Document Title: Enhanced Studies of LC/MS/MS Capabilities to Analyze Toxicology Postmortem Samples

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Enhanced Studies of LC/MS/MS Capabilities to Analyze Toxicology Postmortem Samples

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Final Technical Report

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Abstract

The analysis of blood and urine samples collected at autopsy or as part of DUI investigations for the identification and quantitation of drugs that may be present can be a time consuming and labor intensive process. This project was designed to investigate and develop less time consuming methods of identifying and quantifying drugs using a liquid chromatograph/mass spectrometer/mass spectrometer (LC/MS/MS) to streamline this analysis. The instruments selected for the research were the Applied Biosystems Models 2000 and 3200 QTRAP®. Both of these instruments are triple quadrupole linear ion trap mass spectrometers. The project funded under this 2006 award had several components:

- Investigate new and/or refined methods to determine if the LC/MS/MS instrument and the simple, protein precipitation extraction method could be utilized with opioids, delta-9-tetrahydrocannabinol (THC) and metabolites, barbiturates, and acidic drugs.
- Investigate the stability of commonly encountered drugs in forensic toxicology after storage in liquid blood under normal refrigeration and preservative conditions.
- Provide training workshops to forensic toxicologists from other crime laboratories to disseminate the methods developed from the 2003 project as well as any new information developed during this project.

The drug stability project examined the long term stability of seventy six drugs. The drugs and metabolites studied were those routinely encountered in forensic toxicology analysis including cocaine, opioids, benzodiazepines, and amphetamines. Nine drugs were identified that exhibited substantial levels of degradation within the first 30 days of storage in refrigerated blood samples containing preservatives. The remainder of the drugs did not exhibit any significant levels of degradation over the course of the study.

The method development studies led to simple rapid extraction procedures for the detection and quantitation of opioids as well as THC and two THC metabolites, eliminating the need for lengthy extraction methods. The studies involving barbiturates and acidic drugs demonstrated that although analysis is technically feasible on the LC/MS/MS platform, the specificity is not comparable to that achieved through other analytical techniques such as GC/MS.

Executive Summary

Overview

The role of the forensic toxicologist encompasses the analysis of blood and other biological samples for the presence of drugs that may have contributed to the cause of death or driving under the influence (DUI). In a previous research and development award from the National Institute of Justice (2003-IJ-CX-K007), a generalized liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) method was developed that allowed qualitative and quantitative analysis of over 100 different drugs and metabolites (Herrin, McCurdy, & Wall, 2005) following a simple protein precipitation extraction method in liquid whole blood (Slightom and McCurdy, 1984; Lewellen and McCurdy, 1988, 1994; Cagle et. al., 1997). While the generalized method developed in that project is very successful on a wide range of drugs and has been implemented into routine casework at the Georgia Bureau of Investigation-Division of Forensic Sciences (GBI-DOFS), there were a few commonly encountered drug compounds that were not successfully analyzed using that generalized procedure. Morphine, hydromorphone and 11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid (COOH-THC) were among the drugs and/or metabolites that proved difficult or impossible to adequately identify and quantitate using the generalized method.

The GBI-DOFS adapted immunoassay procedures to alternative biological specimens (Slightom and McCurdy, 1984; Lewellen and McCurdy, 1988, 1994; Cagle et. al., 1997). The acetone based protein precipitation method developed by Lewellen and McCurdy is a standard procedure used for the presumptive identification of drugs in blood and other biological samples by enzyme immunoassay (EIA) for the presence of drug classes/metabolites (cocaine, cannabinoids, opioids, barbiturates, amphetamines, and benzodiazepines). The method developed as a result of the 2003 project was not intended to and does not replace screening via EIA, but serves as a powerful addition by quickly providing specific information as to the drug content of a sample without the need for additional sample consumption and extraction. Utilization of this combined approach within the GBI-DOFS has resulted in the identification of drugs in many casework samples that may otherwise have been undetected based on EIA results alone, especially in DUI cases involving prescription sleeping aids (data not shown). However, one of the shortcomings of the general method developed in the 2003 project was the inability to identify many opioid compounds due to ion suppression. Therefore, this project had three major objectives:

- Investigate new and/or refined methods to determine if the LC/MS/MS instrument and the simple, protein precipitation extraction method could be utilized with opioids, cannabinoids, barbiturates, and acidic drugs.
- Investigate the stability of commonly encountered drugs in forensic toxicology after storage in liquid blood under normal refrigeration and preservative conditions.
- Provide training workshops to forensic toxicologists from other crime laboratories to disseminate the methods developed from the 2003 project as well as any new information developed during this project.

Materials and Methods

All studies except the amphetamine stability study and cannabinoid method development conducted during this project utilized a protein precipitation method (Slightom and McCurdy, 1984) for the extraction of drugs from blood samples. Whole blood was obtained from the Red Cross (Atlanta, GA) or from autopsy and extensively tested to determine that no drugs were present. Blood samples were spiked with pharmaceutical grade drugs at known concentrations in order to perform the various studies. Sample analysis for all drugs other than amphetamine compounds was performed on an Perkin Elmer Model 200 LC linked with an Applied Biosystems Model 2000 or Model 3200 QTRAP® triple quadruple mass spectrometer to form the LC/MS/MS system. Data analysis from the LC/MS/MS was performed using Analyst® version 1.4.1 software. Amphetamine compounds were tested on an Agilent 6890 gas chromatograph coupled with an Agilent 5973N mass selective detector (MSD) quadrupole mass spectrometer to form the gas chromatograph/mass spectrometer (GC/MS) system and the data was analyzed using Chemstation® version D.00.00.38 software.

Project Studies

The first method examined in this project was the feasibility of using LC/MS/MS to qualitatively identify delta-9-tetrahydrocannabinol (THC) and THC metabolites and, subsequently, quantify THC and metabolites from whole blood samples submitted in DUI and postmortem cases. A simplified protein precipitation extraction (McCurdy and Lewellen, 1984) was initially investigated to isolate THC and metabolites from whole blood. A method for LC/MS/MS analysis for identification of THC and metabolites was developed and validated for specificity, sensitivity, and reproducibility. Studies were performed to demonstrate THC and THC metabolites could be successfully quantitated with a degree of accuracy and reproducibility comparable to that obtained from GC/MS methods in use at the GBI-DOFS laboratory.

The second major method investigated during this project was the identification and quantitation of opioid compounds using LC/MS/MS. The general method developed under the 2003 R&D 2003-IJ-CX-K007 project is currently in use in the GBI-DOFS laboratory but has proven incapable of analyzing for these compounds due to ion suppression of the signal. A successful method was developed to overcome the effects of the ion suppression and allow identification and quantitation of opioid compounds on an LC/MS/MS following the simple protein precipitation extraction method discussed earlier. The revised method uses a reverse polarity column as well as modifications to the elution gradient to increase the retention time of the opioids. All common opioids encountered in forensic toxicology were accurately identified using this method based on the retention time on the LC column and the multiple reaction monitoring (MRM) transitions. Quantitative studies using the LC/MS/MS for the opioid compounds demonstrated that the reproducibility and accuracy of this method was equivalent to that obtained from GC/MS analysis. Concordance studies of previously analyzed casework samples were performed to verify that results from the updated LC/MS/MS method were consistent with those obtained using GC/MS analysis.

A third method investigated in this project was the potential of LC/MS/MS analysis to identify and quantify barbiturate compounds and acidic drugs in whole blood. Using LC/MS/MS to characterize barbiturates and acidic drugs has been reported (Feng et al., 2007), but this procedure analyzed urine samples to detect barbiturates and acidic drugs. During this project a solid phase extraction method and the protein precipitation method (McCurdy and Lewellen,

1988) were examined to determine which produced samples more amenable to further analysis. The protein precipitation method proved to be the most suitable because it used less sample and produced more reproducible results; therefore, it was used in subsequent studies. It was possible to quantify the barbiturate compounds using LC/MS/MS; however, qualitative identification proved impractical due to an insufficient number of specific ions per compound in the mass spectra obtained from each compound.

The second major objective of this project was to determine the long term stability of drug and metabolite compounds stored in a biological matrix such as blood. The primary impetus for this study arose from observations during the concordance study completed as part of the 2003 project that indicated levels of some drug compounds had declined during sample storage. In this study whole blood samples were spiked with therapeutic and toxic/overdose levels of drug compounds commonly encountered in routine casework. The stability study was broken into three parts:

- The main stability study consisting of 64 drugs and spanning ~20 months (618 days);
- A supplemental drug stability study of eight drugs and metabolites was started after the initial study was underway due to a delay in acquiring drug and drug metabolite standards from vendors and spanned ~13 months (385 days);
- A stability study of amphetamine and related compounds spanning ~20 months (581 days).

For simplicity sake, these will be referred to as stability study, supplemental stability study and amphetamine stability study, respectively. Aliquots of spiked samples were taken at prescribed intervals ranging from 0 days after drug addition to 618 days of storage. Nine drugs were identified that exhibited significant levels of degradation during storage at 0.5-9°C: cocaine, cocaethylene, benzoylecgonine, clonazepam, mesoridazine, bupropion, diltiazem, ziprasidone and zopiclone. The level of degradation ranged from 27% for benzoylecgonine to approximately 100% for zopiclone. Interestingly and most important from a laboratory standpoint, zopiclone degradation began very rapidly, within the first 40 days of storage. Amphetamines and all other drug compounds did not exhibit a consistent significant level of concentration decrease upon storage.

The last major component of this project was dissemination of the LC/MS/MS methods developed during project 2003-IJ-CX-K007 to forensic toxicologists from other forensic laboratories. To accomplish this goal a series of six hands-on practical workshops were held at the GBI-DOFS facility. Each workshop was attended by four to six toxicologists. The workshop consisted of lectures in the theory and application of the Applied Biosystems LC/MS/MS instrumentation, practical exercises involving extraction and analysis of known samples on the LC/MS/MS, interaction with GBI-DOFS toxicologists to exchange information concerning casework trends and analytical methods, and finally a set of unknown mock casework samples. 34 Toxicologists from 11 states representing 16 laboratories attended the workshops. Each workshop attendee received a certificate of attendance for 40 hours of training. The feedback response from the attendees was overwhelmingly positive. In addition to the workshops, two posters and a seminar were presented at the Society of Forensic Toxicologists (SOFT) meeting held in Phoenix, AZ in October 2008.

Discussion

This research project has been very successful, with the development of new methods for analysis of THC, THC metabolites, and opioids on an LC/MS/MS instrument platform following a simple one to two hour sample preparation and extraction step. By implementing these methods, laboratories have the potential to reduce labor time for sample preparation by up to 60% over more conventional methods such as solid phase or liquid-liquid extraction methods. The analytical methods developed during this project require further validation prior to implementation into routine casework but they have the potential to substantially reduce the labor associated with these types of analysis. Opioid compounds are involved in a significant number of cases each year and any improvements in the analytical method will be a welcome addition to the techniques available. Such improvements could result in faster turnaround times of results to the medical examiner or coroner who is responsible for establishing cause and manner of death or in release of reports in suspected DUI cases.

This project also determined that some drugs and metabolites present in whole blood specimens stored at normal refrigeration temperatures (4°C) do degrade or decompose significantly over time. Having this knowledge is crucial when interpreting the results of testing that occurs months or even years after the sample is collected. There are many cases where the results of initial toxicology findings are challenged or additional testing is required to resolve an issue arising from civil litigation. The knowledge that the later testing may produce results differing from the original analysis allows the toxicologist to make an informed decision as to the conclusions reached in a particular case. Knowing certain drugs degrade fairly rapidly in storage also has significant implications for forensic laboratories experiencing large backlogs or where testing may be delayed for other reasons. Forensic laboratories can utilize the information from this study to modify sample storage policies and analytical testing schema to ensure accurate and reliable results reflective of actual drug content are obtained in all cases.

Finally, through the use of the training workshops conducted as part of this project and presentations at national toxicology meetings, the forensic toxicology community has been exposed to the potential of using LC/MS/MS instrumentation to improve the scope and timeliness of toxicology analysis. This project has produced new knowledge, aided in the development of new methods, and disseminated information in a very effective fashion.

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Chapter 1: Background and Workshops

Background

The role of the forensic toxicologist encompasses the analysis of blood and other biological samples for the presence of drugs that may have contributed to an individual's cause of death or driving under the influence (DUI). Toxicology analysis can be very time consuming and labor intensive because of the number of different compounds that must be identified and in many cases quantitated.

The first step in most forensic toxicology laboratories is the screening of samples via enzyme immunoassay or some other method that will provide general guidance to the toxicologist concerning which drug classes may be present in a sample. Following this initial screening, if the drug screen indicates the sample does contain drugs or if the laboratory has other information that the sample may contain drugs that would not be detected by the particular screening test used, the laboratory must conduct further testing to both positively identify which drugs are present in the sample. One of the most common methods currently used to perform this identification step is analysis by gas chromatography/mass spectrometry (GC/MS). While the GC/MS method is certainly capable of identifying a wide range of drug compounds, there are some limitations to this method. The first limitation is that in order to prepare the sample for injection onto the GC/MS instrument, time consuming and labor intensive extractions of the drugs from the sample often must be performed. Not only are these extractions time consuming and labor intensive, but in many cases separate types of extractions must be used for different drug classes, e.g. opiates vs. amphetamines. A second limitation is the level of sensitivity of the GC/MS instrument itself. Although these instruments are very sensitive, many of the extraction methods require large sample volumes, in the 3-5 milliliter range to recover sufficient drug for detection by the instrument.

Even after all relevant drugs within the sample have been identified, in many cases the quantity of drug must also be determined in order to provide the information necessary for case interpretation, i.e. cause of death or driving impairment. All of these steps from screening to quantitation can take from several days to several weeks to complete, depending on the number of different drugs present in a sample and the staffing available in the laboratory to perform the procedures. A major emphasis of the forensic science R&D programs sponsored by the National Institute of Justice (NIJ) is to develop methods that can enhance current forensic methods and increase the efficiency of crime labs in the United States and abroad. A second emphasis of these R&D programs is the dissemination of findings from the research projects to the general forensic community.

In a previous research and development award from the NIJ (2003-IJ-CX-K007), a generalized liquid chromatography/tandem mass spectrometry (LC/MS/MS) method was developed that allowed qualitative and quantitative analysis of over 120 different drugs following a simple protein precipitation extraction from whole blood (Herrin, McCurdy & Wall, 2005). While the generalized method developed in that project has proven very successful in the analysis of a wide range of drugs, there are a few commonly encountered compounds such as morphine, hydromorphone, and 11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid (COOH-THC) that could not be successfully analyzed using the previously developed procedures because of ion suppression or insufficient sensitivity. Even though the method developed during that project did

have limitations, it was sufficiently robust that the Georgia Bureau of Investigation-Division of Forensic Sciences (GBI-DOFS) implemented the method into routine casework. As a result of this implementation, report turnaround times for many cases were improved. In several cases drugs were identified in samples that would otherwise have remained undetected using GC/MS methods also in use in the laboratory. Perhaps most importantly of all however, is the reduced consumption of casework samples that became possible with the introduction of an LC/MS/MS method into casework.

As a result of the success of that 2003 project, but recognizing the shortcomings outlined above that still existed, a second proposal was submitted to the NIJ to expand upon the previous work. The project outlined in this new proposal had three primary goals and each of these goals was completed during the research project. A more detailed description of the activities undertaken to achieve each goal and the results of the various studies are provided in later sections of this report.

- Investigate new and/or refined methods to determine if the LC/MS/MS instrument and the simple, protein precipitation extraction method could be utilized with opioids, THC compounds, barbiturates, and acidic drugs.
- Investigate the stability of commonly encountered drugs in forensic toxicology after storage in liquid blood under normal refrigeration and preservative conditions.
- Provide training workshops to forensic toxicologists from other crime laboratories to disseminate the methods developed from the 2003 project as well as any new information developed during this project.

Due to the number of different project studies and how different each study was, the analytical projects will be presented in separate chapters of this report. Information and conclusions regarding the training workshops (Goal #3) are included below.

Training Workshops

One of the major missions of the National Institute of Justice's Research and Development programs is the dissemination of new methods or techniques that can enhance the quality of analysis or improve the timeliness of such analysis within forensic laboratories. This project included such a dissemination component. To better communicate and disseminate the methods and conclusions from the 2003 R&D project where a general screening and quantitation method was developed using the LC/MS/MS instrument platform, and to exchange current information regarding the utilization of the Applied Biosystems QTRAP® LC/MS/MS instrument for toxicology analysis a training workshop was developed. All travel expenses for attendees to the workshop were paid from this 2006 award to minimize the barriers to attendance by forensic toxicologists from public crime laboratories to the workshop The primary workshop instructor was a former GBI-DOFS toxicologist who also worked on the 2003 R&D project that originally developed many of the basic LC/MS/MS methods in use for casework at GBI-DOFS. Each workshop was attended by four to six scientists.

Day 1 of the workshop consisted of lectures in the theory and application of LC/MS/MS instrumentation, especially as it related to the Applied Biosystems Model 2000 or 3200 QTRAP® LC/MS/MS instruments. Days 2 and 3 involved exercises with known samples from the protein precipitation method (McCurdy and Lewellen, 1988), followed by analysis on the LC/MS/MS by

the attendees. Each workshop attendee was provided the opportunity to operate the instruments, learning how to perform calibrations, sample injections, and interpretation of data using the Analyst software on the instrument. There was also ample time for interaction with GBI-DOFS toxicologists to exchange information concerning casework trends and analytical methods. On day 4 and part of day 5 the visiting toxicologists completed the analysis of a set of unknown 'competency' samples prepared by the instructor, instrumental analysis and data interpretation.

A total of 34 scientists from 16 different laboratory systems and 11 different states or United States territories attended these workshops. A listing of attendee affiliations is provided below in Table 1. Each attendee who completed the entire workshop was provided with a Training Attendance certificate. The feedback from the attendees at the conclusion of each workshop was uniformly positive. See Appendices A-B for copies of the training syllabus, Powerpoint® presentations and other pertinent information related to these workshops.

Table 1: Workshop Attendees

Attendee Organization	Number of Attendees
Florida Department of Law Enforcement	2
Texas Department of Public Safety	4
Bexar County Medical Examiner Office, Texas	1
Dallas County, Texas	2
Harris County Medical Examiner Office, Texas	2
Alabama Department of Forensic Sciences	5
Arizona Department of Public Safety	2
Tennessee Bureau of Investigate	4
South Carolina Law Enforcement Division	4
Colorado Department of Public Health	2
Federal Bureau of Investigation	1
Los Angeles County Sheriff Office, California	1
Orange County Sheriff Office, California	1
Forensic Science Institute, Puerto Rico	1
Oregon State Police	2
Total	**34**

Literature Cited

McCurdy, H.H. and Lewellen, L.J. (1988) A novel procedure for the analysis of drugs in whole blood by homogeneous enzyme immunoassay (EMIT), *J. Anal. Toxicol.* 12: 260-264.

Chapter 2. THC Analysis

Introduction

According to the National Institute on Drug Abuse, "marijuana is the most commonly used illegal drug in the United States." When prosecuting cases of impaired drivers, that involve marijuana use, solicitors often rely on evidence obtained by a Drug Recognition Expert (DRE) and a corroborating toxicology test result. In Georgia, the DREs have commented that in marijuana cases, a negative toxicology result sometimes conflicts with their observed signs of impairment consistent with marijuana usage in drivers. This conflict is believed to be due in part to the detection limits for COOH-THC of the current solid-phase extraction/gas chromatograph/mass spectrometer (GC/MS) method used and the lack of an analytical procedure for the detection of delta-9-tetrahydrocannabinol (THC) and 11-hydroxy-tetrahydrocannabinol (11-OH-THC) in the GBI-DOFS laboratory. Recent studies indicate that the main active ingredient in marijuana, tetrahydrocannabinol (THC) and its two major metabolites, 11-OH-THC and COOH-THC, can readily be detected using both GC/MS and LC/MS/MS methods (Jamey et al., 2008; Karschner et al., 2009; Maralikova and Weinmann, 2004; Skopp and Pötsch, 2008). However, these methods all rely on some form of selected ion monitoring (SIM) for identification purposes. Although SIM spectra are excellent for identification and quantitation, a SIM spectrum has the disadvantage of being more of a challenge for a jury of non-scientists to understand than a full scan mass spectrum. In this study we determined that a simple liquid-liquid extraction/LC/MS/MS method can be used to generate an enhanced product ion scan (EPI) for THC, 11-OH-THC, and COOH-THC without the use of derivatization.

Materials and Methods

Sample preparation

Samples were prepared by adding various concentrations of THC, COOH-THC, and 11-OH-THC to one, two, or three mL of negative blood (American Red Cross blood or packed red cells screened by immunoassay and LC/MS/MS prior to use). THC-d3 and COOH-THC-d9 were used as internal standards at a final concentration of 25 ng/mL. The samples were extracted by the addition of 3 mL 0.25 N acetic acid followed by 5 mL hexane: ethyl acetate (9:1). The samples were then placed on a multi-tube rocker apparatus for 30 min and then centrifuged at 2500 rpm for 5 min. The organic layer was removed and taken to dryness in a water bath evaporator at 75 ºC. Finally, the samples were reconstituted in 100 µL of 50:50 Mobile Phases A and B. The samples were then transferred to LC/MS/MS vials and analyzed.

Sample analysis

Sample HPLC separation was on a PerkinElmer Series 200 autosampler and column oven. The column was a MetaSil Basic RP (3 µm, 50 x 2.0 mm). Mobile Phase A (MPA) – 0.1% formic acid, 1 g/L (~15mM) ammonium formate in optima grade water; Mobile Phase B (MPB) – 0.1% formic acid, 1 g/L (~15mM) ammonium formate in optima grade methanol. All mobile phases were degassed prior to use. The MS analysis was done on an Applied Biosystems 3200

QTRAP® using TurboIonSpray in positive mode (Herrin, McCurdy, & Wall, 2005). The 1.4.1 version of the Analyst software was used.

Results

Sample Separation and Analysis

Samples containing 50 ng/mL THC, 11-OH-THC, and COOH-THC, and 60 ng/mL THC-d3 and COOH-THC-d9 internal standards were analyzed. Initially a flow rate of 200 µL/min and an injection volume of 20 µL were used with the mobile phase profiles shown in Table 2. Profile 1 is used for the majority of the analysis procedures in our laboratory. The retention times for THC, THC-d3, 11-OH-THC, COOH-THC, and COOH-THC-d9 were 18.55 min, 18.50 min, 17.03 min, 17.35 min, and 17.30 min, respectively. Profile 2 was generated in order to shorten the retention times. The new retention times for THC, THC-d3, 11-OH-THC, COOH-THC, and COOH-THC-d9 were 8.43 min, 8.41 min, 6.91 min, 7.09 min, and 7.05 min, respectively.

The Analyst 1.4.1 software was used to determine compound parameters and to select single transitions for THC (315>193), THC-d3 (318>196), COOH-THC (345>299), COOH-THC-d9 (354>336), and 11-OH-THC (331>193). Both multiple reaction monitoring (MRM) and linear ion trap (LIT) experiments were conducted on each sample (Herrin, McCurdy, & Wall, 2005). All analyses were performed on replicate injections from a single sample resulting in qualitative (EPI) and quantitative (MRM) results. The LIT mode allowed for the generation of enhanced product ion (EPI) scans (Figures 1-5).

Linearity, Limit of Detection, and Limit of Quantification

The Limit of Detection (LOD) was established by determining at which concentration the analyte response is greater than 3 times the baseline of a blank blood sample and gives an Enhanced Product Ion scan. The LOD values were found to be 1.5 ng/mL for THC and 11-OH-THC and 2.0 ng/mL for COOH-THC.

