L/min
Vaporizer temperature
•Optimize with
 flow injection analysis (FIA)
Vcap
•Optimize with FIA (2000-6000)
•Start with 2500 V
Corona current
•Optimize with FIA
•Start with 25 µA (neg) or 4 µA (pos)

Figure 18
APCI spray chamber settings.

ESI	APCI
Ionization: Pre-formed analyte ions transferred to gas phase	Ionization: Charge exchange of gas phase neutral analysis
Mobile phase issues:	Mobile phase issues:
• Organic solvent: little effect on ionization	• Organic solvent: MeOH usually best
• pH: key to pre-formed ions	• neutral analytes
• Buffer concentration: < 25 mM	• Buffer concentration: < 100 mM
• Flow rate: < 0.5 mL/min	• Flow rate: > 0.5 mL/min

Table 1
General comparison—ESI vs APCI.

APCI typically generates just singly charged ions; however, it is possible to get doubly charged ions where the charge sites are separated from each other (usually by a hydrophobic region). For positive-mode APCI, the solvent must be capable of donating a proton and the analyte must have higher proton affinity than the reagent gas. For example, acetonitrile/water is a commonly used mobile phase for LC/ESI-MS, but acetonitrile is not a protic solvent (that is, does not have a proton to donate) and water in the gas phase is a very strong base. Therefore, acetonitrile/water is not a good solvent choice for APCI positive mode. In negative-mode APCI, the reagent gas must be able to abstract a proton or capture an electron. Where ESI is thought of as ionization followed by evaporation, APCI can be thought of as evaporation followed by ionization.

APCI Related to Chromatography Conditions

Typical rigor used in LC applies to LC/APCI-MS. Only highly purified water and organic solvents should be used. Volatile solvents are best for APCI, as the eluent needs to be in the gas phase for ionization to occur. Where ESI requires low buffer concentrations, APCI works in a wide range of buffer concentrations. This is because the APCI process for ionization is based on charge transfer and not the evaporation from the fine particles to give charged analytes. Figure 19 and Figure 20 show the effects of volatile buffer concentrations on the ionization of caffeine and reserpine. For ESI as shown in Figure 19, the buffer ions compete with the analyte about the droplet surface and the analyte has increased difficulty in escaping the droplet as the buffer concentrations increases. Smaller molecules such as caffeine are desorbed earlier in the ionization process. For APCI as shown in Figure 20, at low concentration the ammonium acetate in the buffer aids proton transfer for
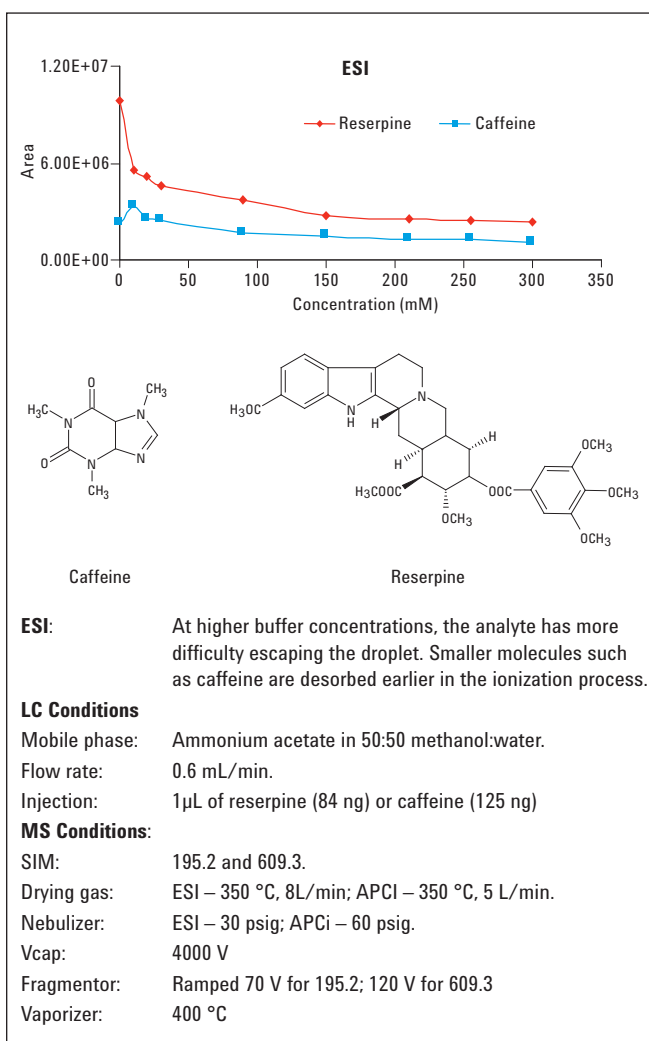
reserpine. However, as the "volatile salt" concentration is increased, it becomes more difficult for the analyte to effectively volatilize. Caffeine, which is a weaker base than reserpine, does not show the same signal increase at low buffer concentrations since ammonia competes with caffeine for protonation.

