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Nota de aplicación

General Unknown Screening for Drugs in Biological Samples by LC-MS

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For forensic toxicology use only.

Abstract

This application note details on the recent development of a unique LC-MS library and ChromaLynx chromatographic deconvolution software, LC-MS can now be considered a powerful and practical alternative to traditional screening methods.

Introduction

Identification of drugs of abuse and toxicants in biological fluids is currently performed by a variety of analytical techniques including immunoassays and chromatographic techniques such as GC-MS and LC with UV detection. Although these techniques are well established and widely used, they suffer from limitations for many toxicologically important compounds. For example, sensitivity is often a limitation with LC/UV techniques as newer drugs are used at lower therapeutic concentrations. In addition, LC/UV methods can require extensive sample preparation. GC-MS is often referred to as the gold standard in toxicology laboratories, but even GC-MS has significant limitations for toxicology screening applications where rapid sample analysis is a requirement. Many substances encountered in toxicology laboratories are non-volatile, polar or thermally labile and cannot be directly analyzed by GC-MS. These compounds usually require time consuming derivatization prior to analysis.

LC-MS, using electrospray ionization (ESI), is ideally suited to polar, non volatile and, thermally unstable compounds and potentially provides a powerful means of identifying many toxicologically relevant compounds rapidly without the need for sample derivatization.

Historically, the lack of availability of LC-MS libraries and reliable LC-MS chromatographic deconvolution software has limited the widespread use of this technique for screening applications. However, with the recent development of a unique LC-MS library and ChromaLynx chromatographic deconvolution software, LC-MS can now be considered a powerful and practical alternative to traditional screening methods.

LC-MS Library Concept

The electrospray ionization process, used in LC-MS systems, is very different from the electron impact (EI) Ionization used in GC-MS systems, thereby preventing the use of commercial EI mass spectra libraries such as NIST, Wiley, and Pfleger-Maurer-Weber.

Electrospray is a soft ionization technique that mainly leads to protonated molecular ions in positive ion mode and to deprotonated molecular ions in negative ion mode. In order to get more specific structural information, it is possible to induce fragmentation of these molecular ions in the source region of a mass spectrometer. This can be achieved by increasing the voltage applied to the sampling cone area where ions transit from a high pressure region to a low pressure region. Molecular ions then collide with neutral molecules in the source region and fragment into characteristic ions. This is referred to as in–source collision induced dissociation (CID). Using this process reproducible LC-MS mass spectra can be used to produce a library of mass spectra .

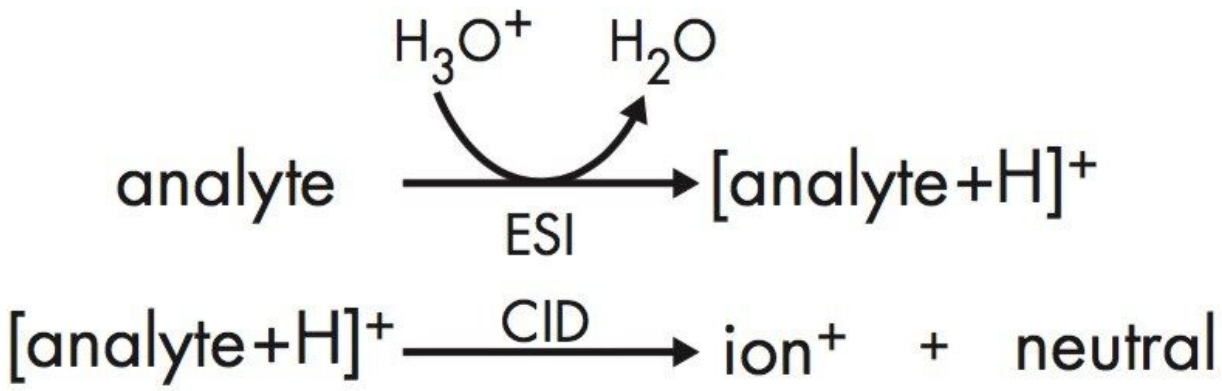

Figure 1. Atmospheric Pressure Ionisation (API) process - This soft ionisation process leads to cations in positive ion mode and anions in negative ion mode which are generally stable. These molecular ions can be fragmented in the source region of LC-MS instruments.

Using in-source CID, it is possible to generate mass spectra exhibiting different fragmentation patterns according to the value of the cone voltage applied in the source. This can be done in both positive and negative ion modes. These spectra can then be used to build a library.