In order to establish the range of linearity, calibration curves for THC, 11-OH-THC, and COOH-THC were generated on three separate occasions using the following concentrations 0, 2, 5, 10, 20, 50, and 100 ng/mL. THC-d3 and COOH-THC-d9 were used as internal standards with THCA-d9 being used as the internal standard for both COOH-THC and 11-OH-THC. The internal standard concentrations were 25 ng/mL. The single transitions listed above were used for detection. All calibration curves generated were linear from 2 to 100 ng/mL and have r^2 values > 0.98 (Figures 6-8). The Limit of Quantitation (LOQ) was established at 2 ng/mL by using similar calibration curves to quantify 10 control samples on three separate occasions (Table 3). Control samples at 10 ng/mL were also analyzed (Table 4).

Concordance Study

Once the LOD, linear range, and LOQ were established, a concordance study began in which five actual case blood samples were analyzed for all three analytes by LC/MS/MS and for COOH-THC only by the current GC/MS method that is used in our laboratory. All three analytes were detected in all five case samples by LC/MS/MS (Table 5). The COOH-THC concentrations were higher than the highest calibrator of 100 ng/mL in four of the five samples by LC/MS/MS and three of the five by GC/MS (Table 5). The recovery of the internal standards in the case samples was 1/3 of that of the calibrator and internal control samples using the LC/MS/MS method (data not shown). We attributed this difference to matrix effects. This

difference was not observed for the GC/MS method.

Discussion

Our laboratory currently uses an LC/MS/MS method that can identify and quantify over 130 prescription and over-the-counter medications in whole blood (Herrin, McCurdy, & Wall, 2005). Most of these medications are identified by full scan mass spectrum. In this study we set out to determine if this same procedure, with minimal modifications, could be used to simultaneously identify and quantify THC and its two major metabolites, 11-OH-THC and COOH-THC. The results presented above indicate that this method has the potential to be extended to include the analysis for THC, 11-OH-THC, and COOH-THC. However, due to matrix effects we have not been able to determine the true LOD, LOQ, or linear range that this method is capable of producing. Since it is difficult to obtain large quantities of relatively fresh whole blood to use for analysis we have been using a 1:4 dilution of packed red blood cells. While this dilution has proven to be suitable for other procedures used within our laboratory, the differences observed for the recovery of internal standards between case blood samples and the diluted packed red blood cells clearly demonstrate that this approach is unsuitable for cannabinoid analysis via LC/MS/MS. Additional studies are still underway to determine the proper dilution factor required for these particular analytes.

The method presented here is not optimized specifically for cannabinoid analysis, but the goal was to determine if the well established method already in use could be extended with minimal modifications to include cannabinoids. A method optimized for cannabinoid analysis would drastically change the current method by requiring the use of solid phase extraction, a different LC column, and different LC mobile phases. Such significant modifications would require extensive instrument setup time, limiting the generalized analytical approach using LC/MS/MS.

Figure 1. Typical Enhanced Product Ion (EPI) scan of THC.

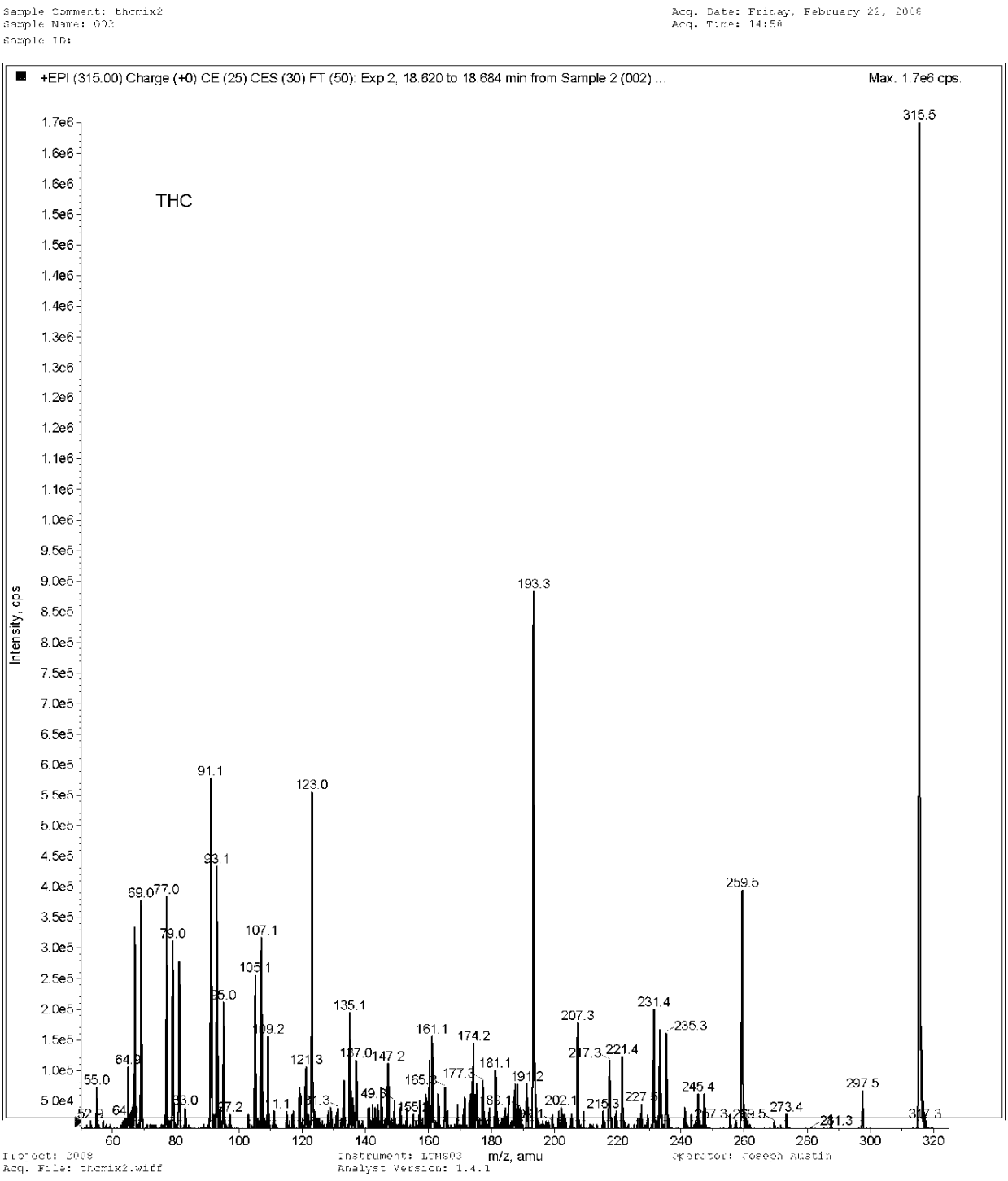

Figure 2. Typical Enhanced Product Ion (EPI) scan of 11-OH-THC.

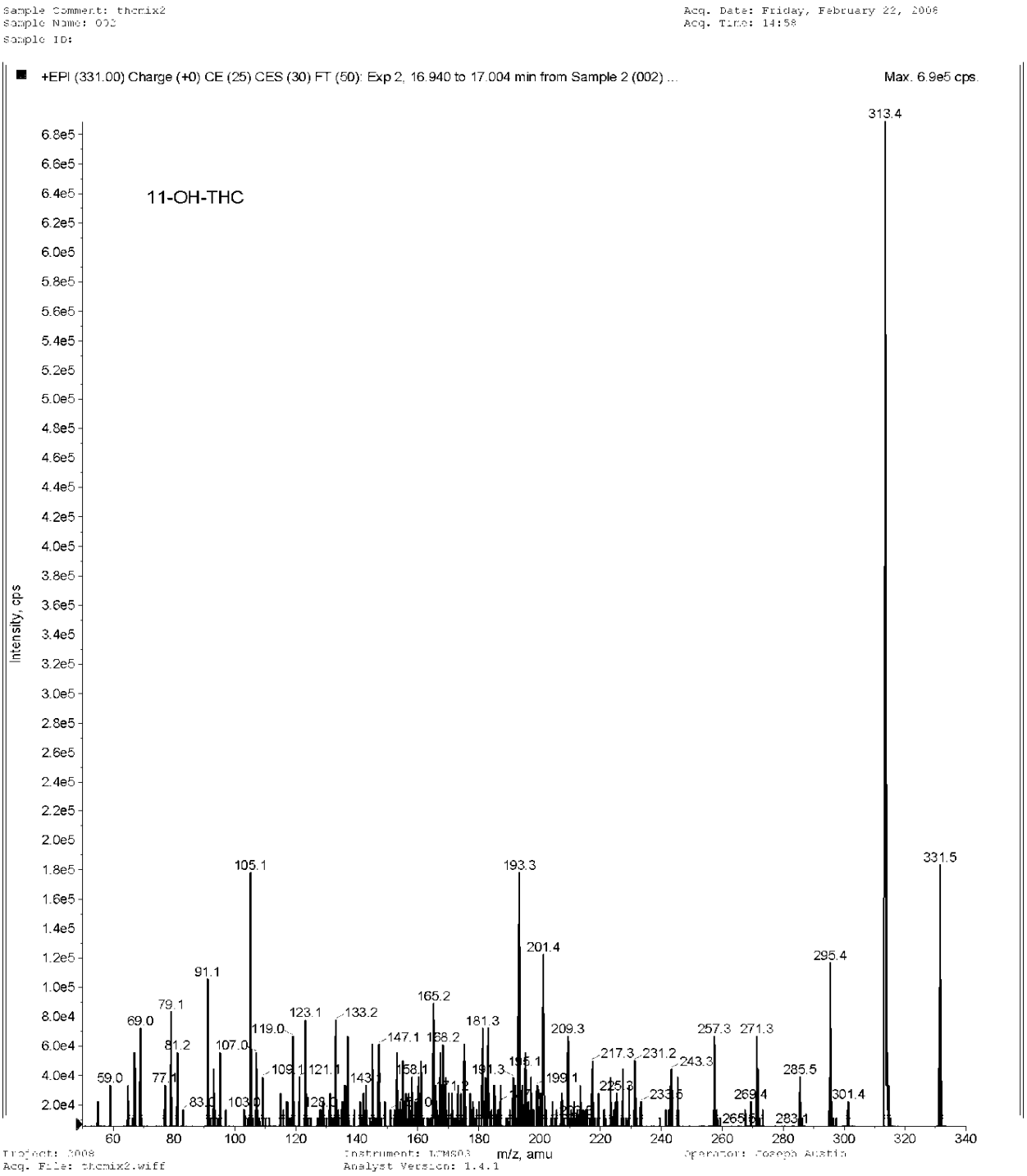

Figure 3. Typical Enhanced Product Ion (EPI) scan of THCA.

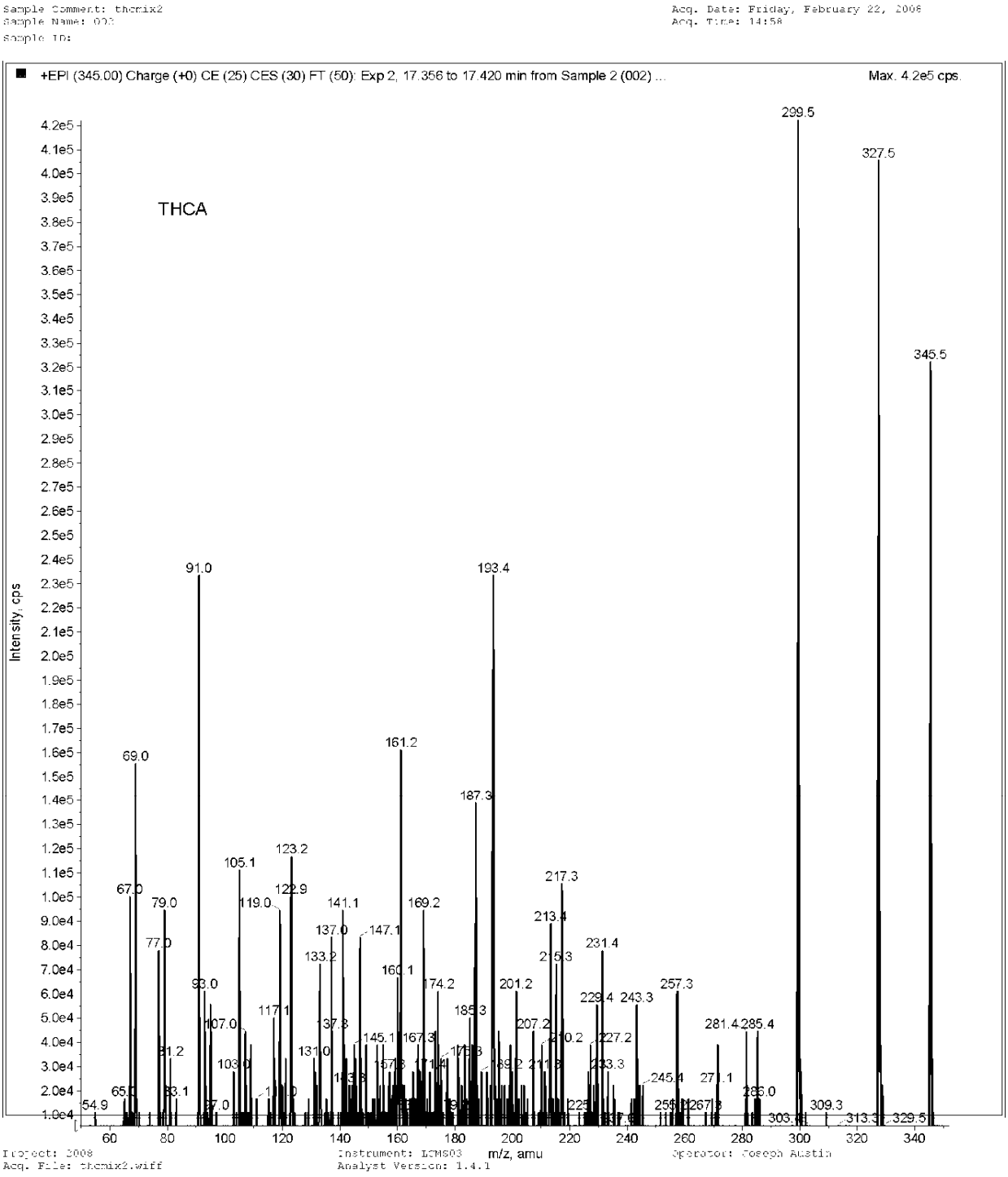

Figure 4. Typical Enhanced Product Ion (EPI) scan of internal standard THC-d3.

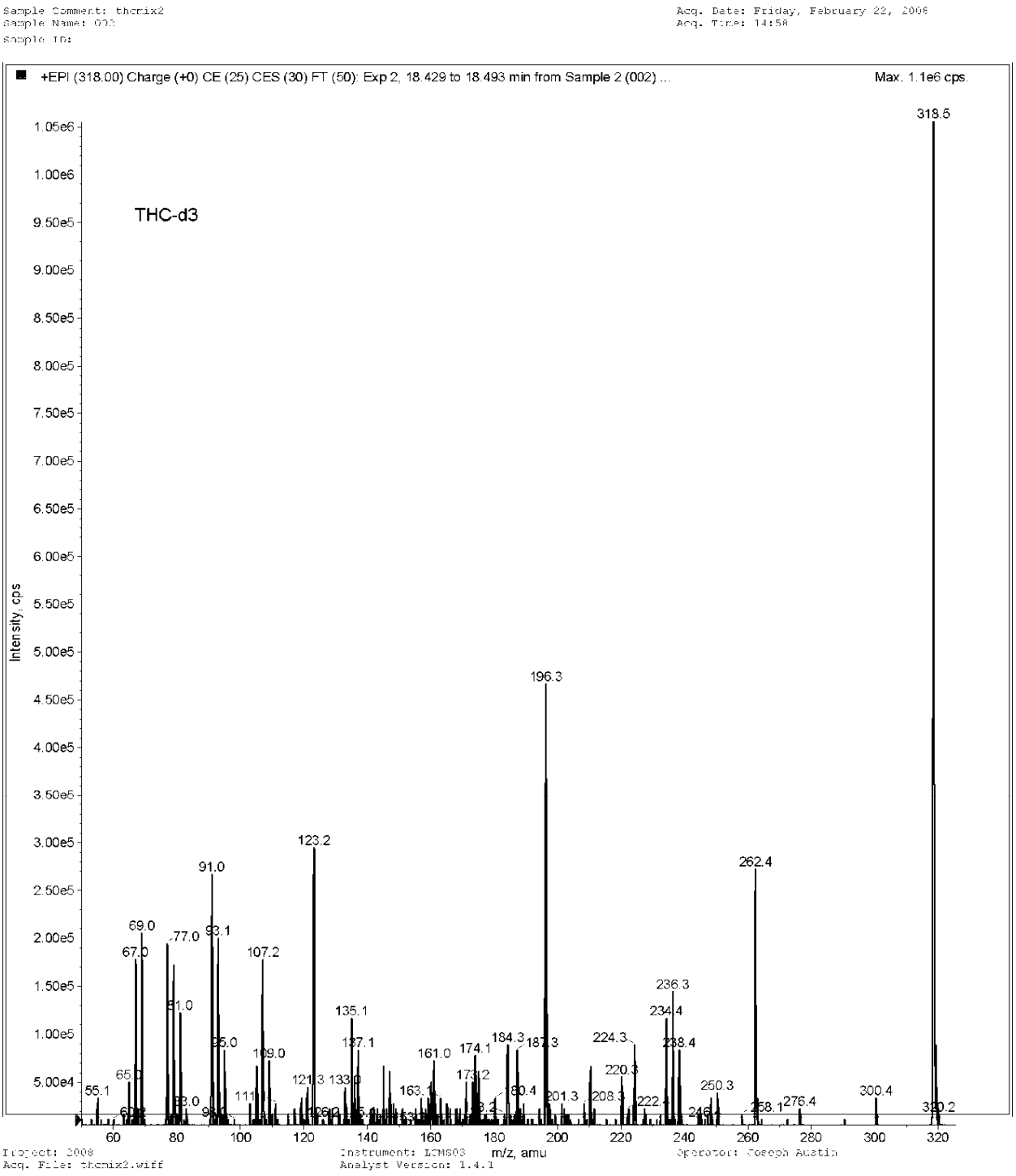

Figure 5. Typical Enhanced Product Ion (EPI) scan of internal standard THCA-d9.

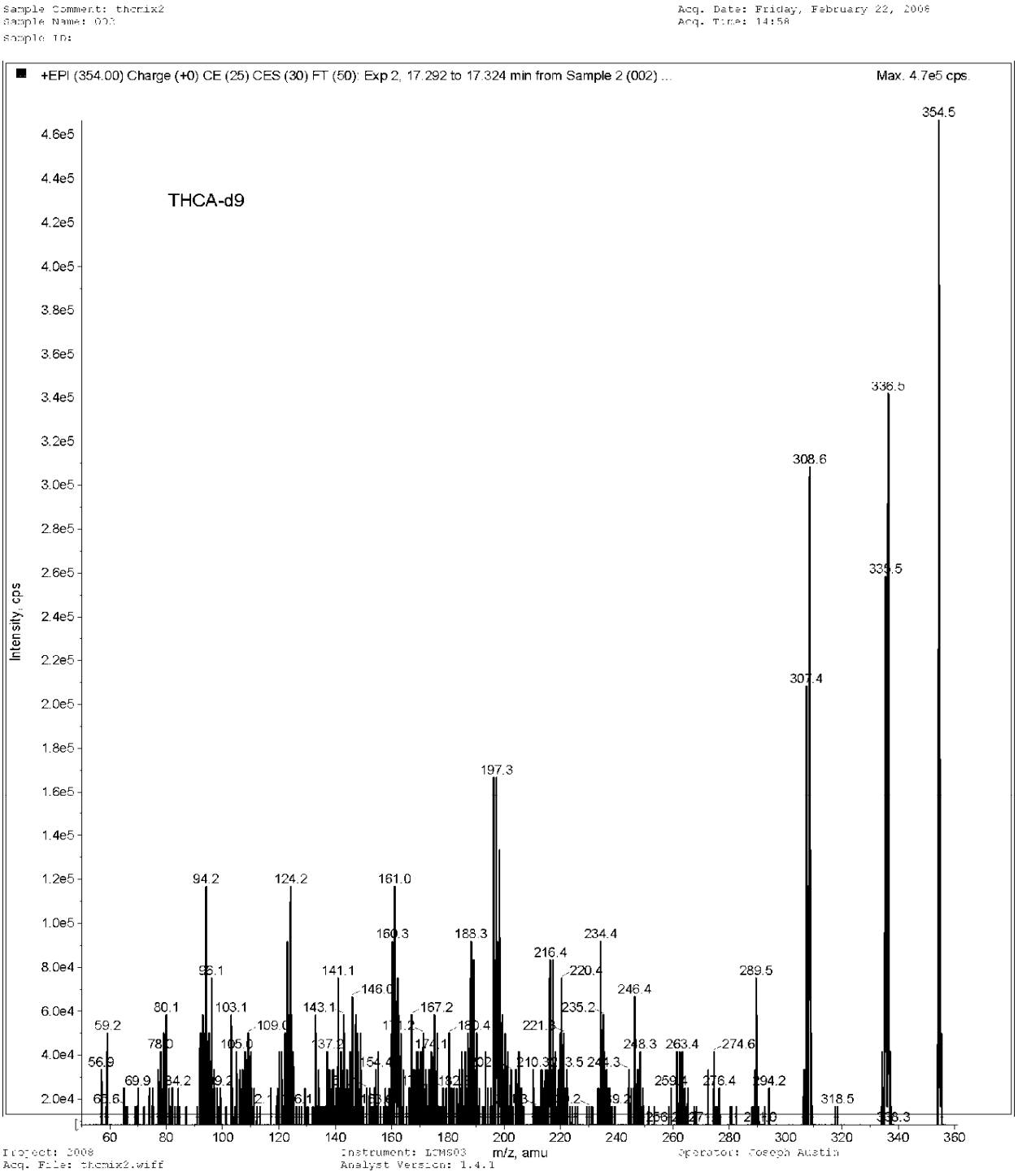

Figure 6. Typical Calibration Curve for THC.