Protic solvents like methanol should be used for the positive ion mode. These solvents have a proton to transfer to the M+H ion. Therefore, methanol is often the better organic solvent choice over acetonitrile. For the negative ion mode, choosing a solvent that can readily capture an electron will make the ionization process more efficient. In general if an ammonium salt is used in the mobile phase, ammonium adducts are likely to form. This is detrimental if monitoring [M+1] but may be beneficial if monitoring for [M+18] as sensitivity can be increased with an ammonium adduct.

APCI can accommodate a very wide flow rate range, up to about 1.5 mL/min, so column dimensions are not as restricted as with some other techniques. Columns of 3.0 mm id are very popular for APCI. These operate around 0.5 mL/min, which is also a flow rate that can be used by ESI with very good sensitivity. However, LC/APCI-MS is also well suited for 4.6 mm id columns which are popular in HPLC/UV methods. So adapting an analytical method from HPLC/UV to HPLC/UV/APCI-MS does not require changing column dimensions or adjusting flow rates. Like LC/ESI-MS, reversed phase chromatography typically precedes APCI along with the use a buffered mobile

ESI: At higher buffer concentrations, the analyte has more difficulty escaping the droplet. Smaller molecules such as caffeine are desorbed earlier in the ionization process.

LC Conditions

Mobile phase:	Ammonium acetate in 50:50 methanol:water.
Flow rate:	0.6 mL/min.
Injection:	1µL of reserpine (84 ng) or caffeine (125 ng)

MS Conditions:

SIM:	195.2 and 609.3.
Drying gas:	ESI – 350 °C, 8L/min; APCI – 350 °C, 5 L/min.
Nebulizer:	ESI – 30 psig; APCi – 60 psig.
Vcap:	4000 V
Fragmentor:	Ramped 70 V for 195.2; 120 V for 609.3
Vaporizer:	400 °C

Figure 19
Effect of volatile buffer concentration on ESI response.

APCI: Ammonium acetate initially aids proton transfer for reserpine. Increasing "volatile salt" concentration makes it more difficult to effectively volatilize the analyte. Caffeine is a weaker base than reserpine and ammonia competes for protonation.
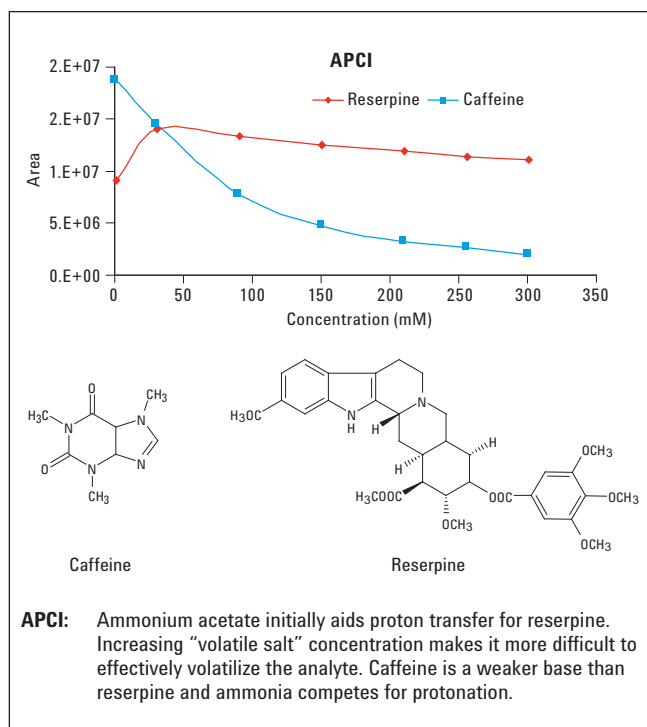
Figure 20
Effect of volatile buffer concentration on APCI response.

15

phase. APCI generally can accommodate a little more salt than ESI, though a volatile buffer is still preferred. APCI is possible with selected normal phase solvents for normal phase HPLC, but highly flammable solvents should be avoided because of the heating process or special caution has to be taken.

Atmospheric Pressure Photoionization (APPI)

Another API technique complementary to ESI is APPI. APPI is typically best for hydrophobic conjugated ring systems (corti-costeroids, PAHs) and may be less susceptible to ion suppression than ESI. APPI is an ionization technique where the analyte and mobile phase is first evaporated then ionized using UV light.

The APPI interface was introduced by Agilent and Syagen at ASMS in May 2001 (Figure 21). Like APCI, it is used for low to medium polarity analytes. The APPI process generates ions by first nebulizing the liquid analyte into small droplets, followed by evaporating the droplets to produce gas phase analyte molecules. The gas phase analyte is then ionized by photoionization from a krypton lamp.