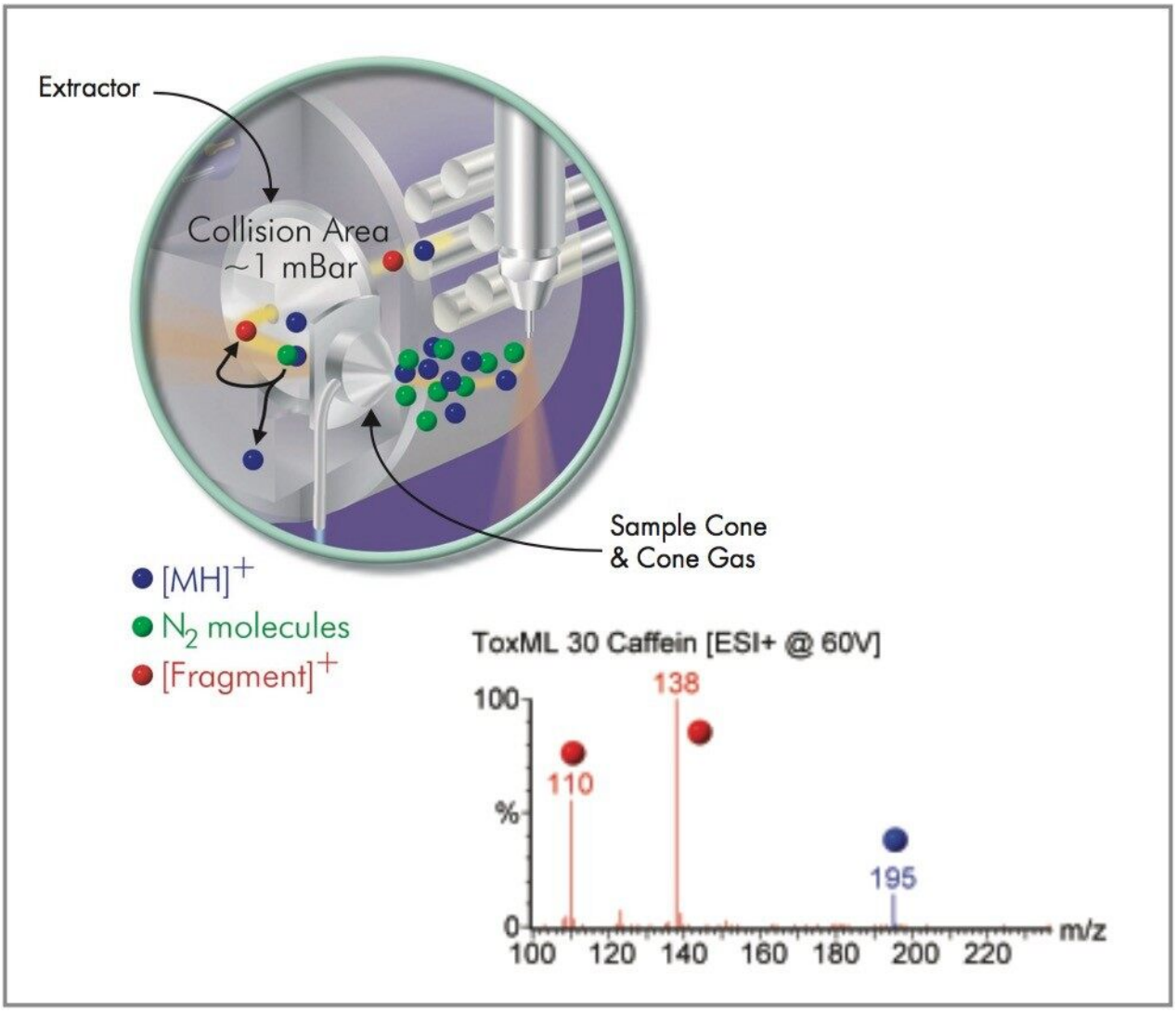

Figure 2. InSource-CID – An example showing fragmentation of the moleculer ion (m/z 195) of caffeine at 60V in the Quattro micro API ion source.