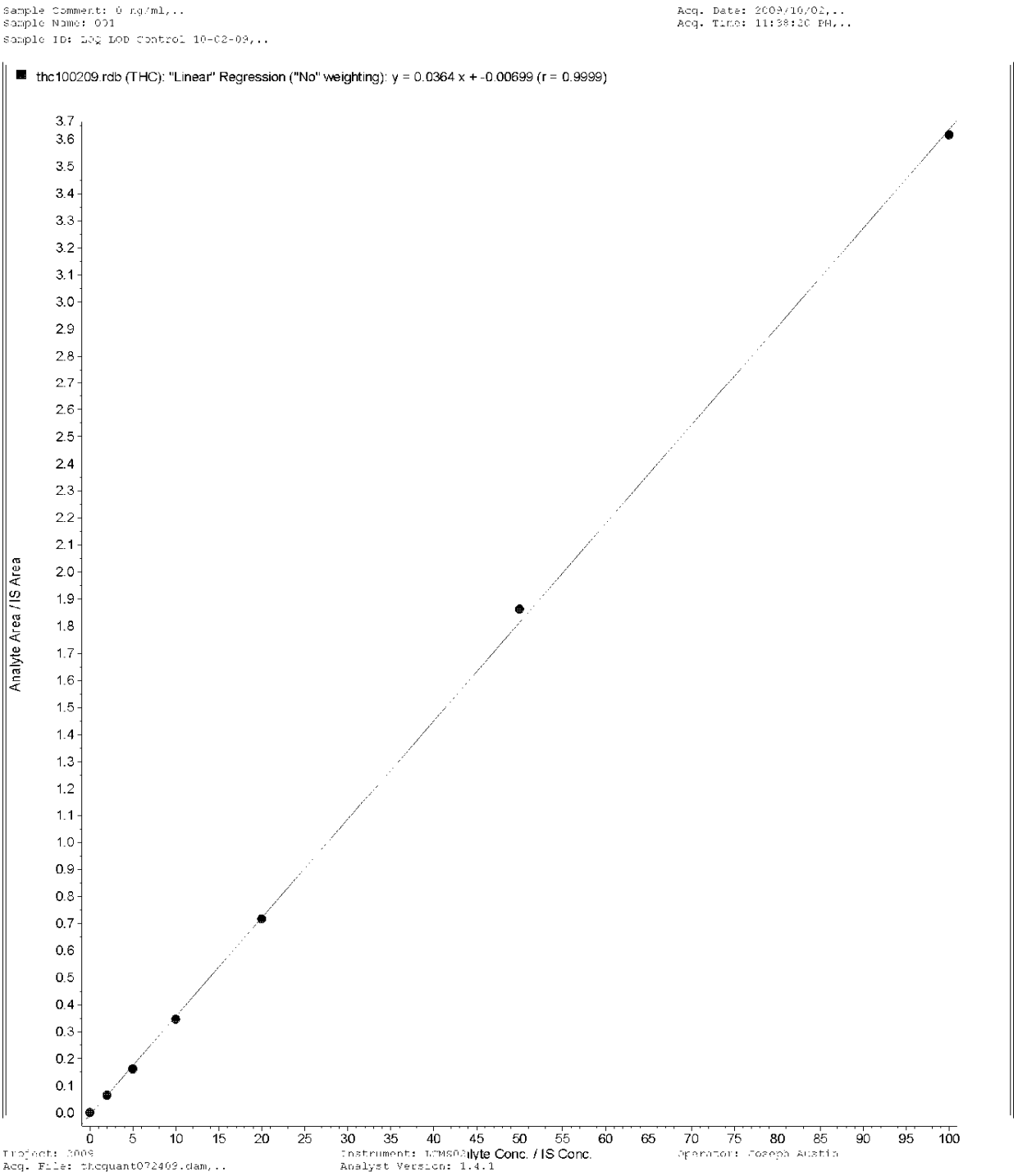

Figure 7. Typical Calibration Curve for 11-OH-THC.

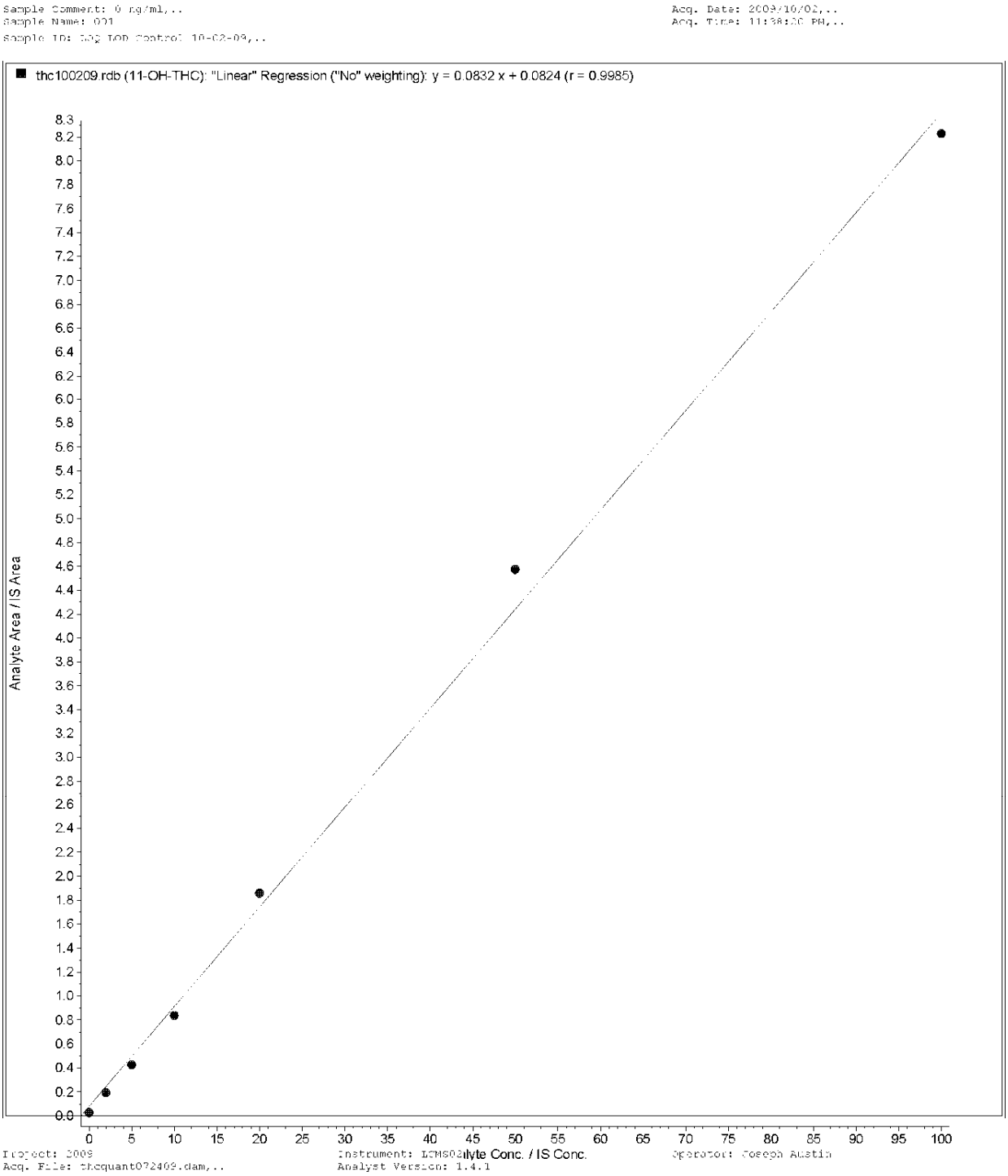

Figure 8. Typical Calibration Curve for THCA.

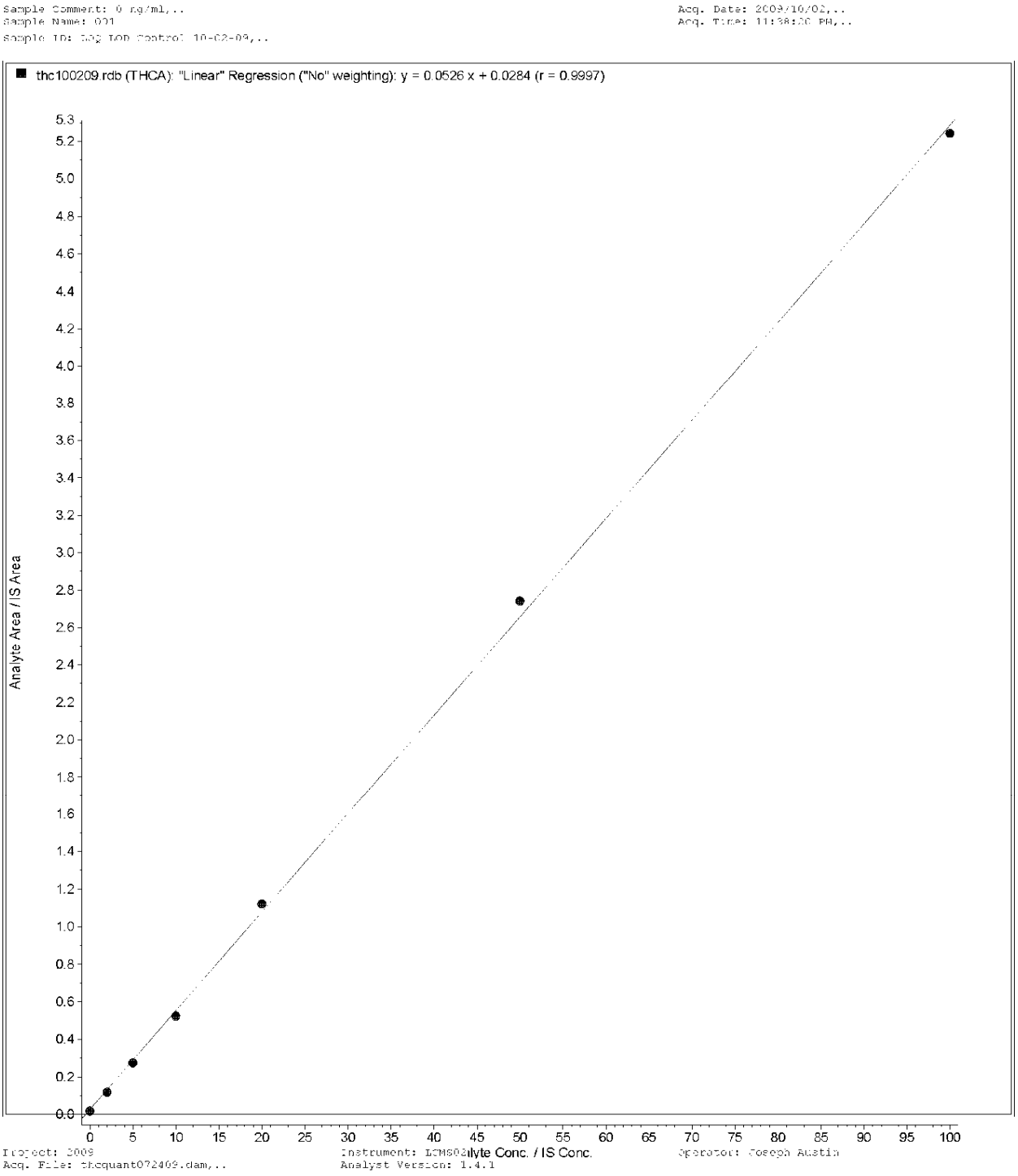

Table 2. Mobile phase profiles for THC Analysis.

Mobile Phase A and B %	Time (min) Profile 1	Time (min) Profile 2
95:5	0-1	0-0.1
20:80	N/A	0.1-4
5:95	1-17.9	4-15.9
95:5	18-20	16-18

Table 3. Data for THC Limit of Quantitation study

	THC	11-OH-THC	COOH-THC
Average	2.02	1.6	1.75
SD	0.37	0.43	0.25
CV	0.18	0.27	0.15
Min	1.397	1.024	1.41
Max	2.969	2.304	2.29

All values are given in ng/mL. The sample size was n=30 and the expected concentration of each analyte was 2 ng/mL.

Table 4. Data for THC control study

	THC	11-OH-THC	COOH-THC
Average	10.05	9.21	9.36
SD	0.52	0.56	0.70
CV	0.052	0.061	0.075
Min	9.164	8.095	8.115
Max	11.184	10.25	11.143

All values are given in ng/mL. The sample size was n=30 and the expected concentration of each analyte was 10 ng/mL.

Table 5. THC concordance data.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
THC	16.293	8.033	12.526	37.554	3.946
11-OH-THC	7.166	5.535	5.999	18.119	1.213
COOH-THC	102.758* (64.89)	178.071* (136.11)*	187.482* (141.05)*	225.627* (221.13)*	49.746 (20.56)

* The highest calibrator is 100 ng/ml.

The data shown above is from case blood samples that had been previously analyzed using GC/MS. All concentrations are in ng/mL. The GC/MS results are in parentheses

Literature Cited

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Karschner, E.L., Schwilke, E.W., Lowe, R.H., Darwin, W.D., Herning, R.I., Cadet, J.L., and Heustis, M.A. (2009) Implications of Plasma, D^9-Tetrahydrocannabinol, 11-Hydroxy-THC, and 11-nor-9-Carboxy-THC Concentrations in Chronic Cannabis Smokers, *J. Anal. Toxicol.* **33:** 469-477.

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Skopp, G., and Pötsch, L. (2008) Cannabinoid Concentrations in Spot Serum Samples 24-48 Hours After Discontinuation of Cannabis Smoking, *J. Anal. Toxicol.* **32:** 160-164.

Chapter 3. Opioid Analysis

Introduction

Opioid testing using certain liquid chromatography–electrospray ionization tandem mass spectrometry (LC/MS/MS) methods can prove difficult with the challenges posed by ion suppression in early eluting compounds (Matsuszewski, Constanzer, & Chavez-Eng, 2003; Dams and Huestis, 2003). Many previous studies employ the use of solid phase extractions to prepare samples for LC/MS/MS opioid analysis (Asmari and Anderson, 2007; Fernandez et al., 2006; Maralikova and Weinmann, 2004; Coles et al., 2007; Maurer, 2005; Dienes-Nagy et al., 1999; Naidong et al., 1999). The protein precipitation sample preparation method that was used in this study has the potential to decrease the costs and time involved with solid phase extractions. It has been shown to work successfully in detection of heroin related opiates in previous literature (Calleux et al., 1999), but has posed some difficulties in wide panel LC/MS/MS screens (Herrin, McCurdy, & Wall, 2005).

Materials and Methods

Instrumentation

The instrument used for this experiment was an Applied Biosystems QTRAP® 3200, with a Perkin Elmer Series 200 HPLC system which was equipped with an autosampler, vacuum degasser, and a column oven set to 35°C. Curtain, source, and exhaust gases were produced by a PEAK gas generator. The software used on the instrument and for analysis of data was Analyst 1.4.1.

Chromatography

Chromatography was performed using a Phenomenex Synergi RP 4 µ 2x150 mm column, with gradient elution performed using Mobile Phase A as a ~15mM ammonium formate buffer in Optima grade water (pH 3) and Mobile Phase B as Optima grade acetonitrile.

Biological Specimens

The analysis was performed with the controls and calibrators being made up in a solution of purchased Red Cross whole blood screened negative by enzyme immunoassay and a wide panel LC/MS/MS screen. The sample analysis for the concordance study between GC/MS and LC/MS/MS results were performed on samples originating from both postmortem and traffic violation cases.

Sample Preparation

Results were observed for both detection and quantification using a quick and simple acetone precipitation procedure, developed initially for enzyme immunoassay and adapted for LC/MS/MS analysis in later publications (Lewellen and McCurdy, 1988; Herrin, McCurdy & Wall, 2005). This procedure requires a 1 ml aliquot of whole blood sample for analysis with addition of 2.5 mL of acetone (vortexing samples during acetone addition), let stand for ten minutes then vortex for approximately 15 seconds, centrifuge samples for 10 minutes, decant supernatant through reservoirs into test tubes containing a glass boiling bead, rinse reservoirs

with 0.5 mL of acetone, remove reservoirs, dry down samples at 75°C for 20 minutes, reconstitute with 1 mL of 97% mobile phase A (ammonium formate buffer) and 3% mobile phase B (acetonitrile) solution, vortex samples until residue is suspended, centrifuge for 10 minutes, and transfer samples to LC/MS/MS vials for analysis.

Instrument Parameters

Quantitative analysis was set to detect only parent to fragment (MRM) transitions yielding no structural data. Qualitative analysis was performed using an enhanced product ion (EPI) scan for full mass spectrum identification to a library match (Figures 9-13). All analysis was performed with the instrument in positive mode with the ionization process being performed using ESI. All analyses were performed on replicate injections from a single sample resulting in qualitative (EPI) and quantitative (MRM) results. See Table 6 for the MRM transition list and specific instrument parameters for each drug or metabolite.

Results

Optimization of Instrumentation

The instrument parameters were adjusted to optimize sensitivity to the various opioids. Each opioid was infused on the instrument at a 10µL/minute flow rate at concentrations of approximately 1µg/mL to determine the most abundant transitions, collision energy, declustering potential, entrance potential, collision entrance potential, and collision exit potential. Transitions and settings can be found in Table 6.

Chromatography

After literature studies (Asmari and Anderson, 2007; Fernandez et al., 2006; Maralikova and Weinmann, 2004; Coles et al., 2007; Maurer, 2005; Dienes-Nagy et al., 1999; Naidong et al., 1999) and experimental analysis a gradient of Mobile Phase A ~15 mM ammonium formate buffer and Mobile Phase B acetonitrile was decided upon (Table 7). Two columns were tested to determine their capability of detecting opioids. The Xterra (Waters MA) reverse phase C18 3.5 µ 2.1x100 mm column was found to produce good responses and chromatography for the analytes of interest (Figure 14), but the selectivity between hydromorphone and morphine and the selectivity between codeine and hydrocodone was not adequate at levels below 50 µg/L for qualitative distinction between the drugs. The second column tested was a Phenomenex Synergi RP 4 µ 2x150 mm. Because this column produced improved sensitivity and allowed discrimination between morphine/hydromorphone, and between codeine/hydrocodone for qualitative analysis it was chosen for the remainder of experiments (Figure 15).

Region of Ion Suppression

Experiments were conducted to determine the region of ion suppression for the finalized method. Analysis was performed by infusing morphine, at a concentration of approximately 1µg/mL at a flow rate of 10 µL per minute, and injecting a sample of the extracted matrix after the infused morphine had an established baseline. The injection for each matrix sample was at a t0 point of 3.2 minutes on the ion suppression graphs. The fact that morphine has a retention time of approximately 6 minutes leads to the conclusion that morphine would have eluted at the 9.2 minute point of the ion suppression graphs. The morphine baseline signal is shown to be stable in the area where morphine is expected to elute and at least 2 minutes removed from the most suppressed signal between 3.5-6 min, demonstrating the finalized methods ability to overcome

potential ion suppression (Figures 16-18).

Limits of Identification (LOI)

A study was done to determine the limit of identification for the analytes of interest. Spiked blood samples measuring from 2.5 µg/L to 12.5 µg/L of each analyte were tested using the described qualitative procedures. The limit of identification was established as the lowest concentration at which the method triggered a transition for the drug of interest and produced a qualitative full mass spectrum identification to a library match while still providing a signal to noise ratio of 3:1 or higher. Results can be found in Table 8.

Interference Study

An interference study using five different mixtures of drugs was performed to determine method specificity and whether or not any cross interference would be observed using the method. As shown in Figures 19-23, no interference was observed. The method under development was sufficiently specific to proceed with additional studies.

Panel 1: olanzapine, haloperidol, metoprolol, bupropion, diazepam, pentazocine, paroxetine, 2-ethyl-5-methyl-3,3-diphenylpyraline (EMDP), alprazolam, hydroxyzine; (Figure 19)

Panel 2: scopolamine, pentazocine, mesoridazine, dextromethorphan, fentanyl, lorazepam, promethazine, haloperidol, clonazepam, cyclobenzaprine, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), olanzapine; (Figure 20)

Panel 3: olanzapine, meperidine, normeperidine, venlafaxine, zolpidem, diphenhydramine, citalopram, diltiazem, nortriptyline, methadone, mirtazapine, maprotiline, nordiazepam, sertraline; (Figure 21)

Panel 4: tramadol, meperidine, oxcarbazepine, buspirone, midazolam, carbamazapine, doxepin, fluoxetine, propoxyphene, verapamil, benztropine, nefazodone, thioridazine; (Figure 22)

Panel 5: gabapentin, ketamine, lamotrigine, chlordiazepoxide, trazodone, metaxalone, quetiapine, temazepam; (Figure 23)

Limits of Quantiation

To determine the limits of quantitation (LOQ) samples of varying concentrations from 2.5 µg/L to 12.5 µg/L were tested. An internal standard mixture was used that contained the deuterated version of each analyte tested, nalorphine, and mepivacaine. This internal standard solution was created to determine if quantitation by deuterated internal standards had an advantage over mepivacaine or nalorphine for quantitation. Concentrations at which a signal to noise ratio proved to be greater than a 10:1 ratio, were recorded to be the limit of quantification for that drug. Results can be found in Table 8.

Reproducibility

To determine the between extract variability of the established method a series of extractions containing five controls were performed twenty separate days yielding a sample population of n=100. The data was analyzed for each internal standard to determine their respective percent variability. The analysis was conducted with a seven point calibration curve with concentrations from 0 µg/L to 200 µg/L. Controls analyzed were to have an anticipated concentration of 50 µg/L. Results of analysis can be found in Tables 9-11. The average variability was also calculated from the data used to determine the limits of quantitation. The sample population is

n=4 for each individual concentration, and only levels that met the limit of quantitation criteria were used for the variability determination at these low level concentrations (Table 12).

Concordance Study

A concordance study was conducted on a combined sample population of 100 postmortem and traffic violation samples to determine intraday and interday reproducibility, and to determine the correlation of results to an industry standard, gas chromatography mass spectrometry (GC/MS). The concordance study was done after initial analysis of samples was completed by GC/MS to produce the best possible distribution of the five different opioids tested. Samples were initially extracted and tested by LC/MS/MS with two separate quantitative analyses of the same sample to produce both intraday reproducibility and correlation to GC/MS results that were previously obtained. A separate extraction and quantitative analysis was done to determine the interday reproducibility to those values obtained previously by LC/MS/MS. Results can be found in Tables 13-15.

Discussion

Detection and quantitation of morphine, hydromorphone, codeine, oxycodone, and hydrocodone in whole blood samples using a simple precipitation procedure is possible, with the caveat of having two separate analysis of the same sample. Opioid reproducibility similar to those determined from deuterated internal standards of the drugs could be achieved using either mepivacaine or nalorphine as the internal standard. Limits of quantitation for the opioids tested ranged from 2.5 µg/L to 11 µg/L. All opioids tested could be identified using an enhanced product ion scan full mass spectrum at concentrations between 2.5 µg/L and 5 µg/L. Concordance studies show that correlation between GC/MS and LC/MS/MS range from 12-21% (deuterated internal standard (I.S.)), 5-32% (Nalorphine I.S.), and 17-37% (mepivacaine I.S.) difference. Intraday reproducibility ranged from 4-10% (deuterated I.S.), 7-12% (Nalorphine I.S.), and 6-12% (mepivacaine I.S.) difference, and interday reproducibility ranged from 7-10% (deuterated I.S.), 11-23% (Nalorphine I.S.), and 12-21% (mepivacaine I.S.) difference. With the potential cost savings, faster extraction procedures, and the reduced amount of sample consumption, LC/MS/MS is a promising alternative to traditional GC/MS analysis of opioids.

Figure 9. Morphine mass spectrum.