The evaporation processes for APPI is similar to APCI and the analyte is not ionized until after evaporation. There are two mechanisms for ionization in APPI (Figure 22). The first mechanism, direct photoionization, results from the analyte absorbing a photon of light from the krypton emission. In order to get this type of ionization, the analyte must have an ionization potential that is less than the 10.6 eV of the lamp. Direct photoionization can only result in positive ion formation. In general, the analyte molecule needs to have only about seven carbon atoms in order to be photoionizable; but it needs to absorb the radiation energy. The second mechanism, as compared to APCI, can be thought of as photo-induced chemical ionization. In this case, a second reagent called a dopant is added to the mobile phase. The dopant is photoionized and the charge is then transferred
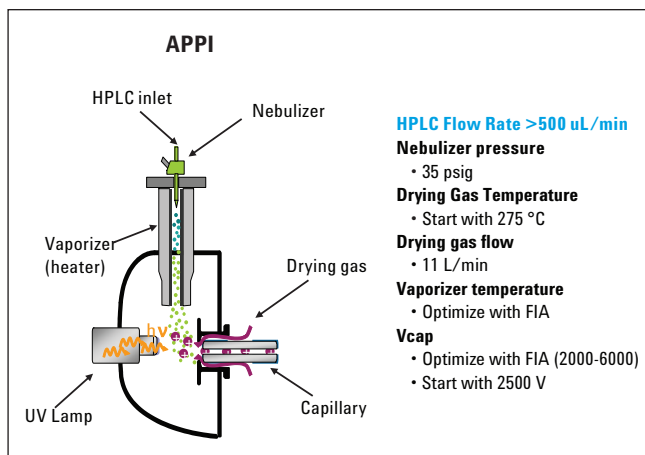

APPI

HPLC inlet
Nebulizer
Vaporizer (heater)
Drying gas
UV Lamp
Capillary

HPLC Flow Rate >500 uL/min
Nebulizer pressure
• 35 psig
Drying Gas Temperature
• Start with 275 °C
Drying gas flow
• 11 L/min
Vaporizer temperature
• Optimize with FIA
Vcap
• Optimize with FIA (2000-6000)
• Start with 2500 V

Figure 21
APPI Sources for LC/MS.

Analyte containing aerosol

Evaporation

Vapor

$h\upsilon$

$h\upsilon$

Photon ionizes analyte

Dopant is photoionized and acts as reagent gas
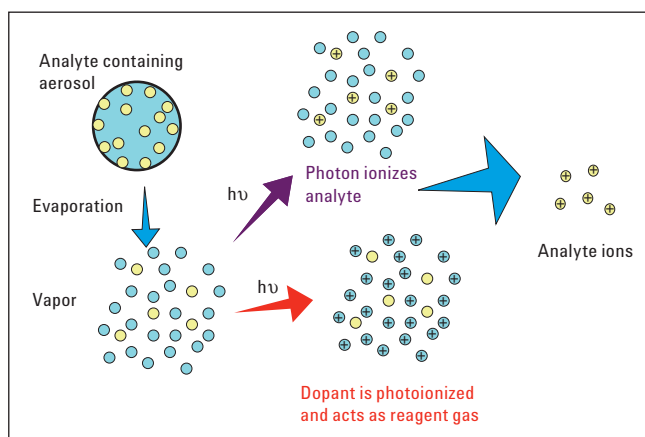
Analyte ions

Figure 22
Theory of atmospheric pressure photo ionization (APPI).

from the dopant to the analyte. Typical dopants are acetone and toluene, although other compounds can be used.

In order to do negative-mode APPI, there must be a source of thermal electrons. While the light striking the metal of the vaporizer barrel can be a source of some electrons, it is far better to use a dopant for this. Acetone is an excellent dopant for negative-mode APPI and does not interfere with positive-mode ionization. As with APCI, the LC mobile phase can interfere with ionization if the solvent has more affinity for the proton than the analyte. Also as with APCI, acetonitrile can be a problem for some analytes and it is recommended to try methanol first.

The Multimode Source: Combined ESI and APCI

The most versatile ion source for the single quadrupoles is certainly Agilent's G1978A multimode source (Figure 23). While ESI and APCI are essentially incompatible processes, each needing its own conditions for aerosol drying and electrical fields, it is possible to form ions simultaneously from ESI and APCI if the two ionization regions are separated in space. The HPLC effluent is nebulized using the same sprayer as for a dedicated ESI source. The droplets are emitted into the "ESI zone," where a high-voltage electrode performs the charging of the droplets and induces the formation of ions. Ions formed in this region pass through the source and enter the capillary. Residual droplets are dried using two IR lamps (not shown) emitting at the absorption frequency of water, and the vapor and analyte(s) enter the APCI zone where they are ionized by this process. Ions are then drawn into the capillary as in the case of the dedicated ESI and APCI sources.