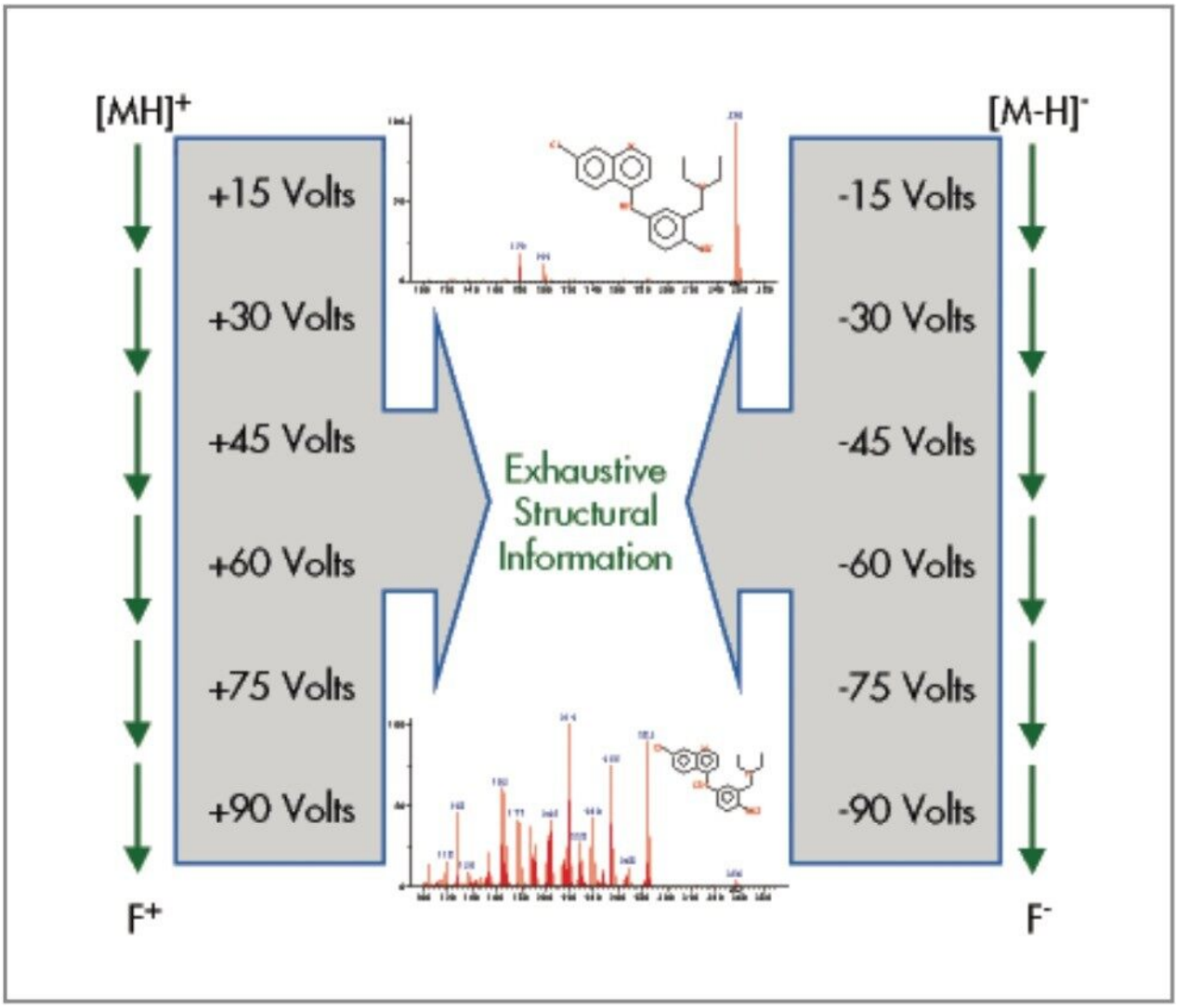

Figure 3. Extensive structural information is stored for each component in the library as mass spectra can be stored at every significant cone voltage in both positive and negative ion mode.