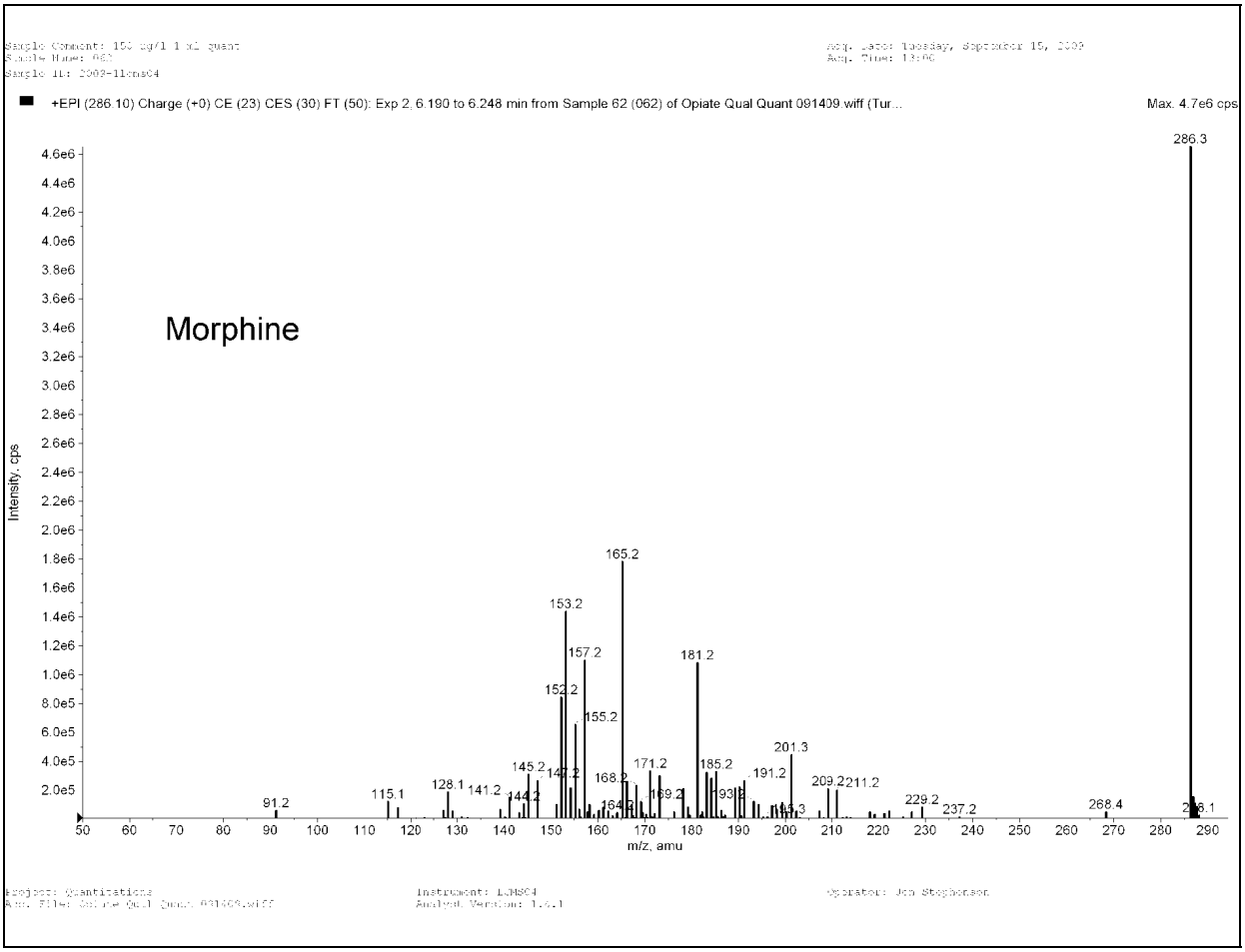

Figure 10. Hydromorphone mass spectrum.

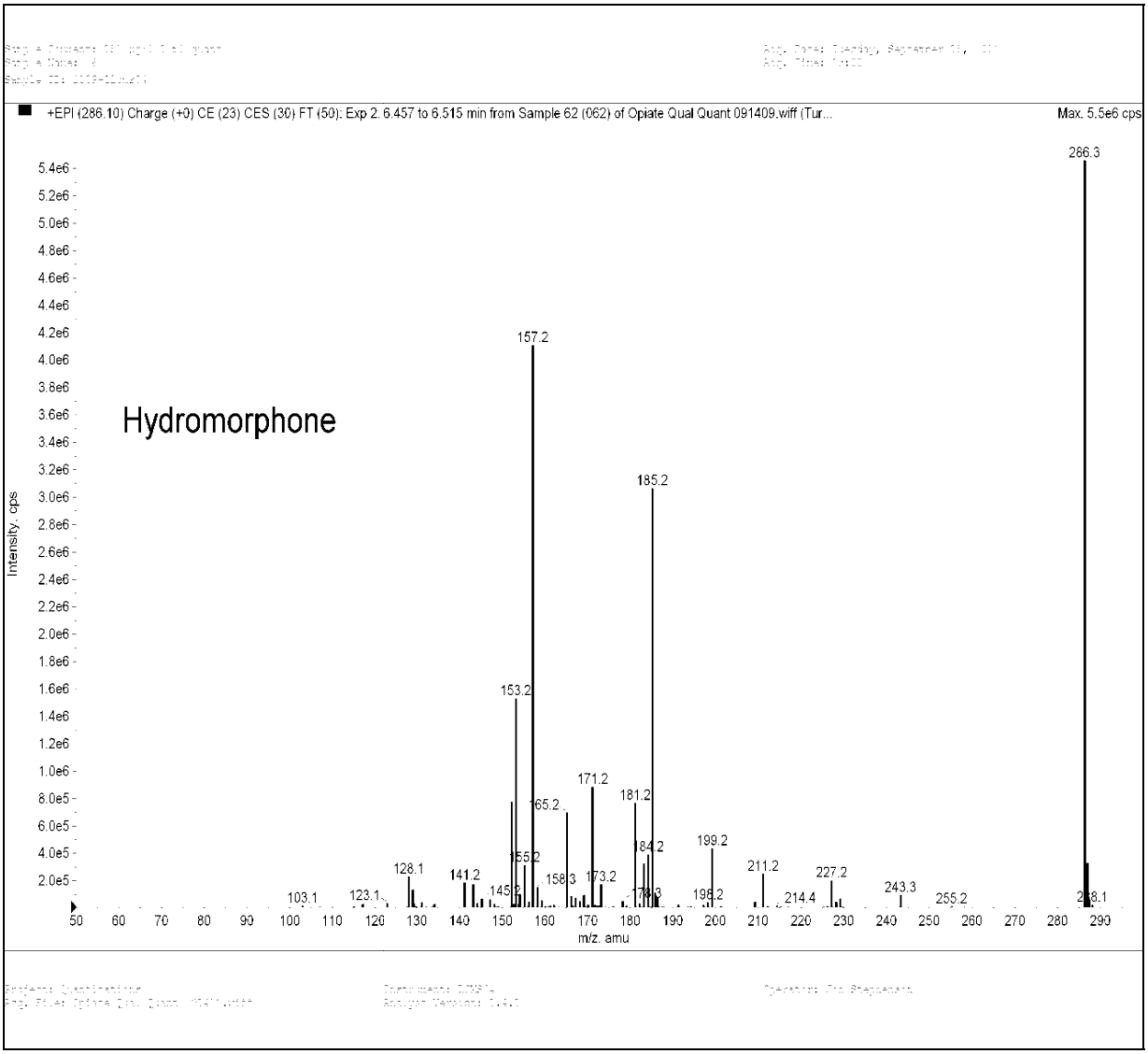

Figure 11. Codeine mass spectrum.

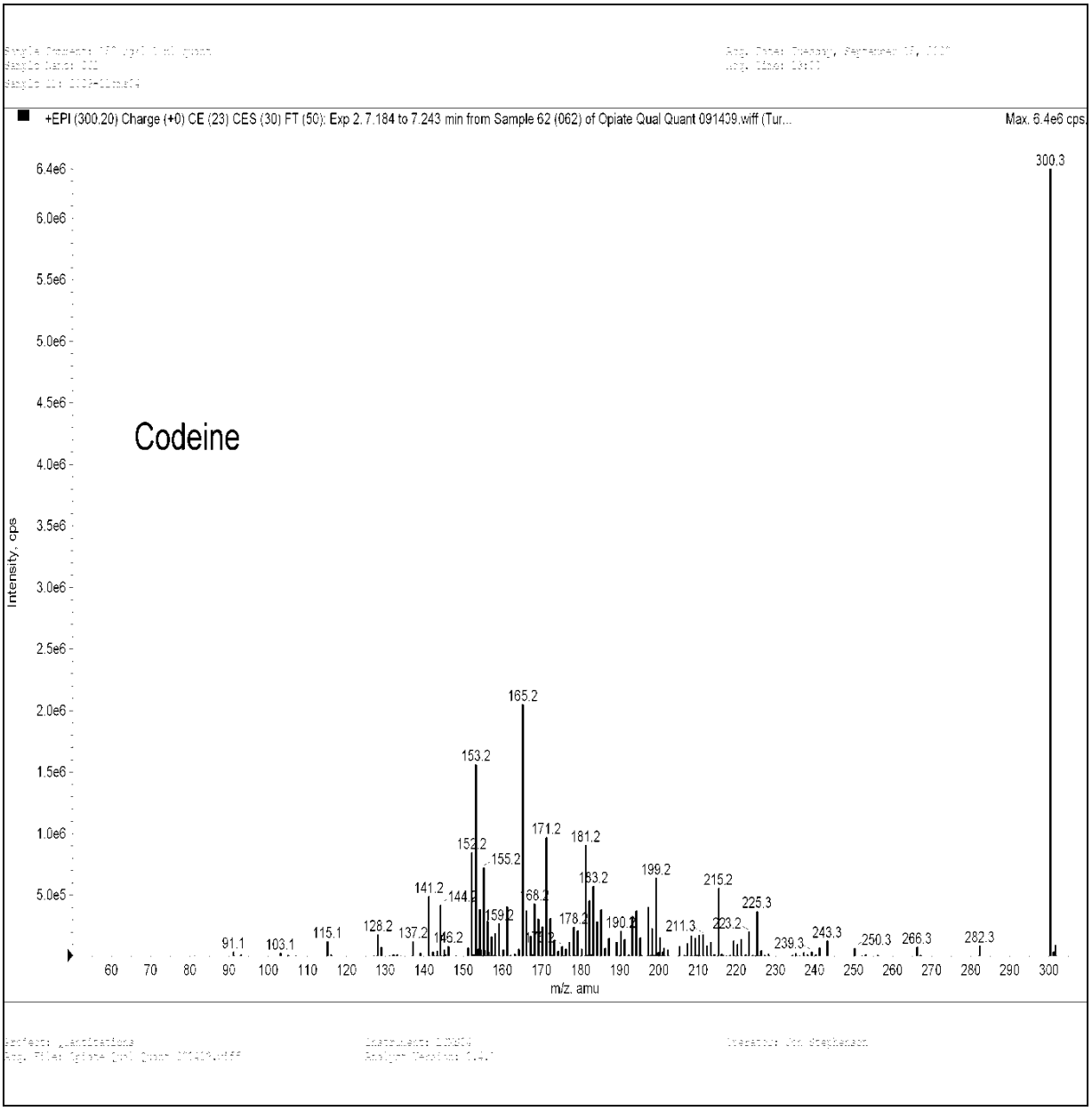

Figure 12. Hydrocodone mass spectrum.

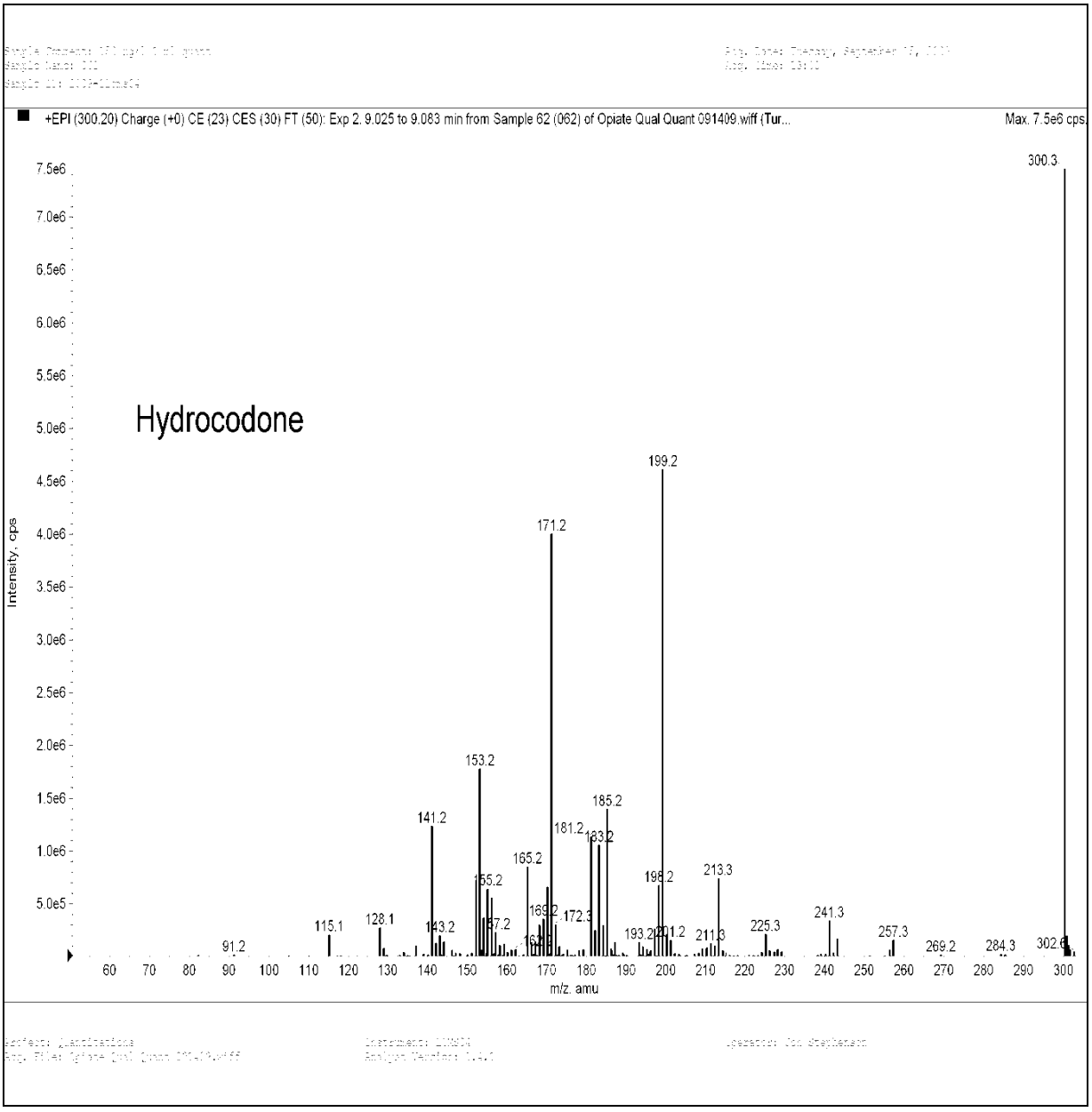

Figure 13. Oxycodone mass spectrum.

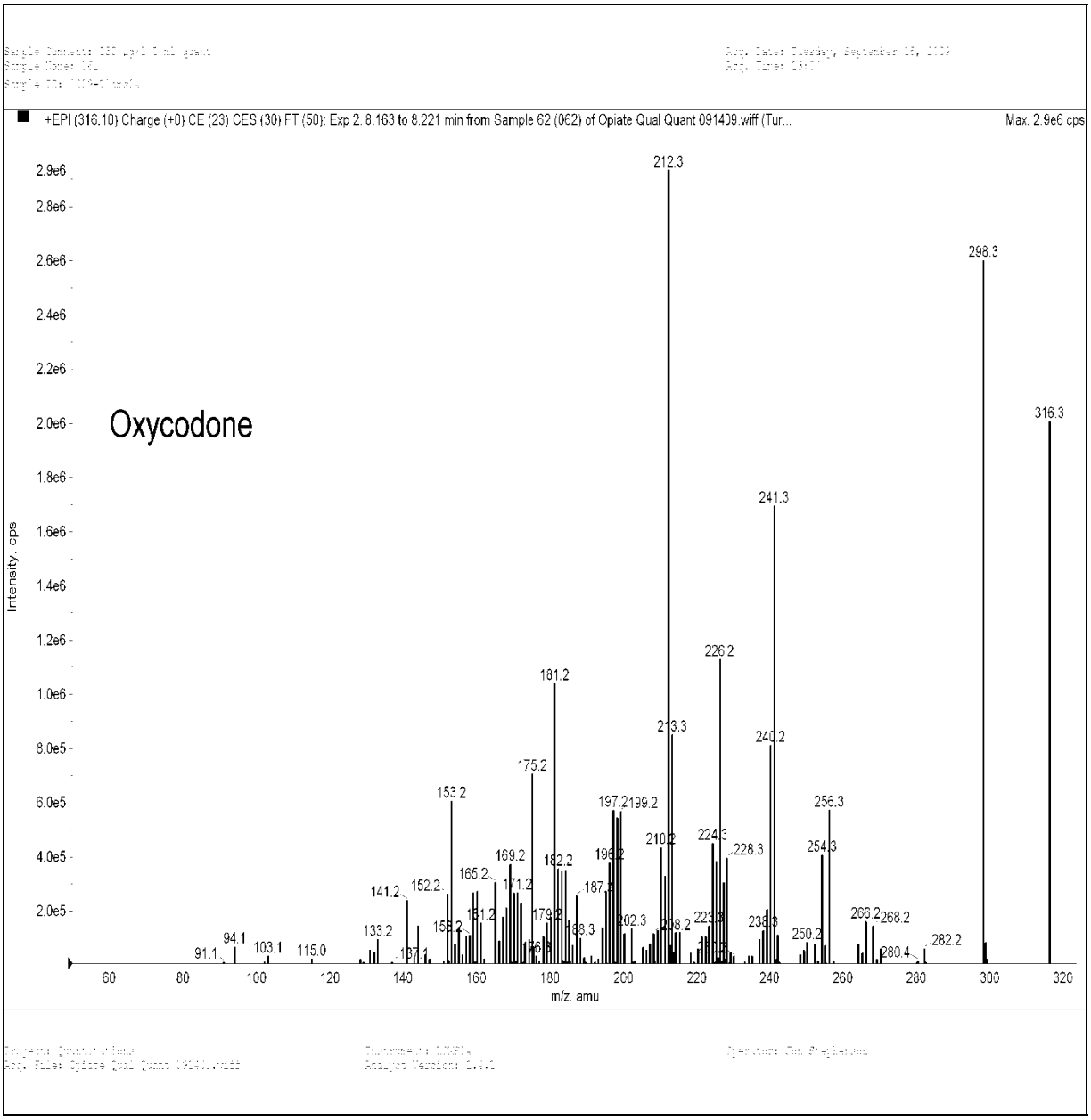

Figure 14. Opioid separation using Xterra column chromatography.

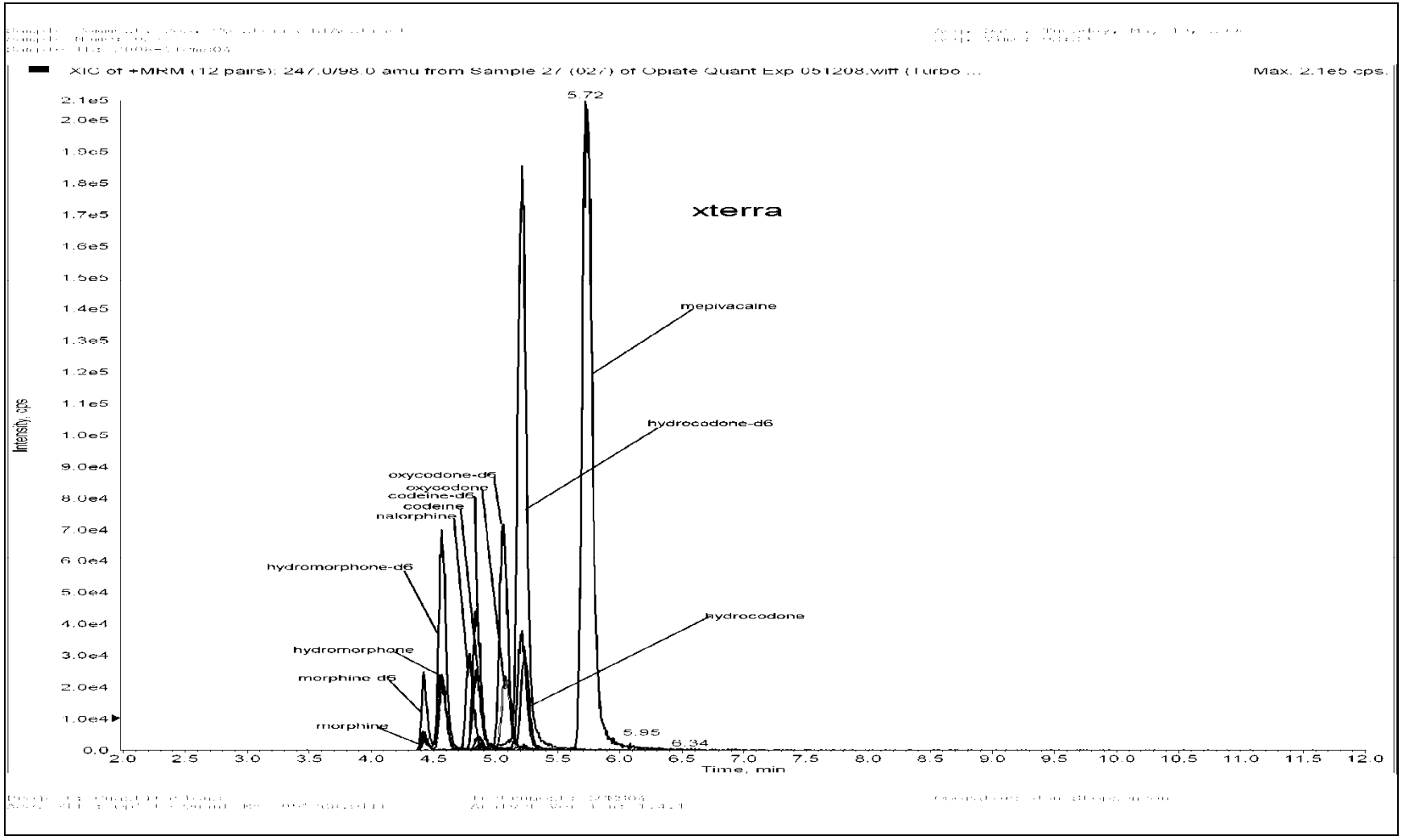

Figure 15. Opioid separation using Synergi column chromatography.

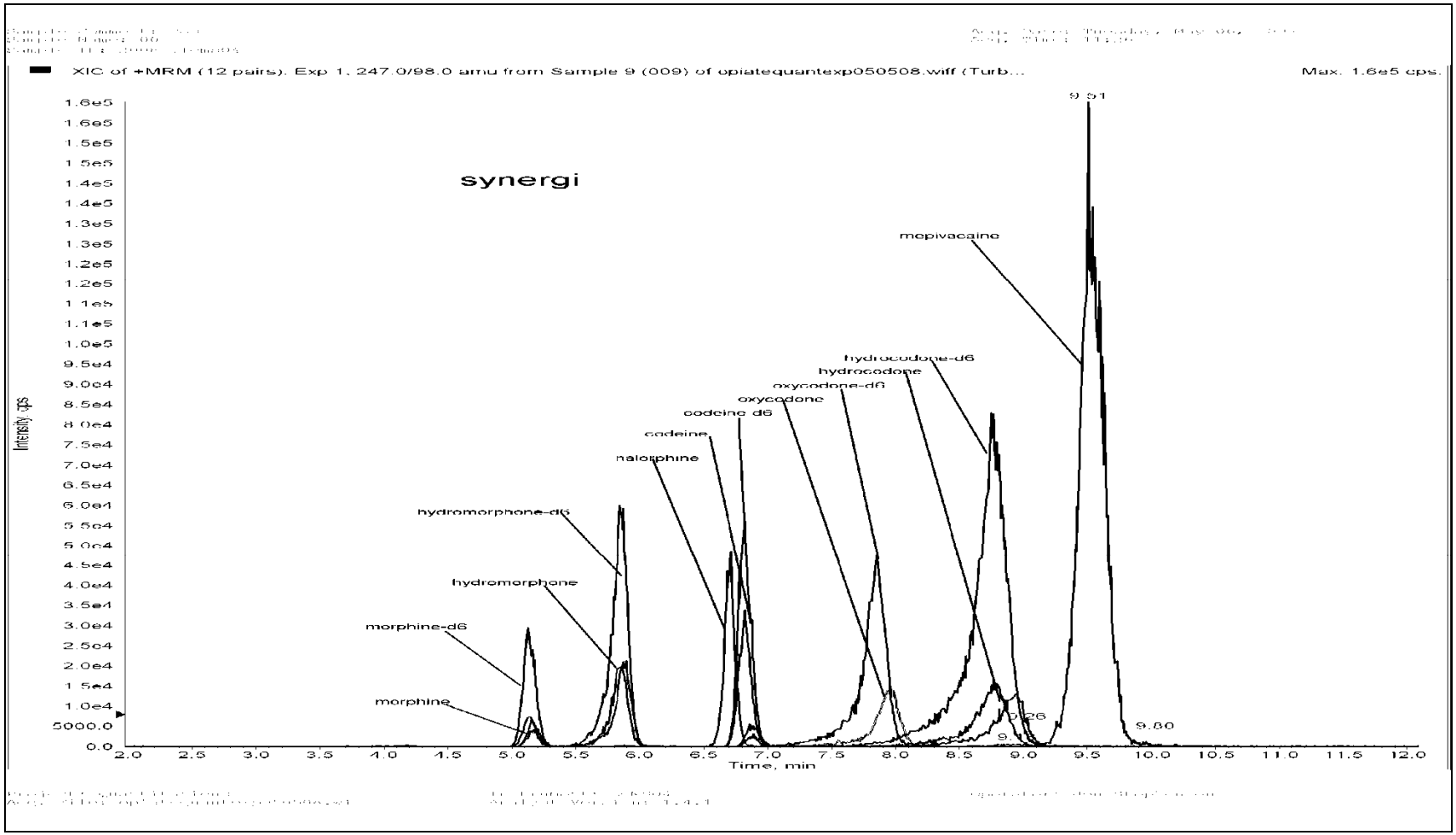

Figure 16. Ion suppression of opioids.