If the multimode source is operated as an ESI or APCI source only, there is no loss in sensitivity. In fact, because the new source uses infra-red lamps for droplet evaporation, the efficiency of the evaporation process actually improves. Increased APCI sensitivity using the multimode source has been documented. This drying technique has also helped in achieving flow rates up to 2 mL/min. The standard APCI source, which used convective heating, works well to flow rates less than 1.5 mL/min. If the multimode source is operated in ESI and APCI simultaneously, sensitivity losses of up to a factor of two for some compounds can occur. Therefore, one must weigh the benefits of running analyses in both modes simultaneously against losses in sensitivity. For most applications, a loss in sensitivity of less than a factor of two is negligible.
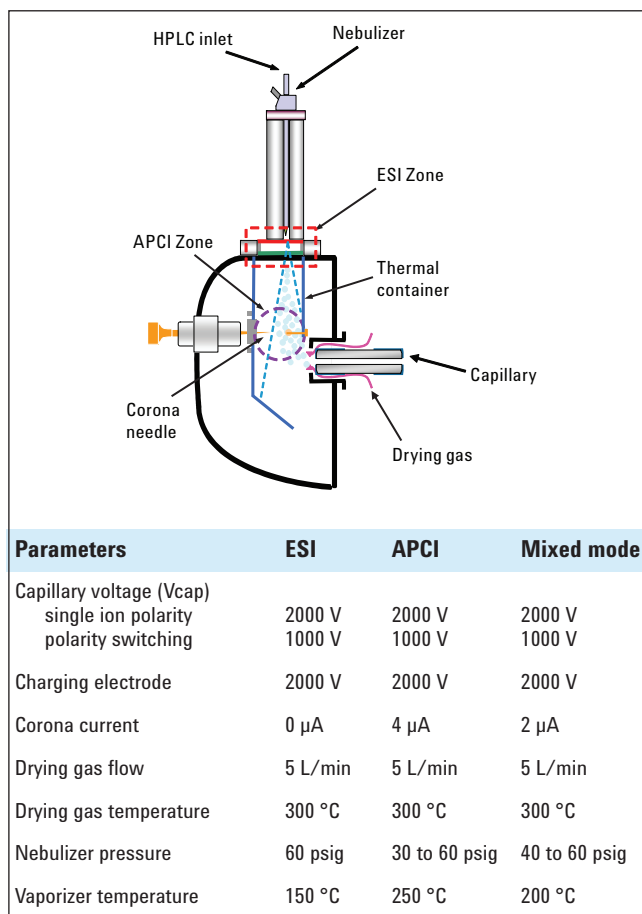

Parameters	ESI	APCI	Mixed mode
Capillary voltage (Vcap)			
single ion polarity	2000 V	2000 V	2000 V
polarity switching	1000 V	1000 V	1000 V
Charging electrode	2000 V	2000 V	2000 V
Corona current	0 µA	4 µA	2 µA
Drying gas flow	5 L/min	5 L/min	5 L/min
Drying gas temperature	300 °C	300 °C	300 °C
Nebulizer pressure	60 psig	30 to 60 psig	40 to 60 psig
Vaporizer temperature	150 °C	250 °C	200 °C

Figure 23
The multimode source: A combination of ESI and APCI (Starting conditions for method development).

Conclusion

The addition of MS to HPLC-UV provides an increase of analytical capability for the chromatographer. The most common atmospheric pressure ionization interface to HPLC is ESI. Not all samples or chromatographic techniques are well suited for ESI. For these applications, two alternative atmospheric pressure ionization techniques have been developed: APCI and APPI.

APCI and APPI both ionize the sample after evaporation while ESI evaporates the sample after ionization. As such, both APCI and APPI are able to ionize the less polar molecules that cannot be ionized by ESI. Since both APCI and APPI are mass dependant analyzers, sample dilution does not reduce sensitivity, so higher flow rates and larger LC column dimensions can be used. APCI is good technique for small molecules (< 1500 Daltons) which can be polar or somewhat non-polar (substituted PAHs and PCBs, fatty acids, phthalates). APPI is a good technique for hydrophobic conjugated ring systems (corticosteroids, PAHs) and compounds that are thermally labil during APCI.

The development of a multi-mode source combining ESI and APCI increases the range of polarity for analyzing samples. When used in solely ESI or APCI modes, the convenience of having both techniques in one source increases productivity. The availability of ESI, APCI, and APPI for HPLC/MS analysis provides options for diverse sample analysis as well as varying HPLC techniques.

www.agilent.com/chem/lcms

Agilent Technologies