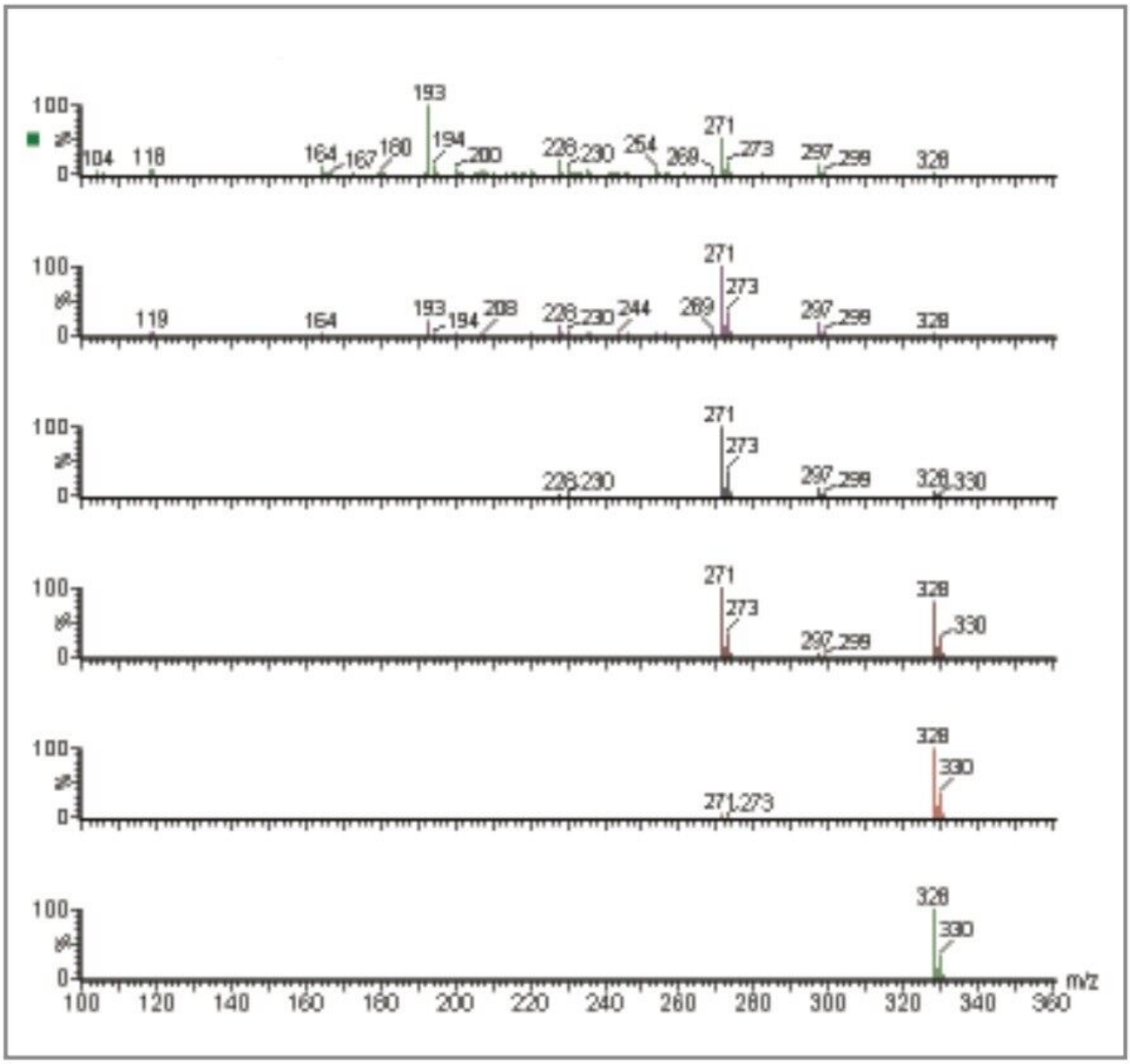

Figure 4. Loxapine, a tranquilizer agent. Mass spectra recorded at 6 different CV values using in-source CID.The degree of fragmentation increases with the cone voltage.

In the current version of the library, this approach has been used for over 500 compounds, which corresponds to approximately 2600 mass spectra. These compounds represent 90% of the intoxication cases encountered in Europe. In addition chromatographic retention times are also stored for each compound in the library. The library is easy to maintain and user appendable.

LC Separation Method

An identical generic LC method is used both to generate the library mass spectra and for sample analysis.

The generic gradient method has been developed based on water and acetonitrile buffered with 5 mM ammonium formate at pH 3. The total run time including system and column re-equilibration is 26 minutes.

ChromaLynx Application Manager

Chromatogram examination is at least as important as the content and structure of the library. The chromatogram from a typical toxicological analysis will usually be complex and exhibit dozens of peaks. Compounds of interest can be difficult to identify especially at low concentrations when they can be hidden in the base line or when they closely elute. ChromaLynx application manager includes a unique algorithm to specifically process multifunctional LC-MS data. The process can be ultimately as exhaustive as analyzing each scan for each cone voltage; this enables the detection of the maximum number of components in a chromatogram.

Unlike other LC-MS/MS screening techniques, ChromaLynx application manager enables a complete and systematic chromatogram examination. This type of data processing is essential for systematic toxicological screening or general unknown screening. ChromaLynx application manager selects a single mass spectrum at a given scan and extracts up to 8 of the most intense ions and reconstructs corresponding ion chromatograms. These ion chromatograms are then examined and components are detected according to user defined parameters. Detected components are then searched against library spectra