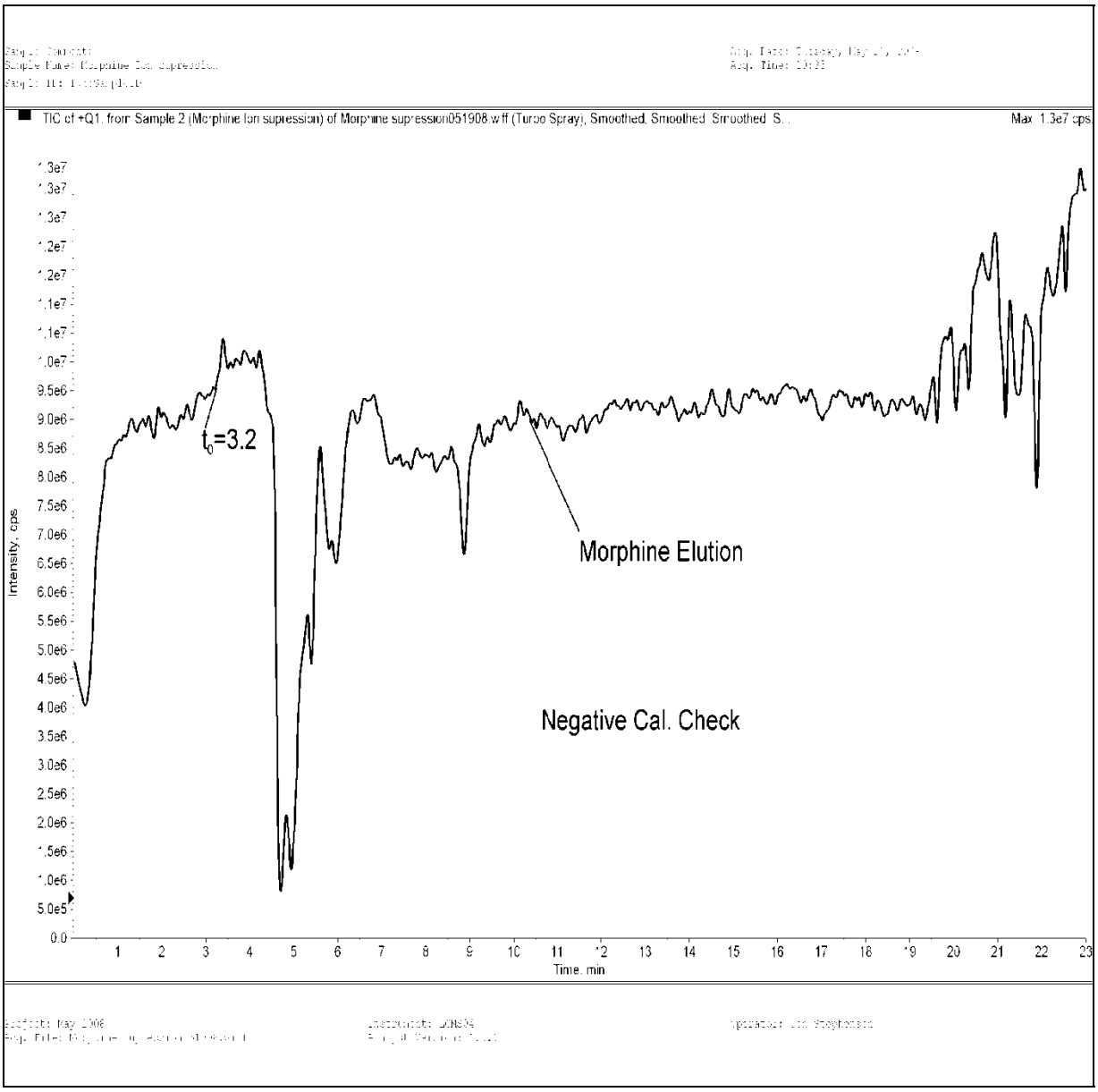

Figure 17. Ion suppression of opioids.

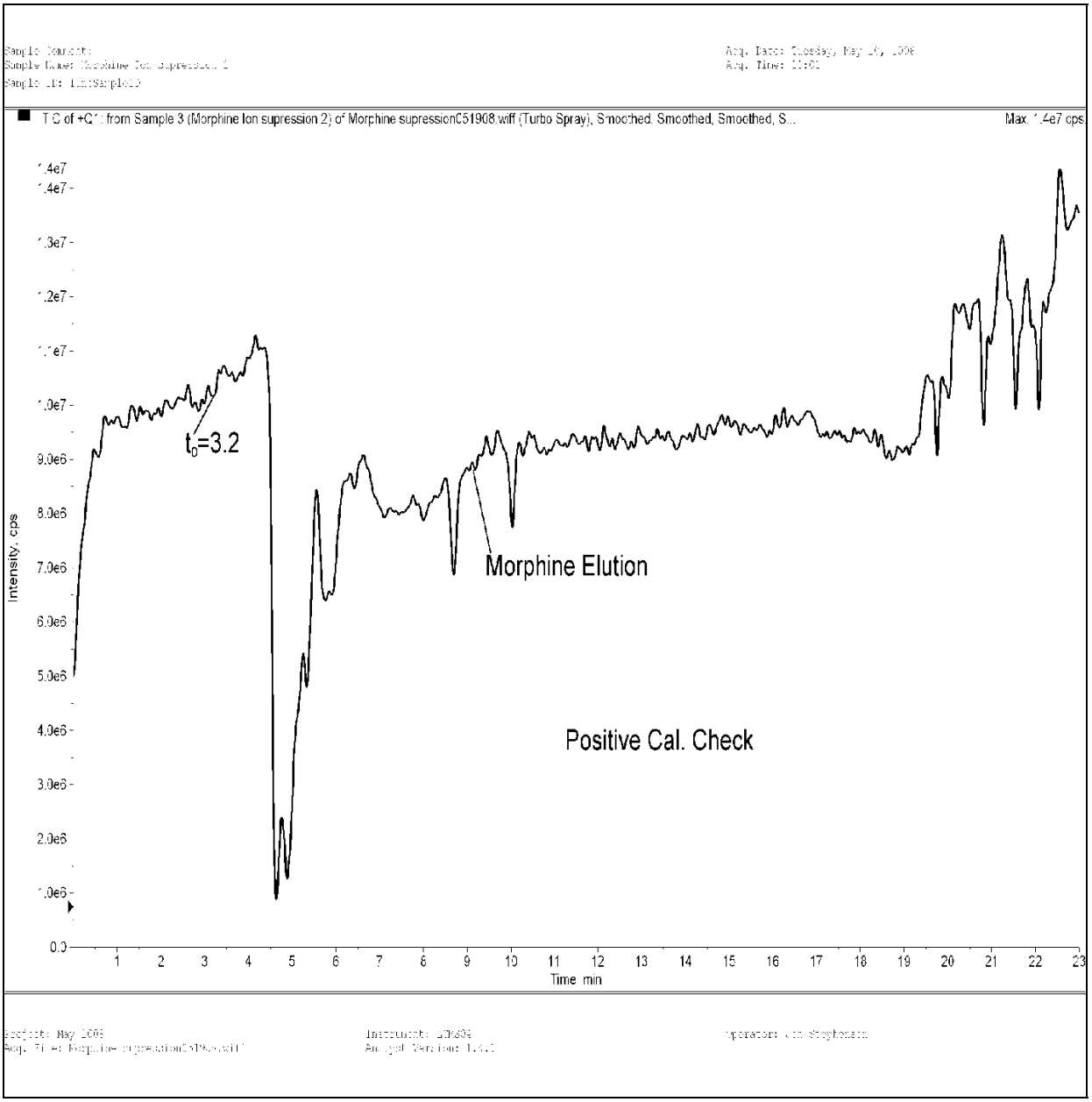

Figure 18. Ion suppression of opioids.

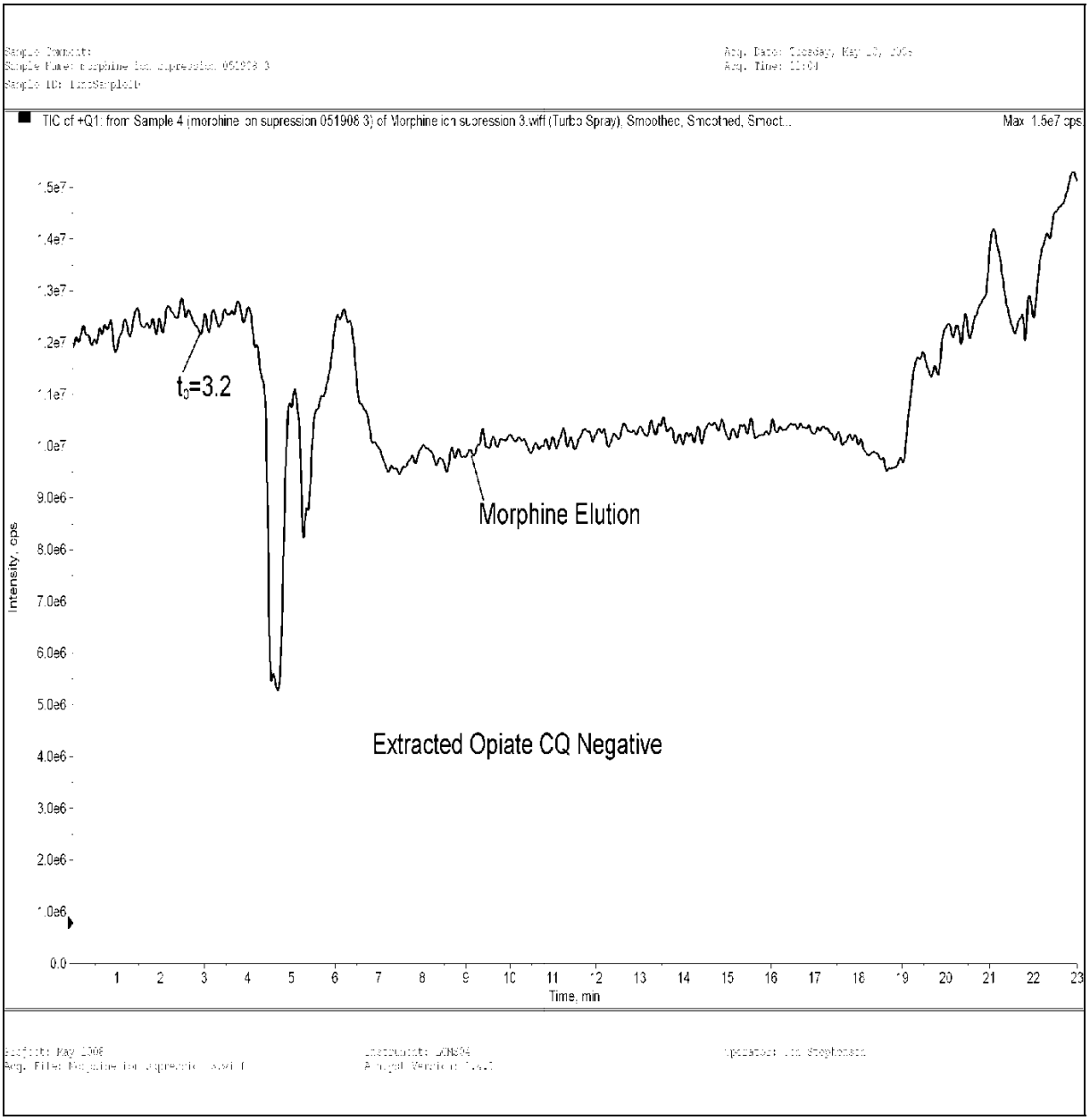

Figure 19. Interference Study Panel 1.

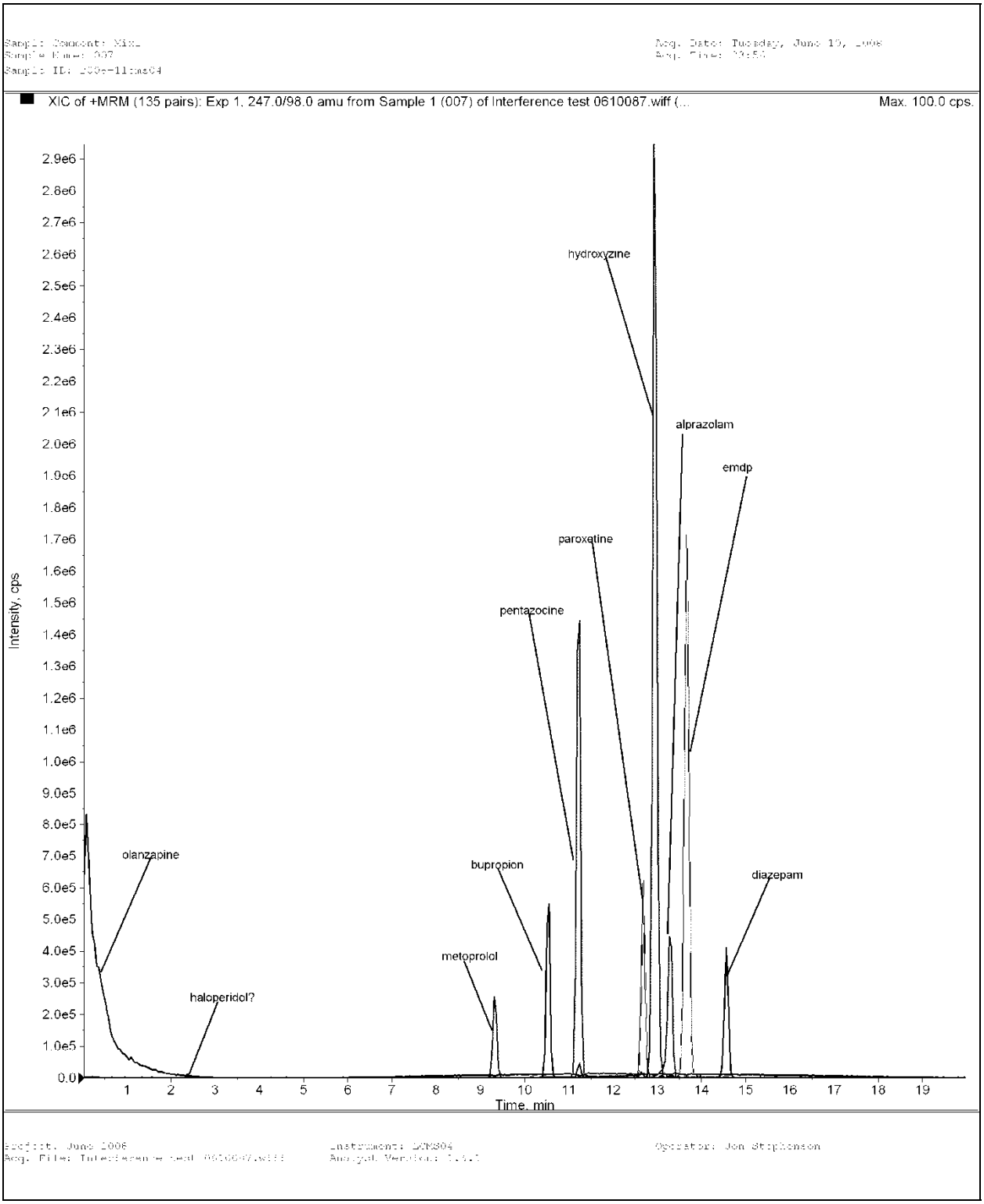

Figure 20. Interference Study Panel 2.

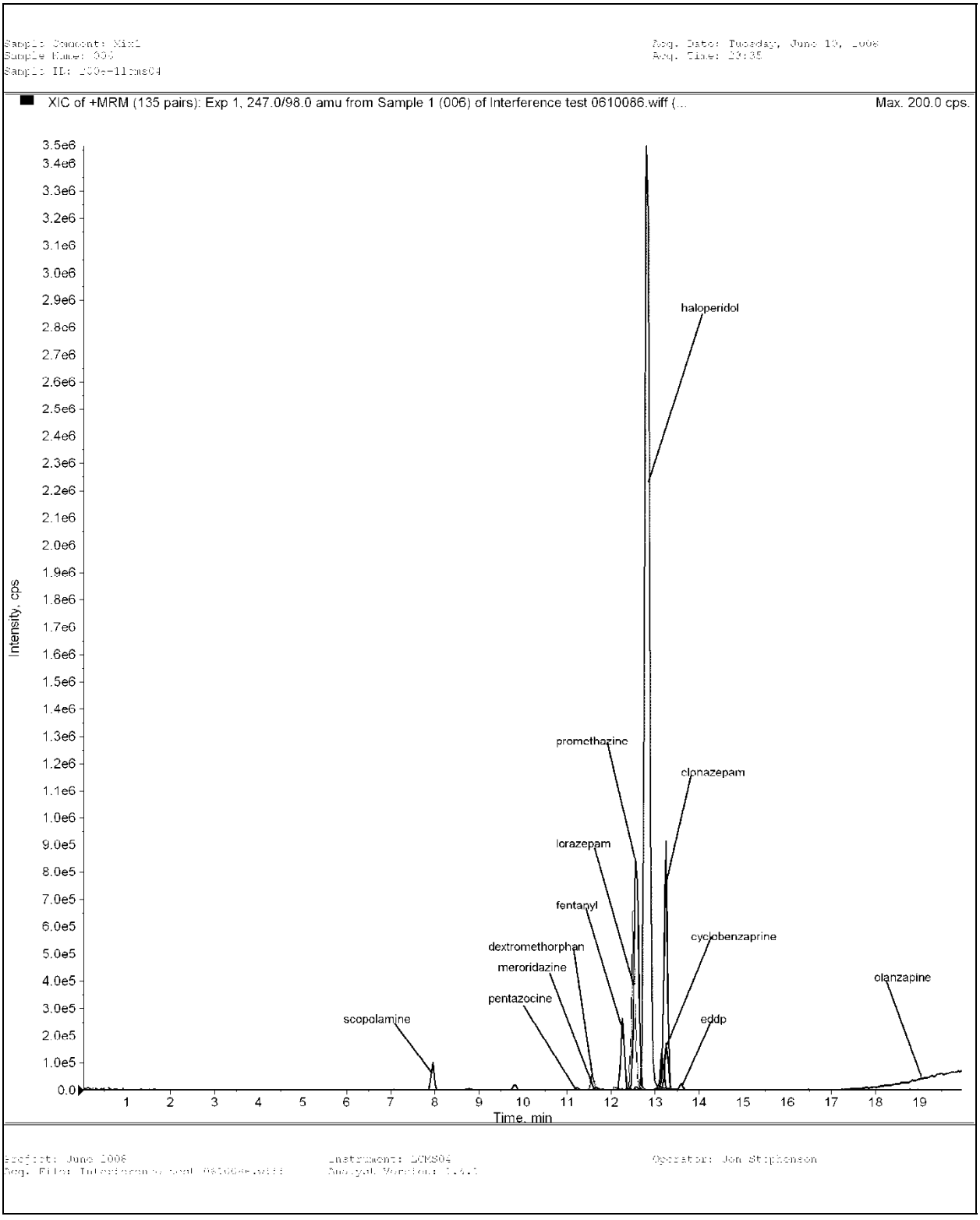

Figure 21. Interference Study Panel 3.

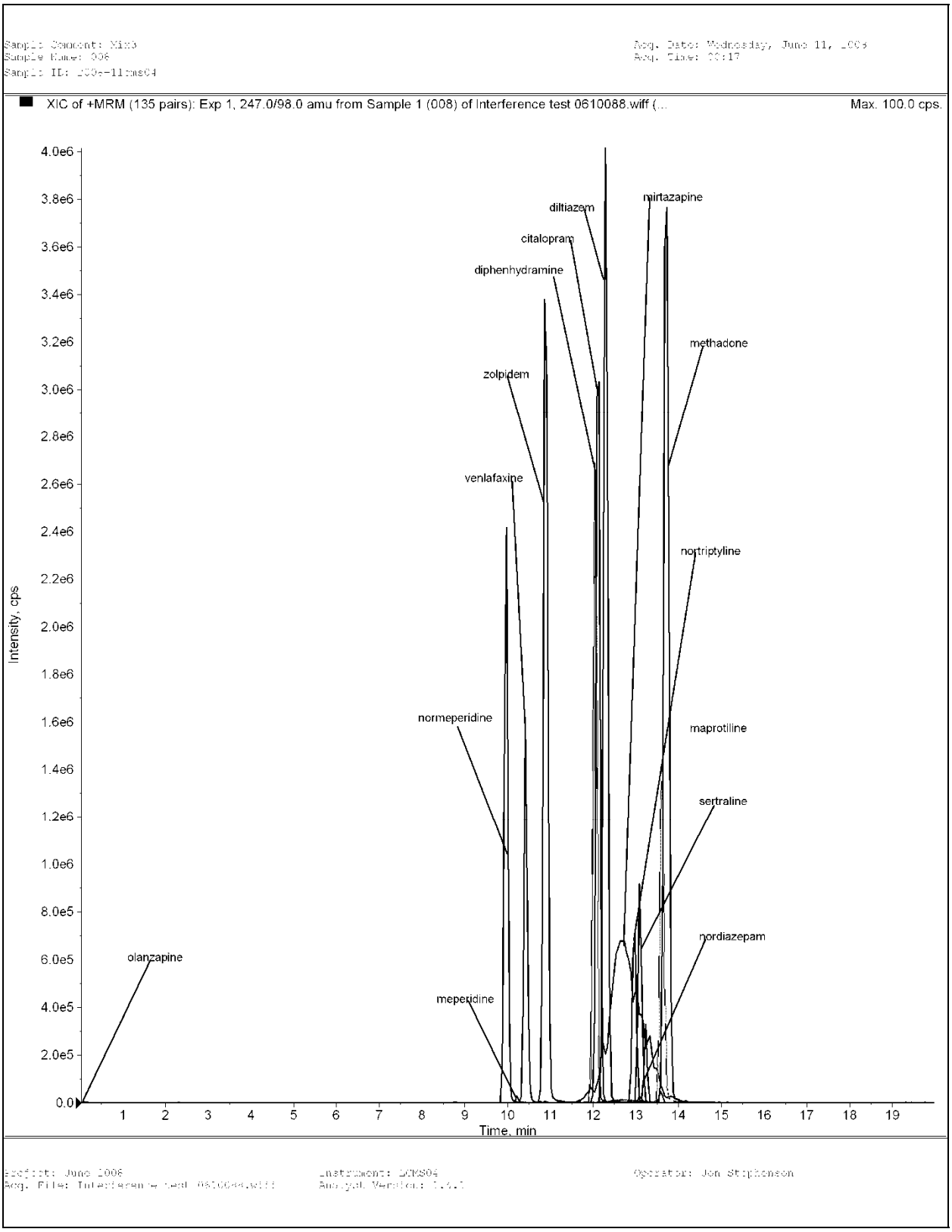

Figure 22. Interference Study Panel 4.

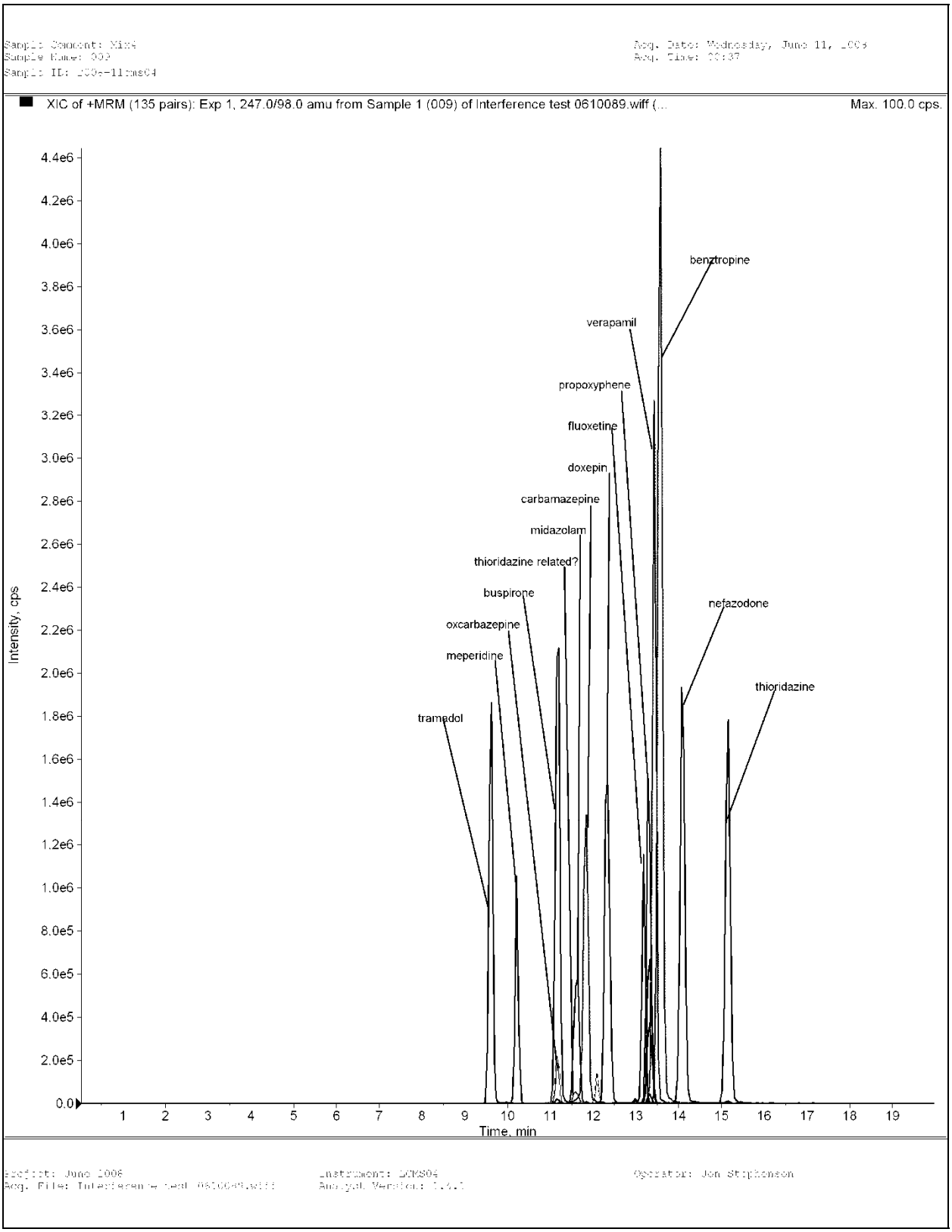

Figure 23. Interference Study Panel 5.

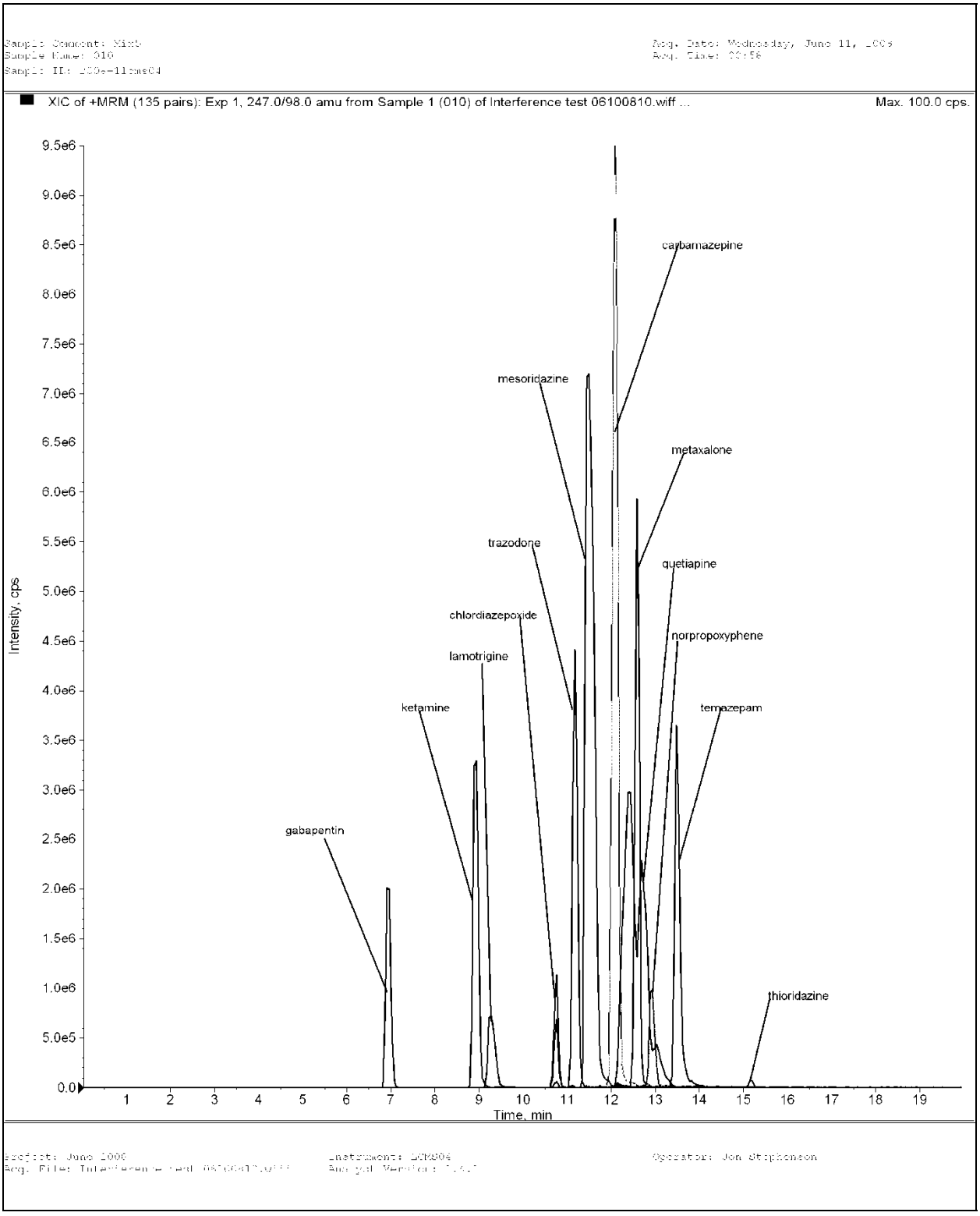

Table 6. Instrument Parameters for Opioid Analysis.

Analyte	MRM Transition (m/z)	Declustering Potential (DP) (V)	Entrance Potential (EP) (V)	Collision Entrance Potential (CEP) (V)	Collision Energy (CE) (%)	Collision Exit Potential (CXP) (V)	Dwell Time (msec)
Morphine	286.1→201.0	61	10	12	33	4	45
Morphine-d6	292.2→152.2	61	7.5	14	81	4	25
Hydromorphone	286.1→185.0	61	10.5	14	37	4	25
Hydromorphone-d6	292.2→185.1	61	10.5	14	39	4	25
Codeine	300.2→152.0	56	4.0	14	81	4	45
Codeine-d6	306.2→152.1	61	8.5	14	89	4	25
Hydrocodone	300.2→199.0	58.5	9.75	24	40	4	25
Hydrocodone-d6	306.2→202.2	61	9.5	14	39	4	25
Oxycodone	316.1→241.0	51	6.75	21	39	4	25
Oxycodone-d6	322.2→247.2	46	8	14	37	4	25
Nalorphine	312.2→152.0	56	7.75	20	89	4	10
Mepivaciane	247.0→98.0	41	3	14	27	4	10

Table 7. Elution Gradient for Opioids.

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	97	3
3	84.5	15.5
12	82	18
16	5	95
18	5	95
18.1	97	3
20	97	3

Table 8. Opioid Limits of Identification and Quantitation.

Compound	Parent Ion	Product Ion	RT (min)	Qualitative LOI	Quantitative LOQ
morphine	286.1	201	5.93	5 µg/L	6 µg/L
hydromorphone	286.1	185	6.38	2.5 µg/L	2.5 µg/L
codeine	300.2	152	7.28	5 µg/L	11 µg/L
oxycodone	316.1	241	8.51	3 µg/L	6 µg/L
hydrocodone	300.2	199	9.54	3 µg/L	6 µg/L

Table 9. Quantitation variability using nalorphine internal standard.

Analyte	STDEV	Mean (µg/L)	Variability (%)
morphine	5.28	52	19.89
hydromorphone	4.26	48.86	17.07
codeine	6.82	51.91	25.75
oxycodone	4.01	53.08	14.7
hydrocodone	4.05	48.75	16.3

Table 10. Quantitation variability using deuterated internal standard.

Analyte	STDEV	Mean (µg/L)	Variability (%)
morphine	3.41	50.52	13.25
hydromorphone	2.36	48.28	9.59
codeine	6.19	52.04	23.32
oxycodone	2.48	52.25	9.32
hydrocodone	2.29	49.01	9.15

Table 11. Quantitation variability using mepivacaine internal standard.

Analyte	STDEV	Mean (µg/L)	Variability (%)
morphine	5.58	52.08	21.01
hydromorphone	4.01	48.95	16.04
codeine	6.7	51.68	25.43
oxycodone	4.22	53.04	15.61
hydrocodone	3.51	48.92	14.07

Table 12. Average variability using Limits of Quantitation data.

	Morphine	Hydromorphone	Codeine	Oxycodone	Hydrocodone
Average Variability (%) Deuterated I.S.	29.3	18	47.6	17.9	14.6
Average Variability (%) Mepivacaine I.S.	27.3	18.6	35.7	21.6	15.7
Average Variability (%) Nalorphine I.S.	29.7	13.2	38.1	18.1	15.2

Table 13. Correlation of GC/MS with LC/MS/MS

Analyte	Internal Standard	Range % Difference	Average % Difference	Median % Difference	Cases Pos. for Analyte
Morphine	Morphine-d6	3-54%	15%	11%	26
Hydromorphone	Hydromorphone-d6	0-24%	12%	8%	6
Codeine	Codeine-d6	3-29%	16%	14%	13
Hydrocodone	Hydrocodone-d6	0-62%	20%	16%	49
Oxycodone	Oxycodone-d6	2-59%	21%	21%	28
Morphine	Nalorphine	0-54%	17%	14%	26
Hydromorphone	Nalorphine	3-37%	25%	26%	6
Codeine	Nalorphine	3-30%	15%	19%	13
Hydrocodone	Nalorphine	0-68%	23%	17%	49
Oxycodone	Nalorphine	0-60%	21%	18%	28
Morphine	Mepivacaine	2-62%	17%	11%	26
Hydromorphone	Mepivacaine	7-51%	28%	24%	6
Codeine	Mepivacaine	0-81%	37%	27%	13
Hydrocodone	Mepivacaine	0-90%	24%	16%	49
Oxycodone	Mepivacaine	0-69%	22%	11%	28

Table 14. Intraday Reproducibility.

Analyte	Internal Standard	Range % Difference	Average % Difference	Median % Difference	Cases Pos. for Analyte
Morphine	Morphine-d6	0-18%	7%	5%	26
Hydromorphone	Hydromorphone-d6	3-14%	8%	6%	6
Codeine	Codeine-d6	0-23%	10%	6%	11
Hydrocodone	Hydrocodone-d6	0-21%	5%	3%	49
Oxycodone	Oxycodone-d6	0-13%	4%	3%	28
Morphine	Nalorphine	0-36%	12%	9%	26
Hydromorphone	Nalorphine	0-21%	11%	10%	6
Codeine	Nalorphine	0-29%	11%	10%	11
Hydrocodone	Nalorphine	0-26%	8%	5%	49
Oxycodone	Nalorphine	0-20%	7%	6%	28
Morphine	Mepivacaine	0-26%	9%	7%	26
Hydromorphone	Mepivacaine	2-23%	11%	11%	6
Codeine	Mepivacaine	0-24%	12%	11%	11
Hydrocodone	Mepivacaine	0-42%	6%	3%	49
Oxycodone	Mepivacaine	0-23%	6%	5%	28

Table 15. Interday Reproducibility.

Analyte	Internal Standard	Range % Difference	Average % Difference	Median % Difference	Cases Pos. for Analyte
Morphine	Morphine-d6	0-39%	10%	4%	21
Hydromorphone	Hydromorphone-d6	2-14%	8%	7%	5
Codeine	Codeine-d6	0-29%	9%	7%	7
Hydrocodone	Hydrocodone-d6	0-36%	7%	5%	45
Oxycodone	Oxycodone-d6	0-22%	10%	6%	26
Morphine	Nalorphine	1-80%	21%	13%	21
Hydromorphone	Nalorphine	2-90%	11%	24%	5
Codeine	Nalorphine	9-45%	23%	23%	7
Hydrocodone	Nalorphine	0-47%	15%	10%	45
Oxycodone	Nalorphine	0-63%	20%	14%	26
Morphine	Mepivacaine	3-58%	17%	13%	21
Hydromorphone	Mepivacaine	2-37%	13%	9%	5
Codeine	Mepivacaine	5-35%	21%	25%	7
Hydrocodone	Mepivacaine	1-48%	12%	9%	45
Oxycodone	Mepivacaine	2-37%	14%	11%	26

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Chapter 4. Barbiturate and Acidic Drug Analysis

Introduction

The acidic properties of barbiturates and other acidic drugs, such as salicylic acid and ibuprofen, prohibit analysis with the current positive mode LC/MS/MS methods being used at the GBI-DOFS laboratory. Currently these drugs are analyzed by GC/MS after sample pretreatment including extraction and derivatization. This study investigated the potential of negative mode LC/MS/MS analysis to identify and quantify barbiturate compounds and acidic drugs in whole blood.

The quantitative analysis of barbiturates in urine by LC/MS/MS in negative mode scan has been reported (Feng et al. 2007). These parameters and a protein precipitation method were examined in this study using blood samples for analysis of barbiturates. If successful, the LC/MS/MS methods developed in this study would allow samples to be analyzed after a simple protein precipitation procedure without derivatization reducing costs and time.

Materials and Methods

Drug Standards

All standard drug compounds were of pharmaceutical purity obtained from various pharmaceutical companies and/or vendors. Standard solutions of each drug (typically at 1 mg/mL) were prepared in methanol or other appropriate solvent (e.g. deionized water) for dilutions and use in subsequent studies.

Sample Preparation

Samples of the drugs acetaminophen, ibuprofen, salicylic acid and valproic acid were made in the concentration range of 20-120 mg/L in 1 mL of Red Cross negative whole blood and extracted using a protein precipitation procedure. Two sample pretreatment procedures were evaluated for the barbiturates; a solid phase extraction (Feng et al., 2007) and a protein precipitation using acetone (McCurdy and Lewellen, 1988). Samples of the drugs butabarbital, secobarbital, pentobarbital, amobarbital, phenytoin, phenobarbital, and butalbital were prepared in the concentration range of 2.5-25 mg/L in 1 mL of Red Cross negative whole blood and were tested using both methods. The internal standards used were hexobarbital, pentobarbital-d5, secobarbital-d5, butalbital-d5, and phenobarbital-d5. The solid phase extraction used is from Feng et al. (2007), substituting Red Cross negative whole blood instead of urine. The solid phase extraction procedure is as follows: 20 µL of internal standard stock solution was added to 500 µL of the blood samples. Then 500 µL of phosphate buffer (pH 7.4) and 20 µL of concentrated ammonium hydroxide solution is added. The samples are equilibrated on a shaker for 20 minutes. They are then poured into 1 mL conditioned Waters Oasis HLB columns; the columns are conditioned by washing them with 1 mL of 5% solution of methanol in water and then eluted with 1 mL of methanol. Once eluted from the column the samples were dried down and then reconstituted with 20 µL of the acetonitrile 50:50 mix. The protein precipitation procedure (McCurdy and Lewellen, 1988) was used to extract the barbiturate and acidic drug samples. For the protein precipitation procedure 1 mL of sample and 100 µL of each of the internal standards was used. The samples were reconstituted with 0.5 mL of the 50:50 Mobile Phase A (0.385 g of

ammonium acetate in 1000 mL of optima grade water with the addition of 0.5 mL of concentrated ammonium hydroxide) and Mobile Phase B (acetonitrile) buffer. The protein precipitation method proved to be the most suitable because it used less sample and produced more reproducible results.

Instrumental Methods

Separation and subsequent analysis was preformed utilizing a Perkin Elmer Series 200 binary high performance liquid chromatography (HPLC) system equipped with an autosampler, solvent degasser, and column heater coupled with an Applied Biosystems QTRAP® 3200 using Analyst 1.4.1 software.

Mobile Phase A consisted of 0.385 g of ammonium acetate in 1000 ml of optima grade water with the addition of 0.5 ml of concentrated ammonium hydroxide. Mobile Phase B was acetonitrile. The column was a MetaSil Basic RP (3 μm, 50 x 2.0 mm)

Two methods were used for analysis to determine the optimum method for detecting acidic drugs and barbiturates. The first method was developed by Feng et al. (2007), which consisted of the following parameters for negative ion detection: The curtain gas was set at 30 L/min, and Gas 1 and Gas 2 were both set at 40 L/min. The desolvation temperature was set at 550°C and the collision-assisted dissociation gas was set at 5. Due to using negative mode, the capillary voltage was set to -4500 V, and dwell times were set at 0.05 s. The second method was the same except for a reduction in the desolvation temperature to 500°C.

Results

Instrument Optimization

Standard solutions of acetaminophen, ibuprofen, salicylic acid and valproic acid were made to a 1 mg/mL solution and then were infused as a 10 mg/L solution in the acetonitrile 50:50 mix. The barbiturates were made at a concentration of 10 mg/L using the standard solution (1 mg/mL) of each barbiturate and infused using the acetonitrile 50:50 mix. The optimized instrument parameters are shown in Table 16.

Identification and Quantitation

Extracted samples containing acetaminophen, ibuprofen, salicylic acid and valproic acid were analyzed using the LC/MS/MS method described in the Materials & Methods section. No internal standards were used in the initial experiments to determine if any of the drugs could be successfully detected. Only salicylic acid and ibuprofen were detected using this method. The barbiturates were analyzed using the above method in a screening mode (generation of EPI spectra) and a quantitation mode (MRM only). The screen was to determine if the mass spectra would contain detail that could be used for identification. The barbiturates were able to be successfully separated and quantitated. The calibration curves and quantitative results for positive controls (expected concentrations of 10 mg/L) were within 30% of expected values which are considered to be acceptable for the purposes of this analysis. The barbiturates were quantitated using both hexobarbital and deuterated barbiturate standards. Both methods produced acceptable results. The chromatogram for the positive control analyzed under the quantitative method is shown in Figure 24.

While the barbiturates were able to be successfully quantitated using this new method, the main limitation was the lack of detail in the mass spectra produced, preventing positive identification

in an unknown sample. The total ion chromatogram of the samples analyzed qualitatively looks smoother than that of samples analyzed using the quantitation method, but the mass spectra produced do not provide enough detail to positively confirm identification. GC/MS analysis following extraction and derivatization would still be required. An example of the mass spectrum produced by the qualitative method is shown for phenobarbital (Figure 25) and secobarbital (Figure 26).

Discussion

Studies conducted to determine the feasibility of identifying and quantifying barbiturate and acidic drugs using LC/MS/MS were relatively unsuccessful. Although the method could easily separate the various barbiturate compounds, the level of detail in the mass spectra was insufficient to allow a positive confirmation. Due to the lack of detail, the barbiturate drugs could not be identified by LC/MS/MS without additional testing by GC/MS, per our current quality policies at the GBI. The actual extraction procedure for LC/MS/MS did not save any time when compared to the current extraction method for GC/MS and the GC/MS method allows for both qualitative and quantitative analysis in one step. The additional testing required for LC/MS/MS analysis defeats the goal of saving cost and time. Analysis of acidic drugs like ibuprofen and salicylic acid was not successful. At this point in time, the preferred method for analysis of these compounds remains GC/MS.

In addition, with barbiturates being analyzed in negative mode scan on the LC/MS/MS and the use of different solutions, one instrument would need to be dedicated to the analysis, reducing available instrumentation for other casework. If one instrument was not dedicated to negative mode scan, time would be wasted preparing the instrument each day. With the low volume of casework requiring this analysis, it would not be time or cost effective to dedicate an instrument for this analysis.

Figure 24. Chromatogram for positive barbiturate control of 10 mg/L.

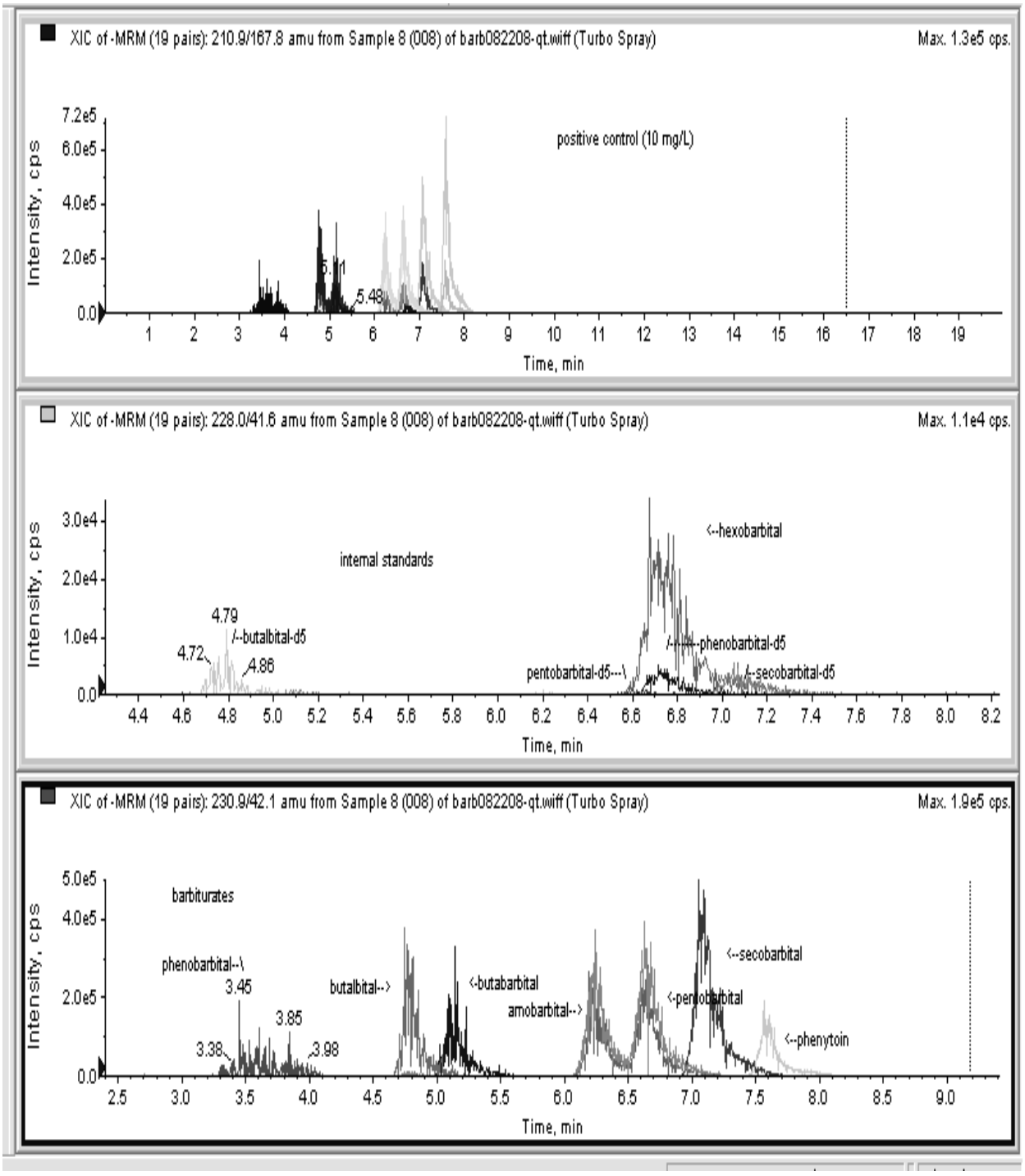

Figure 25. Phenobarbital mass spectrum.