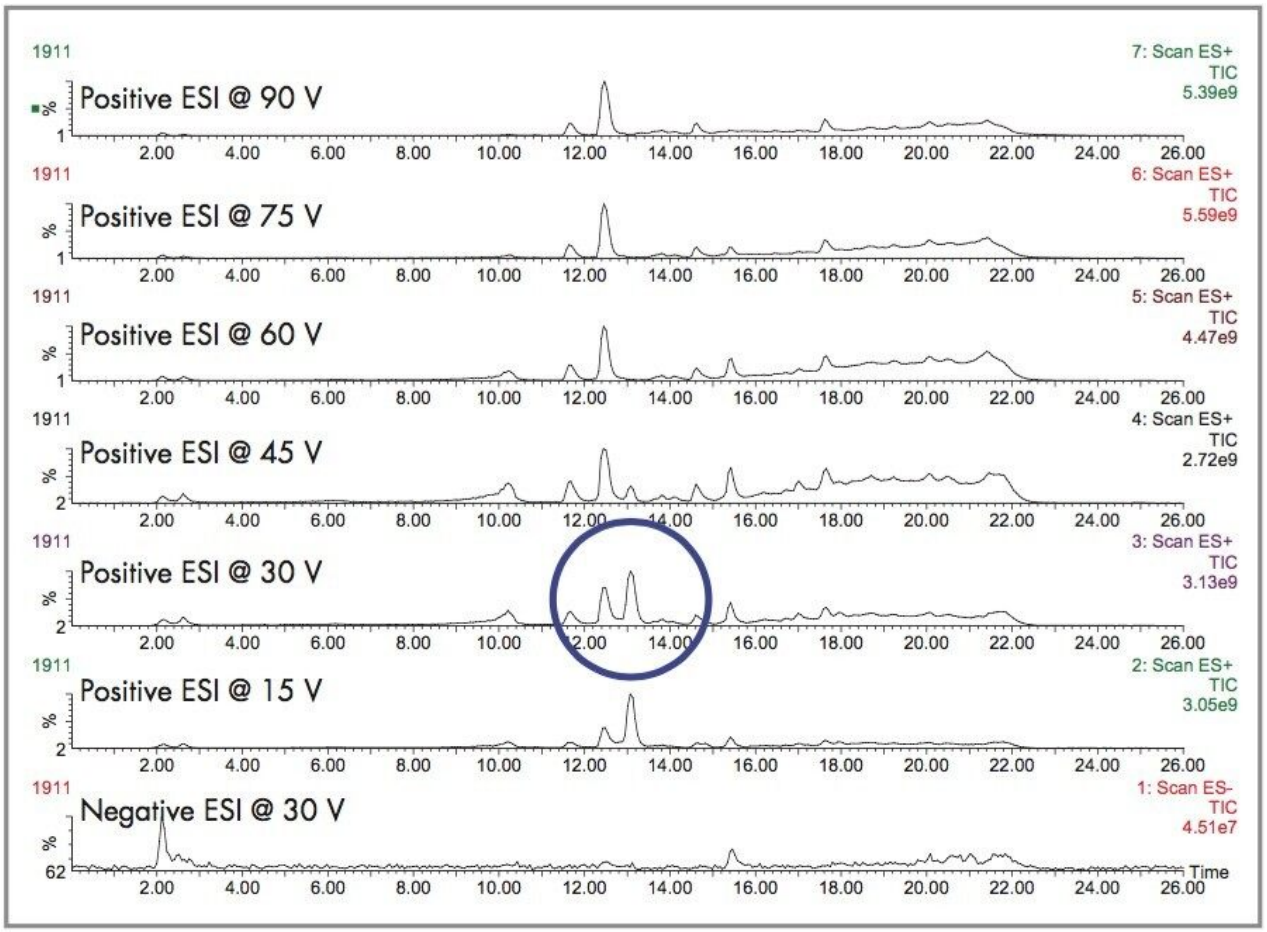

Figure 5. Urine extract - one single sample analysis leads to 7 chromatograms. Manual examination of each chromatogram would be time consuming and not feasible for a routine toxicology laboratory. Analysis of the area circled in the chromatogram above by ChromaLynx (figure 6b) illustrates the presence of several peaks that would be missed on examination of the total ion chromatogram.

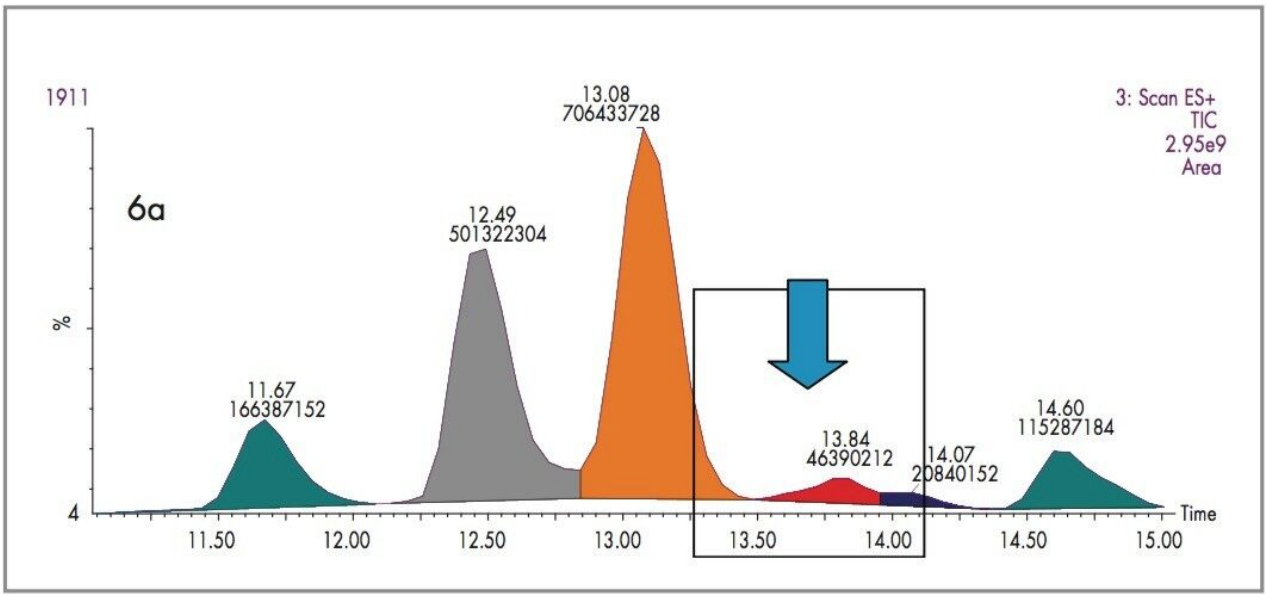

Figure 6a. Close-up view of the 11-15 minutes section of chromatogram area for function 3 acquired in positive ESI @ 30 V. Here the total ion chromatogram (TIC) indicates that only one component elutes at 13.8 minutes.