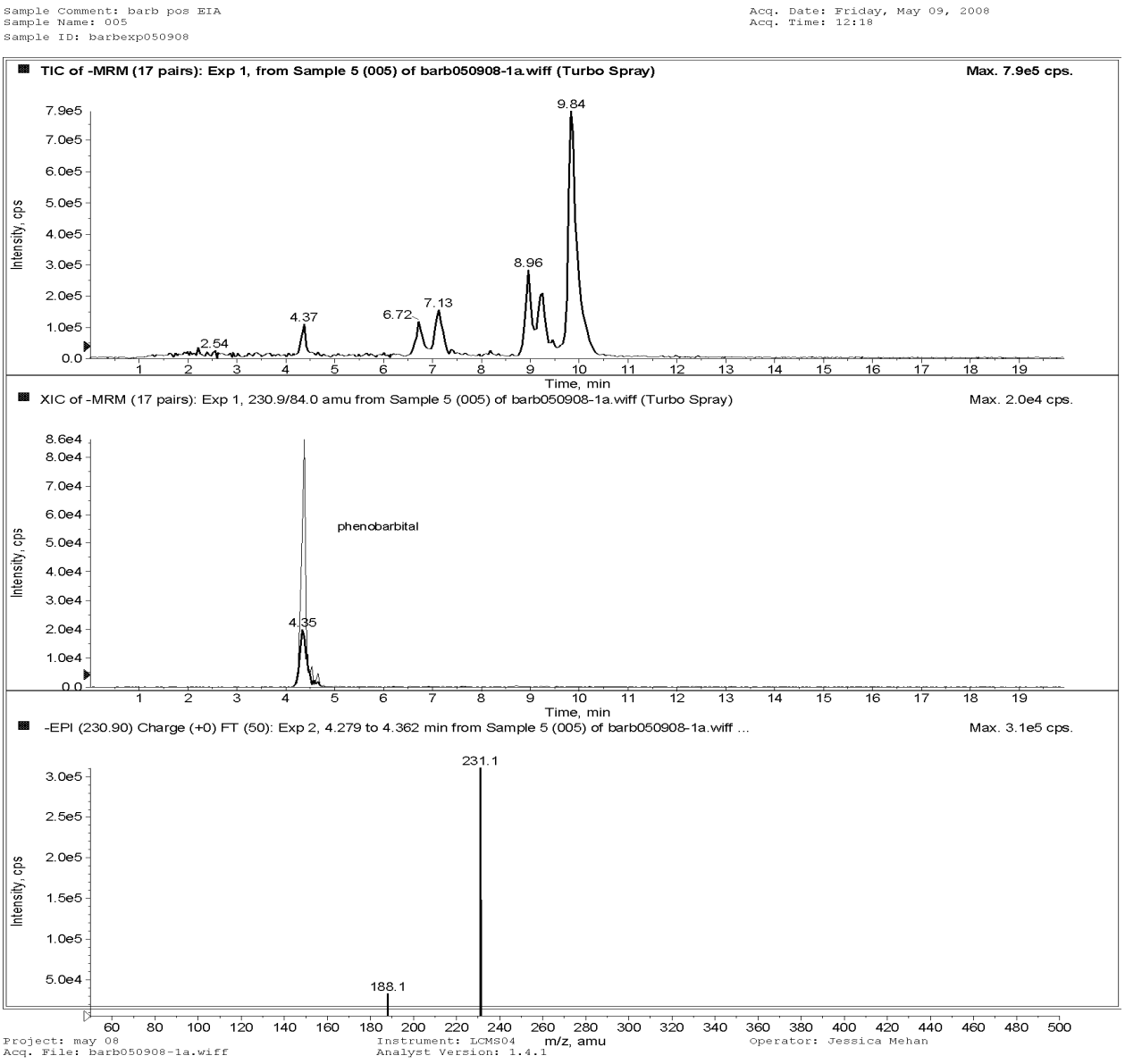

Figure 26. Secobarbital mass spectrum.

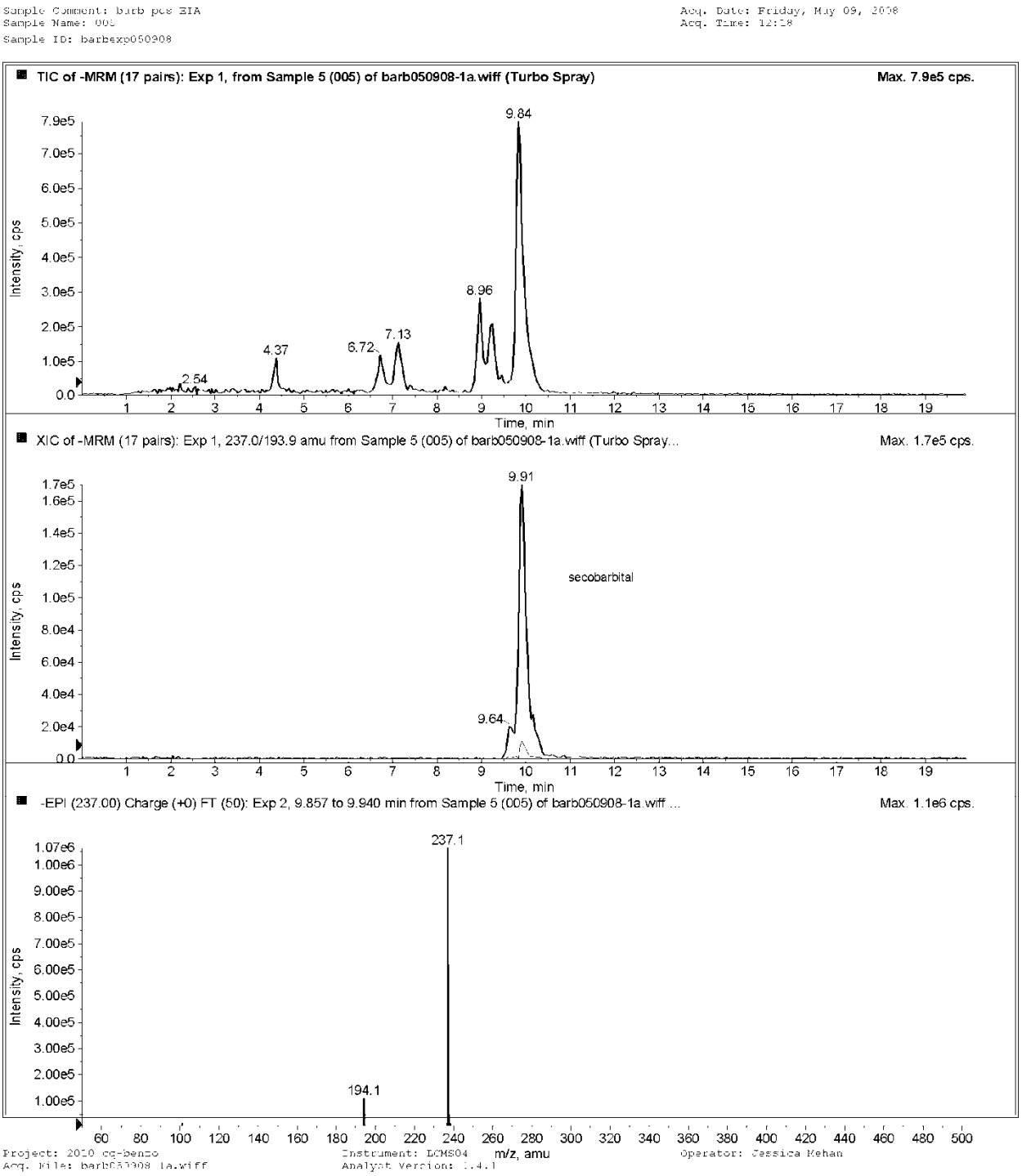

Table 16. Instrument Parameters for Barbiturate and Acidic Drug Analysis.

Analyte	Transitions	Declustering Potential (DP)	Entrance Potential (EP)	Collision Entrance Potential (CEP)	Collision Energy (CE)	Collision Exit Potential (CXP)
Butabarbital	210.9/167.8 210.9/42.1	-30.00	-4.00	-14.00	-14.00	-4.00
Secobarbital	237.0/193.9 237.0/42.1	-30.00	-2.50	-10.00	-14.00	-4.00
Pentobarbital	225.4/181.8 225.4/42.1	-25.00	-2.00	-16.00	-14.00	-4.00
Amobarbital	225.0/181.8 225.0/42.1	-25.00	-5.00	-16.00	-14.00	-4.00
Phenytoin	250.9/101.6 250.9/42.1	-40.00	-3.00	-16.00	-30.00	-2.00
Phenobarbital	230.9/84.0 230.9/42.1	-20.00	-5.00	-12.00	-16.00	-2.00
Butalbital	223.1/180 223.1/42.1	-30.00	-1.00	-16.00	-14.00	-4.00
Pentobarbital-d5	23.0/41.7	-35.00	-2.50	-14.00	-30.00	-6.00
Hexobarbital	236.2/41.3	-115.00	-1.00	-14.00	-30.00	-4.00
Secobarbital-d5	242.0/41.7	-36.00	-2.00	-20.00	-34.00	-4.00

Analyte	Transitions	Declustering Potential (DP)	Entrance Potential (EP)	Collision Entrance Potential (CEP)	Collision Energy (CE)	Collision Exit Potential (CXP)
Butalbital-d5	228.0/41.6	-30.00	-3.50	-18.00	-30.00	-4.00
Phenobarbital-d5	235.9/41.8	-20.00	-9.00	-20.00	-32.00	-6.00
Valproic Acid	143.0/98.6	-30.00	-2.50	-10.00	-8.00	-2.00
Ibuprofen	204.8/159.2	-20.00	-2.50	-10.00	-10.00	-2.00
Salicylic Acid	136.8/92.6	-30.00	-2.50	-10.00	-22.00	-0.00

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McCurdy, H.H. and Lewellen, L.J. (1988) A novel procedure for the analysis of drugs in whole blood by homogeneous enzyme immunoassay (EMIT), *J. Anal. Toxicol.* 12: 260-264.

Chapter 5. Stability Study, Supplemental Stability Study, and Amphetamine Stability Study

Introduction

The primary impetus for this study arose from observations during the concordance study completed as part of the 2003 Research and Development grant from NIJ (2003-IJ-CX-K007) which indicated levels of some drug compounds had declined during sample storage. While sample degradation is not unexpected, this type of information could prove important in cases where a significant time interval has elapsed between specimen collection and subsequent analysis or reanalysis. There are some drugs with well documented degradation; however, degradation of commonly detected prescription drugs found in forensic toxicology casework is less well known. The stability study and supplemental stability study goal was to determine how much degradation occurs with drugs commonly found in forensic toxicology casework utilizing a simple protein precipitation followed by analysis utilizing liquid chromatography tandem mass spectrometry (LC/MS/MS).

The amphetamine stability study objective was to test the stability of amphetamine, Methamphetamine, 4-methylenedioxyamphetamine (MDA), and 3,4-methylenedioxy-methamphetamine (MDMA) in blood samples using GC/MS.

Amphetamine and methamphetamine are stimulants and common drugs of abuse. They are also routinely used in the treatment of ADHD, obesity, and narcolepsy. MDMA is known as "ecstasy" and is also a stimulant, however, with psychedelic characteristics. MDA is both a metabolite of MDMA and an obtainable drug by itself with similar characteristics as MDMA, only less potent.

Our interest in studying the stability of compounds stems from the fact that courts can request that a sample be retested months after they arrive at our laboratory. If there is any notable deterioration of the drugs' quantity in the blood sample, then reproduction of original results may be problematic. Another point of interest in determining the stability of these drugs is evaluating the time frame for necessary testing. If these compounds begin deteriorating soon after a sample is drawn from a human subject, then it compels our laboratory to test the samples as soon as possible.

Materials and Methods

Drug Standards

All standard drug compounds were of pharmaceutical purity obtained from various pharmaceutical companies and/or vendors. Standard solutions of each drug (typically at 1 mg/mL) were prepared in methanol or other appropriate solvent (e.g. deionized water) for dilutions and use in subsequent studies.

Stability and Supplemental Stability Instrumentation

The instrument selected for this research was the Applied Biosystems, Inc. QTRAP® 2000 or QTRAP® 3200 in multiple reaction monitoring (MRM) mode with an attached Perkin Elmer

Series 200 binary high performance liquid chromatography (HPLC) system equipped with an autosampler, solvent degasser, and column heater. A PEAK nitrogen gas generator was used to supply the curtain, source, and exhaust gases and to fill the collision cell for the mass spectrometer system. All analysis was performed with the instrument in positive mode using electrospray ionization (ESI) as the ionization method. The instrument software used for data collection and analysis was Analyst version 1.4.1. The primary difference between the QTRAP® 2000 and QTRAP® 3200 is increased sensitivity in the 3200 model. See Table 17 for the MRM transition list for each drug or metabolite in the stability study and Table 18 for those in the supplemental stability study. See Table 19 for the LC parameters and Table 20 for the source and MS parameters

Instrumentation for Amphetamine Stability Studies

An Agilent model 6890 gas chromatograph (GC) paired with a model 5973N mass selective detector (MSD) quadrupole mass spectrometer was used for analysis of amphetamine compounds. 6890 GC Method: The oven's initial temperature was 80° C. The initial time was 1.00 min. with an equilibration time of 0.50 min. The first ramp was at a rate of 50.0° C/min to a final temp of 120°C with a hold time of 0.0 min. The second ramp was at a rate of 20.0° C/min to a final temp of 285° C with a hold time of 14.0 minutes. The front inlet was run in the pulsed splitless mode with at a temperature of 250° C. The pulse time was 0.80 min. with the purge time of 1.50 min. The total helium flow was 39.3 mL/min. The capillary column had an initial flow of 1.3 mL/min. with an average velocity of 43 cm/sec. 5973N MSD Detector: The mass spectrometer acquisition parameters were set at full scan mode scanning from 40 – 550 a.m.u. The mass spectrometer quadrupole temperature was set at 150° C with the mass spectrometer source temperature set at 230° C. The gas used was Ultra Pure Grade helium and the column was a HP5-MS.

HPLC Column and Mobile Phase

The column was a MetaSil Basic RP (3 µm, 50 x 2.0 mm). Mobile Phase A (MPA) – 0.1% formic acid, 1 g/L (~15mM) ammonium formate in optima grade water; Mobile Phase B (MPB) – 0.1% formic acid, 1 g/L (~15mM) ammonium formate in optima grade methanol. All mobile phases were degassed prior to use.

Preparation of Stability and Supplemental Stability Study Specimens

Postmortem whole blood was obtained from the Georgia Bureau of Investigation Medical Examiner's office. The blood samples from four individuals were used. Each sample was tested using cloned enzyme donor immunoassay (CEDIA) for six classes of drugs with the following cut-offs: barbiturates (1000 ng/mL), opioids (50 ng/mL), benzodiazepines (200 ng/mL), cocaine (50 ng/mL), amphetamines (75 ng/mL), and cannabinoids (25 ng/mL). All specimens were negative by CEDIA without any elevated results (>10 ng/mL). Additionally all the blood specimens were screened by LC/MS/MS for 130 drugs and were found not to contain any of the drugs in the study. Because of the limited volume of postmortem blood available from the medical examiner, two specimens were combined to form a single lot of negative postmortem blood to prepare study specimens for the stability specimen blood. The negative blood was sonicated and forced through cheesecloth immediately prior to specimen preparation to breakup and remove blood clots. For the supplemental drug stability study, the two blood specimens were not combined to form a single lot. Blood obtained from the Red Cross was used for preparation of standards and controls during the study period. The blood was tested in the same manner as

the postmortem blood used to make study specimens. Calibration/control blood was diluted 50:50 with deionized water prior to preparation of calibration standards in order to create viscosity in the calibration standards consistent with that observed in casework whole blood samples.

Seventy-six drugs commonly encountered in forensic toxicology casework were selected to be tested during the stability study (64 in the stability study, eight in the supplemental stability study and four in the amphetamine stability study). The non-amphetamine drugs were grouped into seven mixtures based primarily on concentration and the amphetamine drugs into two mixtures. Study specimens were prepared by dispensing the appropriate level of stock solution into a test tube, and drying down the solvent. Each drug group was tested at four concentration levels in an attempt to reflect low therapeutic, therapeutic, high therapeutic/toxic and overdose levels. The drugs tested and target concentrations are listed in Tables 21-25. 10 mL of postmortem blood was added to the tube containing the evaporated drug standard solutions, the tube was vortexed for 30 s and transferred to gray stopper Vacutainer® tubes. For each study specimen two gray stopper tubes were filled with approximately 5 mL of blood. The samples were stored in plastic gray stopper blood collection tubes (6 mL BD Vacutainer® 15 mg Sodium Fluoride/ 12 mg Potassium Oxalate) and placed in refrigerated storage (0.5-9°C).

Preparation of Amphetamine Stability Study Specimens

Negative blood and packed red blood cells were obtained from the American Red Cross for the purpose of preparing study specimens, calibrators and controls during for analysis. Blood was screened for drugs in the study prior to use and determined to be negative for anything other than caffeine or nicotine. A 1:4 dilution of packed red blood cells with deionized water was used to provide a sample viscosity similar to that of whole blood samples submitted as routine casework samples.

Four amphetamine and related compounds commonly encountered in forensic toxicology casework were selected to be studies studied; amphetamine, methamphetamine, 3,4-methylendioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA). Study specimens were prepared by adding the appropriate concentration of drug standards to 75 mL of drug-negative blood. Two different mixes were prepared and care was taken to not include a metabolite of a drug and its parent in the same mix. These mixes were made up at four different concentrations to approximate low, mid, high and three times high therapeutic concentration. Five mL of each mix, at each concentration, were aliquoted into gray stoppered Vacutainer tubes for refrigerated storage. See Table 26 for mixes and concentrations for the amphetamine stability studies.

Preparation of Stability and Supplemental Stability Drug Mixes and Calibrators

For the purpose of quantitation of drugs in the study specimens, seven standard mixes were prepared. (Tables 27-28). Calibration standards were prepared from working standards mixes, by aliquoting the appropriate volume, evaporating the solvent, and reconstituting with 2 mL of 50:50 deionized water: negative Red Cross blood. Positive and negative controls were also prepared. Some groups were broken over multiple mixes to avoid overloading any particular study specimen and to ensure a compound that was a breakdown product or metabolite was not in the same sample as the parent drug. For example, clonazepam and 7-aminoclonzepam were both considered group B drugs for target test sample concentrations but were included in two different mixes (Mix 1 and Mix 2) in order to monitor possible conversion from one to the other.

Preparation of Amphetamine Stability Calibrators

Controls and calibrators were prepared fresh with each extraction by adding the appropriate concentration of a mixed drug standard to 2 mL of drug-negative blood. See Table 26 for the calibration range of each mix.

Testing of Stability and Supplemental Stability Study Specimens

Study specimens were removed from cold storage and allowed to warm to room temperature prior to sampling. Calibration standards and controls were prepared (see Preparation of Drug Mixes and Calibrators Section). To each tube 10 µL of an internal standard solution containing mepivacaine (1.5 mg/L) was added. Specimens were extracted using an acetone precipitation procedure followed by reconstitution in 50:50 ratio of mobile phase buffers A and B described above. At each sampling, each specimen was aliquoted twice (200 µL), and each aliquot extracted and injected twice, totaling four injections per specimen. The study specimens were extracted and quantitated on the day of preparation to establish the true starting concentration. The study specimens were tested weekly for one month, then biweekly for two months, then approximately monthly for 4 months, then bimonthly to the one year interval and then once 8 months later (Table 29).

Due to the shorter time frame for the supplemental stability study, an abbreviated timeline for testing was used. The testing was performed weekly for one month, then biweekly for 2 months, then approximately monthly for four months, and the testing was concluded with two tests at approximately one year from the initial day of preparation (Table 30).

Testing of Amphetamine Stability Study Specimens

The extraction method utilized for the amphetamine study was a liquid-liquid extraction. To 2 mL of study specimen, calibrator or control, d-11 methamphetamine, d-11 amphetamine, mepivacaine were added as internal standards. After the specimens were buffered and made basic by adding 1 g of NaCl, followed by 1 mL of pH 9.0 ammonium chloride buffer and 100 µL of concentrated ammonium hydroxide (vortex), the drugs were extracted from the blood by adding 10 mL of n-butyl chloride and mixed on a rotary apparatus for at least 30 minutes. After centrifuging the mixture, the organic layer was transferred; 0.20 mL of a 2% solution of HCl in methanol was added and then taken to dryness. The residue was resuspended by adding 3 mL of 0.5 N H2SO4, followed by 3 mL of hexanes. After mixing and centrifuging, the hexanes were aspirated to waste and this wash step was repeated with 3 mL of hexanes. The analytes were back extracted into 3 mL of n-butyl chloride by adding 0.5 mL of concentrated ammonium hydroxide. After mixing and centrifuging, the n-butyl chloride was transferred; 0.05 mL of acetic anhydride is added for the purpose of derivatizing the amphetamines and the contents taken to dryness. In order to reconstitute the drugs, 0.075 mL of ethyl acetate was added to each sample. The ethyl acetate was then transferred to GC/MS vials.

For quantitation purposes, d-11 amphetamine, d-11 methamphetamine, and mepivacaine were used as internal standards for amphetamine, methamphetamine and MDMA/MDA, respectively. The dates of sampling and extraction can be seen in Table 31.

Results

For most drugs in Mix 1 the lowest concentration did not produce consistent results. Tizanidine was included in the study design for Mix 1, but analysis proved unsuccessful because the

extraction and analysis scheme did not consistently recover tizanidine at any level. Initial analysis of both study specimen tubes of the Mix 4 group showed that they were not the same concentration. In subsequent extractions, analysts were careful to record which tube was used. Due to a sampling error, no data was recorded for the 9/27/2008 point. For the supplemental stability study, the response for carbamazepine-10, 11-epoxide resulted in several irregular-shaped peaks that were detected at a similar retention time, making quantitative analysis difficult. The areas of response for all of the peaks around the expected relative retention time were integrated. This was done in a similar manner for each sample and resulted in linear calibration curves.