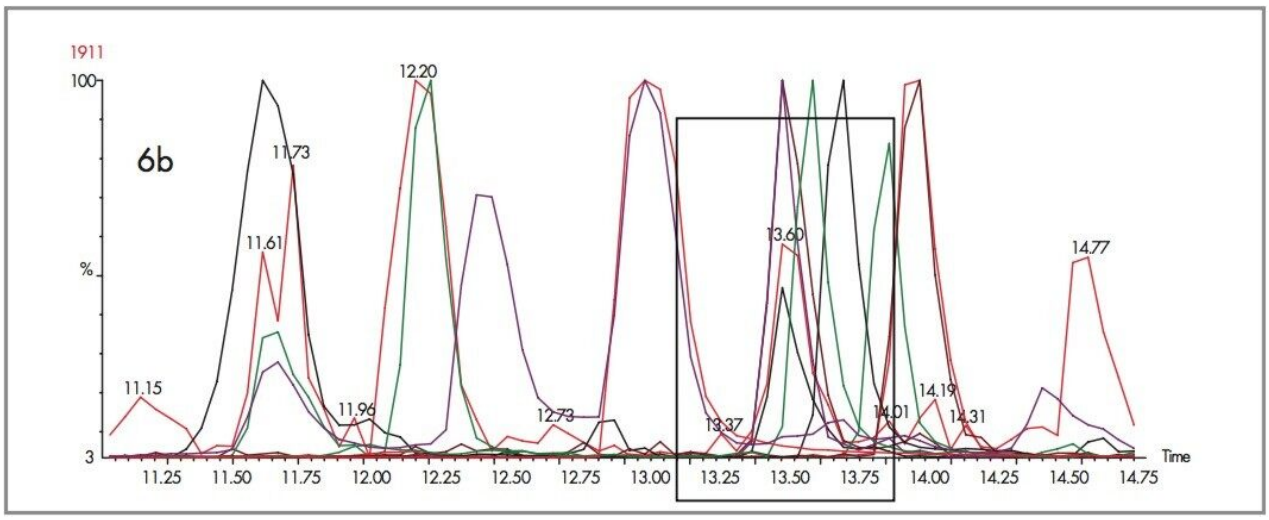

Figure 6b. Close-up view of the 11 - 15 minutes section of chromatogram area for function 3 acquired in positive ESI @ 30 V. Using extracted ion chromatograms shows that at least three components elute between 13.5 and 13.8 minutes.

This area represents only 4 minutes out of the 26 minutes of the whole chromatogram for function 3 recorded in positive ion mode at 30V. ChromaLynx will process all chromatograms to achieve a detailed and

efficient screening. ChromaLynx application manager automatically processes data in minutes that would take hours manually.

Mass Spectrum Extraction and Library Search Process

Once chromatographic components have been detected, ChromaLynx automatically extracts mass spectra of the individual components. This is performed taking into account possible interferences due to closely eluting peaks. It is possible to customize parameters in order to get precise background subtraction depending on the peak width and tolerance on apex determination. Extracted mass spectra of detected components are then compared to library mass spectra.

In order to improve the specificity of the screening technique, additional filters have been developed to enhance the quality of the screening and to get more relevant results. Retention time filters as well as cone voltage filters are available and user defined tolerance parameters can be implemented.

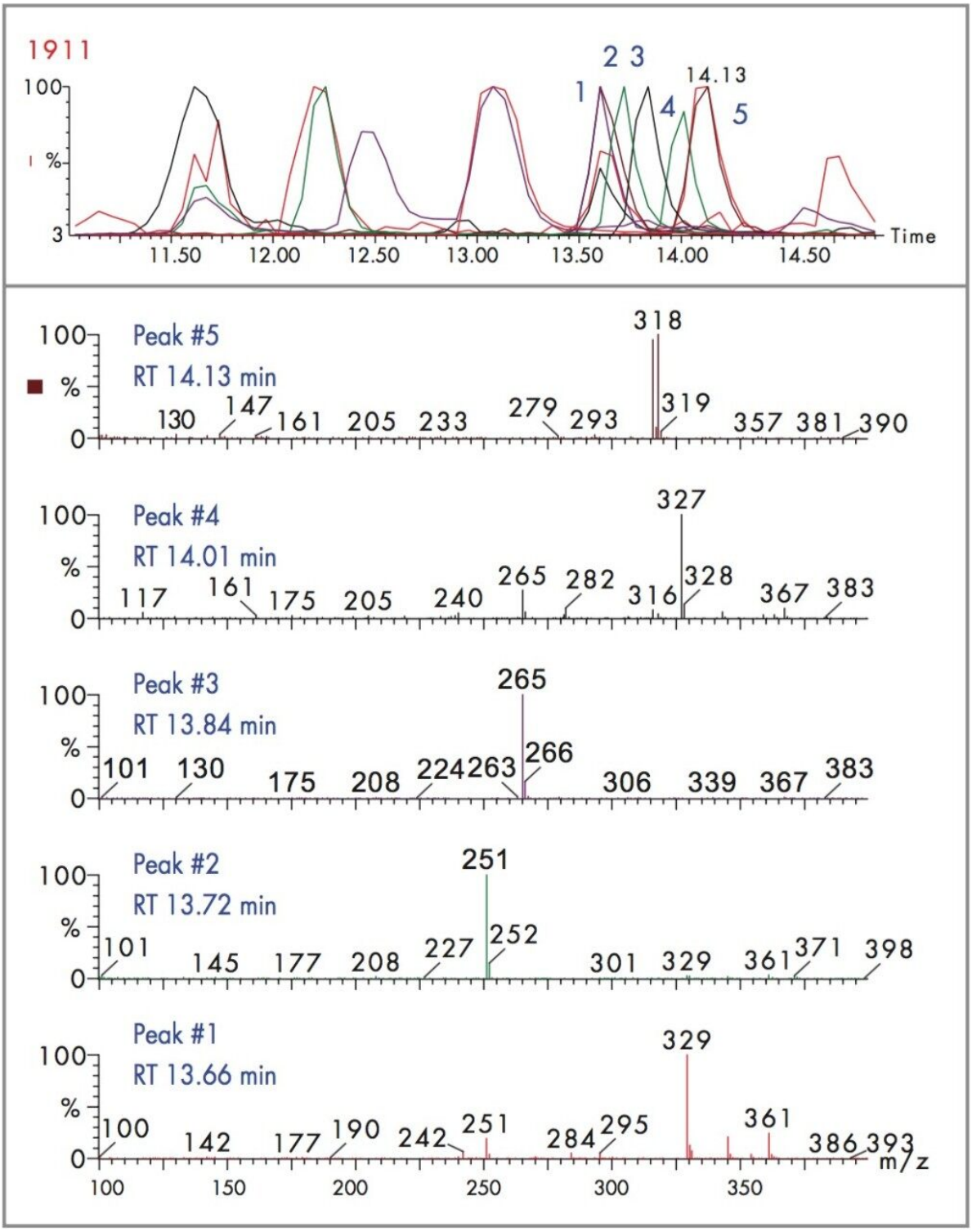

Figure 7. Chromatogram acquired in positive ESI @ 30 V and corresponding mass spectra of 5 components detected by ChromaLynx. Automated spectral deconvolution allows extraction of clean mass spectra that can be used for library searching.

Application Example – Polyintoxication

A urine sample was taken from a suspected intoxication. It was known that the person was taking a number

of prescribed drugs. The urine samples were analyzed by LC-MS to identify the cause of intoxication. Toxicologists were looking for both expected compounds due to the regular treatment and unexpected active substances that may have been taken accidently or deliberately.

Experimental

Analytical Equipment and Instrumentation

Waters Toxicology Screening LC-MS System comprising of:

- ZQ Single Quadrupole Mass Spectrometer
- Alliance 2695 Separations Module
- MassLynx 4.0 Data Station
- ChromaLynx 4.0 Application Manager

Sample Preparation

Liquid/liquid extraction at 2 pH (4.5 & 9.0) using dichloromethane/ether/ hexane [30:50:20] + 0.5% isoamylic alcohol.

LC Separation Method

- XTerra MS Column & Precolumn: C_{18}, 3.5 µm, 2.1 mm id x 150 mm (10 mm for precolumn)
- Column Oven Temperature: 30 ˚C
- Mobile Phase based on Water/Acetonitrile with Ammonium Formate 5 mM @ pH 3
- Gradient: 5% organic to 90% organic from 2 min. to 16 minutes

MS Operating Conditions

- Capillary 3.5 kV in both positive and negative ion modes
- Source Temperature @ 120 ˚C & Desolvation Temperature @ 250 ˚C
- Desolvation Gas Flow Rate @ 350 l/h & Cone Gas Flow Rate @ 100 l/h
- Function 1: Full Scan - Negative ESI from 100 to 650 amu in 250 ms @ 30 Volts

- Functions 2 to 7: Full Scan - Positive ESI from 100 to 650 amu in 250 ms @ from 15 Volts to 90 Volts

Results and Discussion

From the resultant analysis, 8 out of 9 expected components were successfully identified by the ChromaLynx data processing library search process. Tramadol was the only expected compound that was not detected. In addition, three unexpected compounds were also Detected-Meprobamate, Acepromazine and Bromazepam. It was highly likely that these three compounds were the cause of the intoxication.

				Candidate Average Fit (%)
#	Analyte Name	Status	Origin	6 Functions
1	Nicotine	Unexpected molecule	Smoker / Contamination	56.1
2	Trimetazidine	Expected molecule	Medication	63.3
3	Acetaminophen	Expected molecule	Medication	62.3
4	Caffeine	Expected molecule	Medication	74.0
5	Quinine	Expected molecule	Medication	70.3
6	Zolpidem	Expected molecule	Medication	94.7
7	Meprobamate	Unexpected molecule	Unknown	55.3
8	Mianserin	Expected molecule	Medication	67.1
9	Acepromazine	Unexpected molecule	Unknown	57.6
10	Bromazepam	Unexpected molecule	Unknown	53.1
11	Hydroxyzine	Expected molecule	Medication	88.2
12	Propoxyphene	Expected molecule	Medication	62.1
13	Tramadol	Expected molecule	Medication	Not found

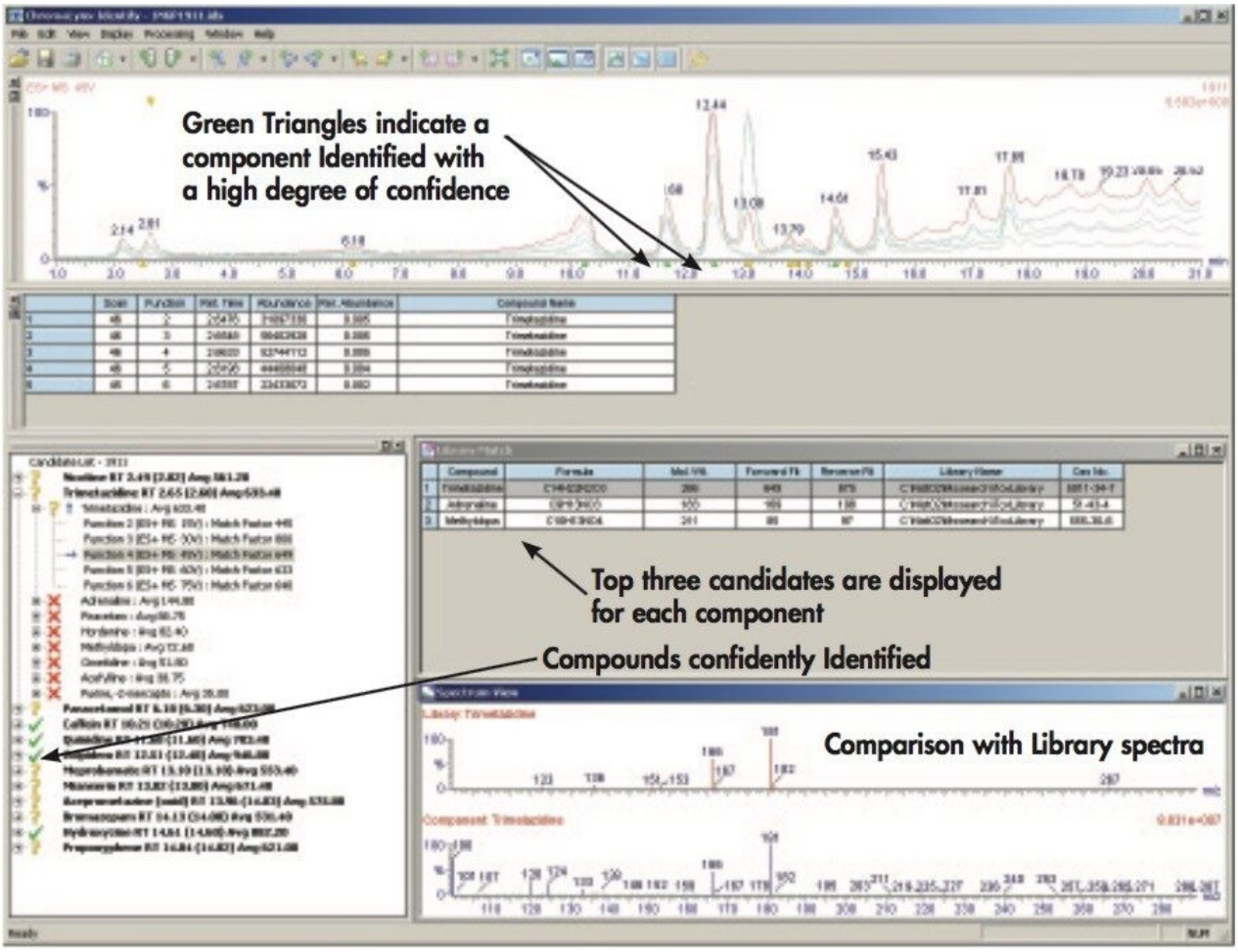

Figure 8. ChromaLynx browser showing a list of candidate compounds, chromatogram recorded at different cone voltages and comparison of a unknown spectra against library spectra.

Conclusion

Using the combination of in-source CID at multiple cone voltages and retention time data results in a library containing detailed information for each compound. With the development of ChromaLynx data chromatographic deconvolution software, LC-MS can now be considered a powerful tool for toxicology screening applications.

The unique ChromaLynx deconvolution algorithm ensures that the maximum number of components are detected. The unique algorithm enables low intensity and closely eluting peaks to be detected and identified.

The accuracy of the library search process is enhanced by utilizing multiple mass spectra per component and retention time.

Featured Products

Alliance HPLC System <https://www.waters.com/534293>

MassLynx MS Software <https://www.waters.com/513662>

ChromaLynx <https://www.waters.com/513759>

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