Evaluation of the study results at the one month and one year mark are of particular importance since former represents the goal for toxicology testing for most forensic toxicology laboratories and the latter is the minimum duration specimens are retained by the GBI-DOFS laboratory before being discarded. The methodology used for quantitation has an established variability of 21% at the 95% CI using mepivacaine as the internal standard. Many drugs exhibited small changes (positive and negative) from the initial concentration result. If those changes were within 21%, and did not show a definite trend during the study period then the losses/gains were considered within the normal procedural variance. Of interest were those study drugs that exhibited losses greater than 21% at one year, had shown similar losses at all concentration levels and had shown a downward trend at nearly every data point. Cocaine, cocaethylene, benzoylecgonine, bupropion, clonazepam, diltiazem, mesoridazine, ziprasidone and zopiclone showed significant reduction of concentration during the first year of the study period. Tables 32-33 summarize the loss of these drugs during the study period and Figures 27-35 illustrate these losses.

Analysis of the cocaine specimen for benzoylecgonine showed that 44% of the cocaine had been converted to and remained benzoylecgonine in the 370 day sample (Figure 31). Analysis of the clonazepam containing specimen showed that 92% of the clonazepam had been converted to and remained 7-aminoclonazepam (Figure 27). The presence of benzoylecgonine and 7-aminoclonazepam were confirmed using LC/MS/MS in enhanced product ion mode. Beyond one year several drugs continued to decline. Cocaethylene, and mesoridazine continued to decline, and zopiclone was undetectable in the final sample. Cocaine, benzoylecgonine, clonazepam and diltiazem did not continue to decline after one year. The apparent increase in the specimen concentrations may be due to experimental variability, switching to secondary specimens and new standard solution. Nevertheless, all continued to show losses greater than 21% from the initial specimen concentration.

Lorazepam and scopolamine showed losses throughout the study period and warrant additional attention as the losses were steady. Analysis of scopolamine resulted in highly irregular peak shapes, making proper integration and therefore quantitation difficult. Lorazepam was not detectable in the lowest concentration, 0.001 mg/L (an expected limitation) and was intermittently detected in the second low level, 0.01 mg/L. Results at the 0.05 and 0.1 mg/L levels showed losses, though not consistent between levels. At one year the 0.05 mg/L lorazepam specimen showed a 47% decrease whereas the 0.1 mg/L specimen showed only a 12% loss. By 618 days both exceeded 21%.

Ziprasidone rapidly decreased in concentration for all levels within the first week since preparation and continued to decrease for up to a year at which time the study concluded. The decrease was observed for both sources of negative blood, but the extent of degradation was

different. One source of negative blood had an observed change of concentration of 65% after the first week and a change of 98% after a year; the other source of negative blood showed changes of 42% and 75%, respectively (Table 33).

Amphetamine stability studies showed no signficant changes in concentrations over the course of the study for amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine and 3,4-methylenedioxymethamphetamine (Figures 36-37).

Discussion

Drug decomposition was monitored for seventy two drugs prepared in postmortem or Red Cross blood for more than one year. Specimens were stored in grey stopper tubes and held in refrigerated conditions to simulate common blood evidence collection and storage. During the study period nine drugs showed a significant reduction (>21%) in drug concentration. After approximately one month from the date of preparation, benzoylecgonine concentrations remained effectively unchanged, but five drugs showed a small though measurable loss in concentration.. Zopiclone, ziprasidone and diltiazem concentrations had already dropped by more than 21%. At approximately one year the average percentage loss for all concentration levels tested was greater than 80% for cocaine, bupropion, diltiazem, and zopiclone. Analysis of the cocaine specimen predictably revealed benzoylecgonine concentrations increased as cocaine hydrolyzed to benzoylecgonine (Figure 31). Cocaine degradation is consistent with previously reported studies (Isenschmid, Levine & Caplan, 1989; Isenschmid, 2002). The clonazepam loss is consistent with loss found by Mahjoub and Staub (2000). Robertson and Drummer (1998) reported loss of nitrobenzodiazepines stored at 4 °C in postmortem blood and significant, rapid loss when incubated at 22 °C with bacteria. Bacterial contamination was not checked in this study, but since the matrix used was blood obtained at autopsy, bacterial contamination is not unreasonable. 7-amino-clonazepam concentrations increased as clonazepam concentrations decreased (Figure 27). Zopiclone degradation was much greater than found by Holmgren et al (2004), but their study specimens were kept much colder (-20 °C). Zopiclone instability has been reported by other authors (Pepin, Dubourvieux & Gaillard, 1998; Volgram and Khodasevitch, 2007). Diltiazem degradation has previously been reported and the loss observed here appears consistent (Koves, Lawrence & Mayer, 1998).

The goal of the GBI laboratory is complete analysis of specimens within 45 days of arrival within the laboratory; significant losses within that period would have the greatest effect on interpretation of toxicology findings for the majority of cases. For zopiclone and diltiazem fast analysis of the case specimen would seem to be necessary. Analysis of cocaine, cocaethylene, benzoylecgonine, bupropion, clonazepam, ziprasidone and mesoridazine containing cases should be performed with time constraints considered since they decomposed greatly by one year.

To further evaluate the observations of drug loss, a secondary study was designed focusing on the drugs which showed significant degradation during the initial study period. Lorazepam, clonazepam, bupropion, diltiazem, zopiclone, ziprasidone, cocaine cocaethylene, benzoylecgonine as well as metabolites norcocaine, ecgonine methyl ester, hydroxybupropion and 7-aminoclonazepam were included in the study. The sample preparation, extraction and analysis were conducted in the same manner as the initial study with three important variations. The secondary study used Red Cross whole blood, the study focused on a single concentration level, and when possible deuterated internal standards were used for quantitation. The study was designed to run for approximately thirty to forty five days. Unfortunately instrument and method

difficulty during the first three weeks made continuation of the experiment unviable and prevented any conclusions from being drawn. The secondary study is worth investigating in the future to examine degradation of drugs with respect to potential increases in metabolites.

This stability study of methamphetamine, amphetamine, 3,4-methylenedioxymethamphetamine (MDMA), and 3,4-methylenedioxyamphetamine (MDA) demonstrated that there were no reproducible changes in concentration over the course of the 83 weeks of the study. These results are consistent with those found by Giorgi and Meeker (1995) with respect to methamphetamine and amphetamine. The normal length of time that a toxicology specimen is held by this laboratory is one year. Within that time period, courts are able to request retesting of samples with confidence that results should be reproducible.

This study also gives laboratories the confidence that the time intervals among the drawing of the specimen, initial testing, confirmation testing, and any re-testing is not absolutely crucial to the reproducibility of the results within the normal operations of a forensic laboratory.

Figure 27. Loss of clonazepam and increase of 7-aminoclonzepam over study period.

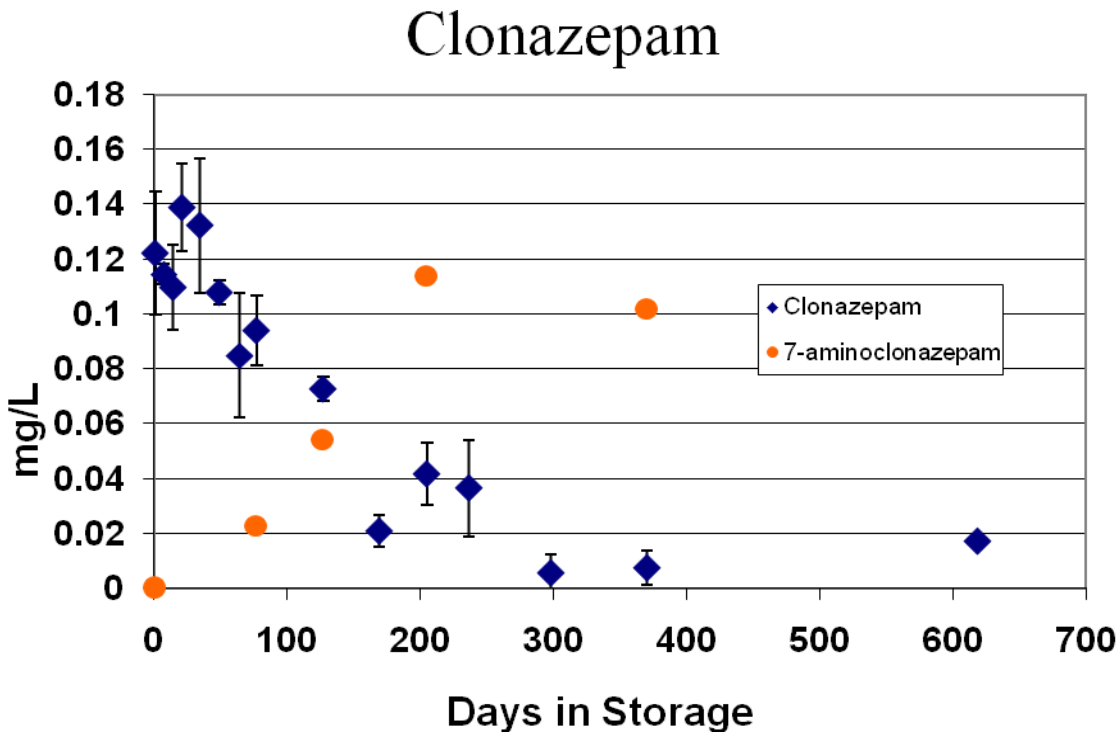

Figure 28. Loss of bupropion over study period.

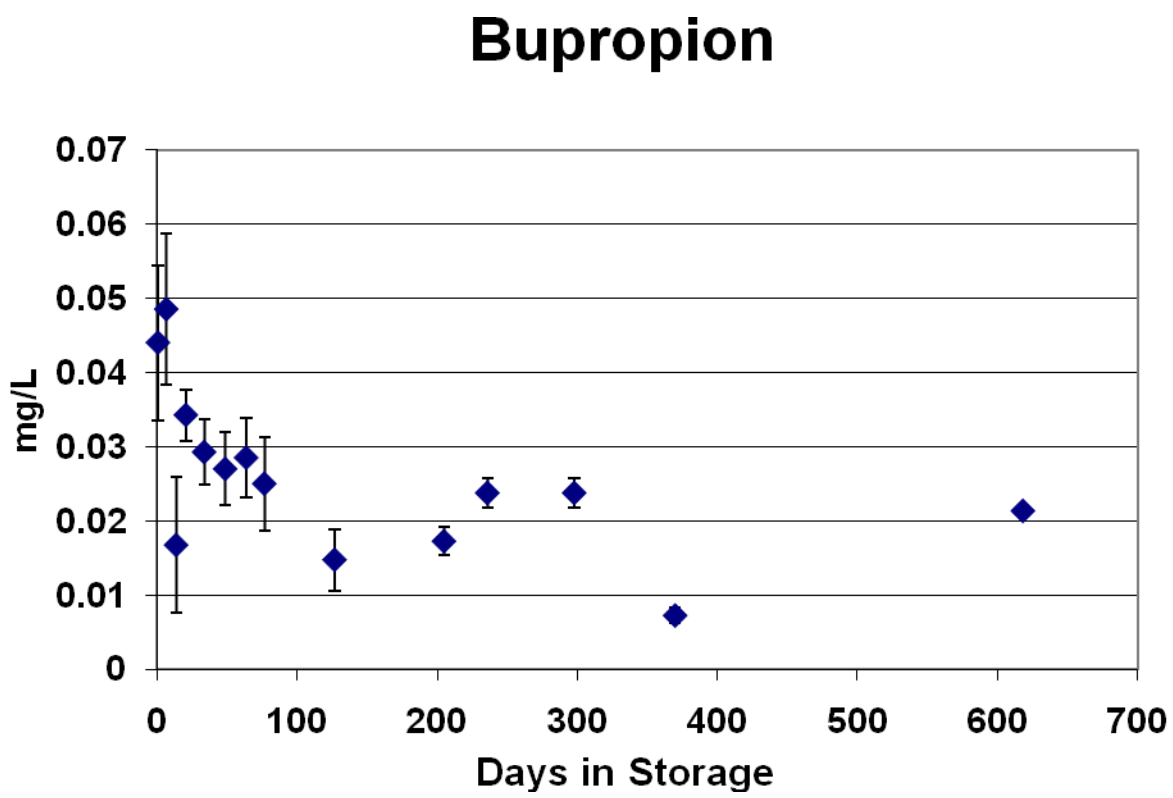

Figure 29. Loss of cocaethylene over study period.

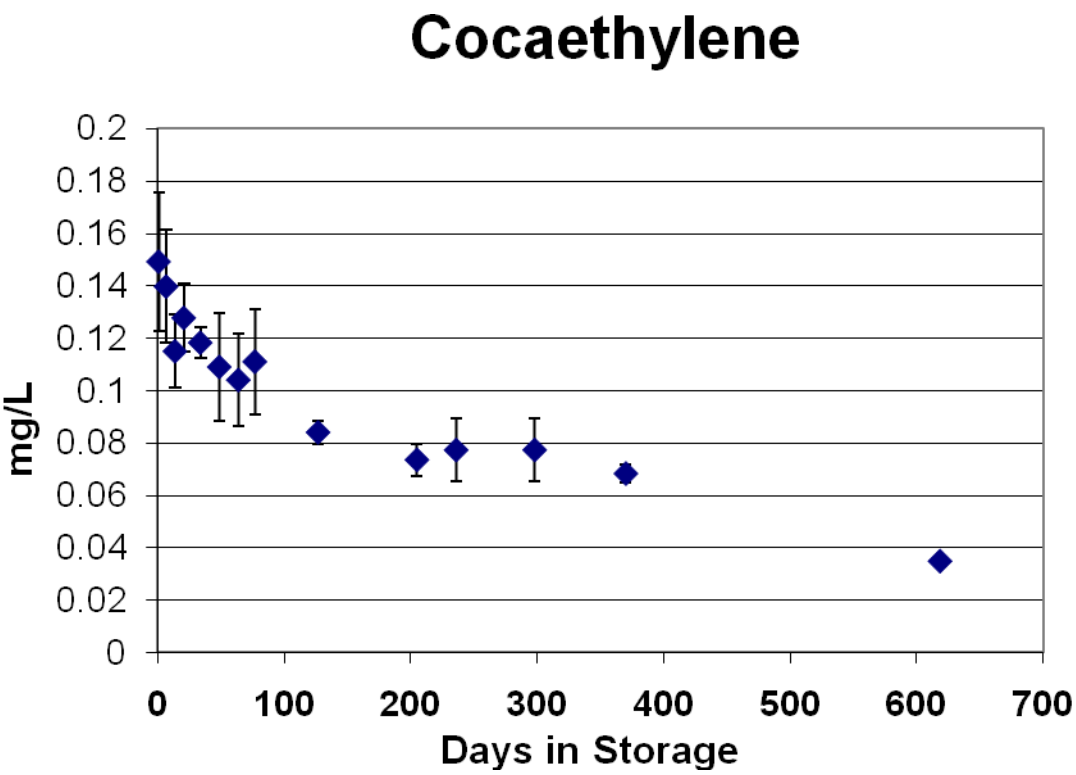

Figure 30. Loss of zopiclone over study period.

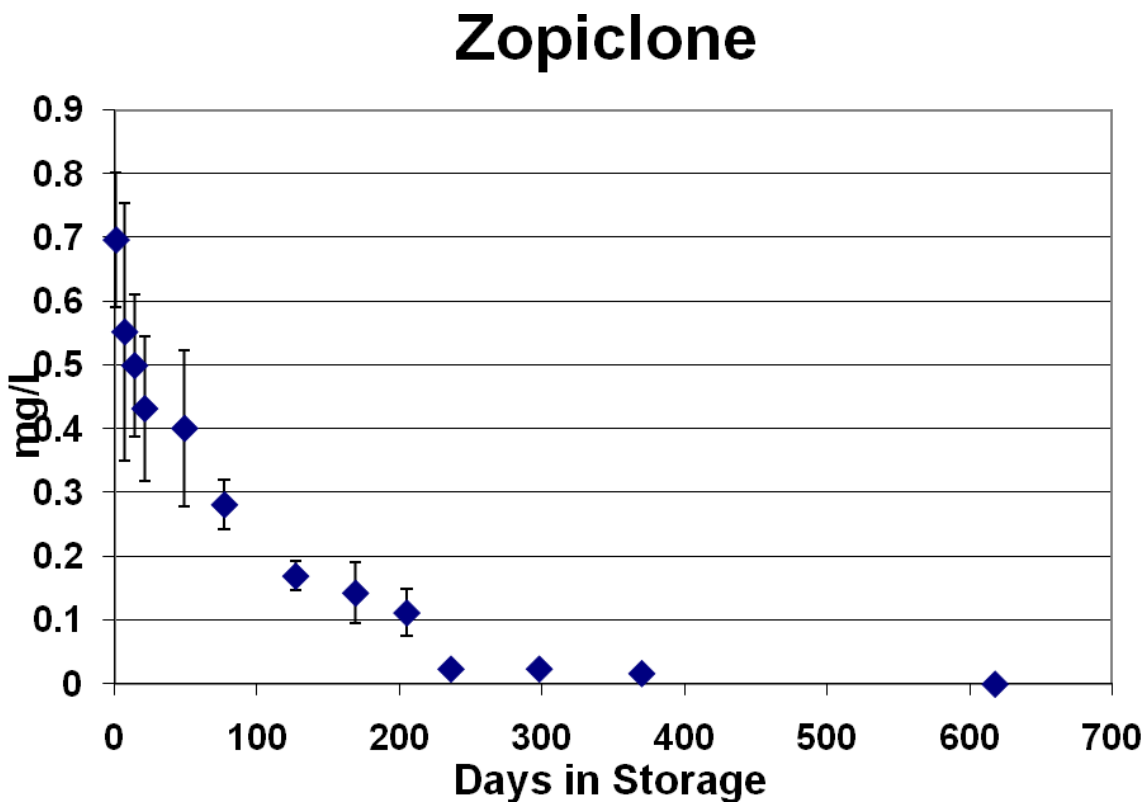

Figure 31. Loss of cocaine over study period.

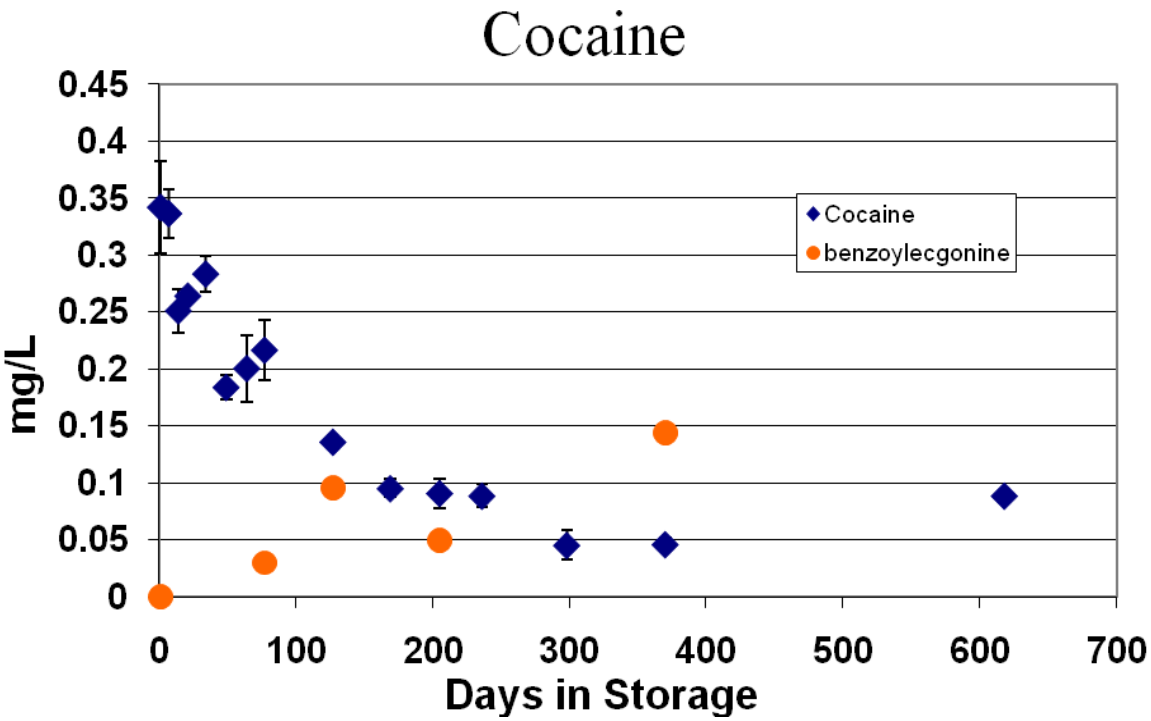

Figure 32. Loss of benzoylecgonine over study period.

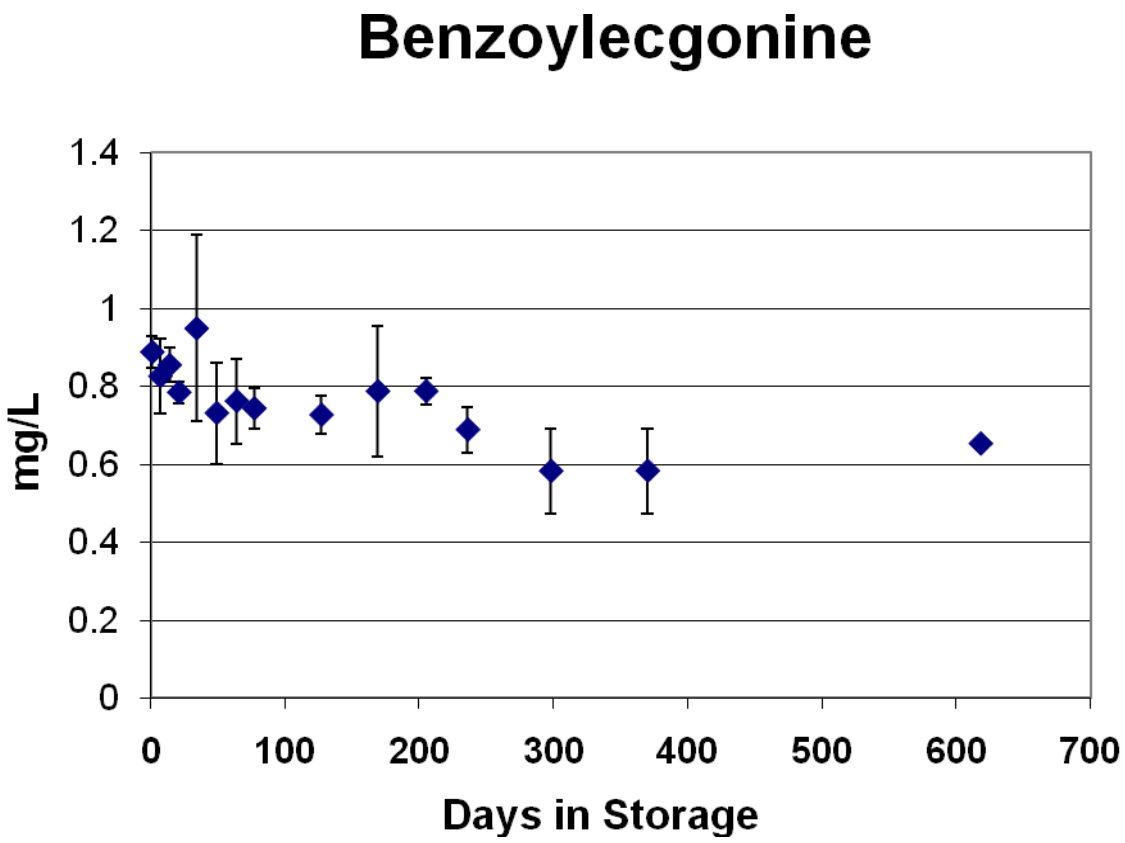

Figure 33. Loss of diltiazem over study period.

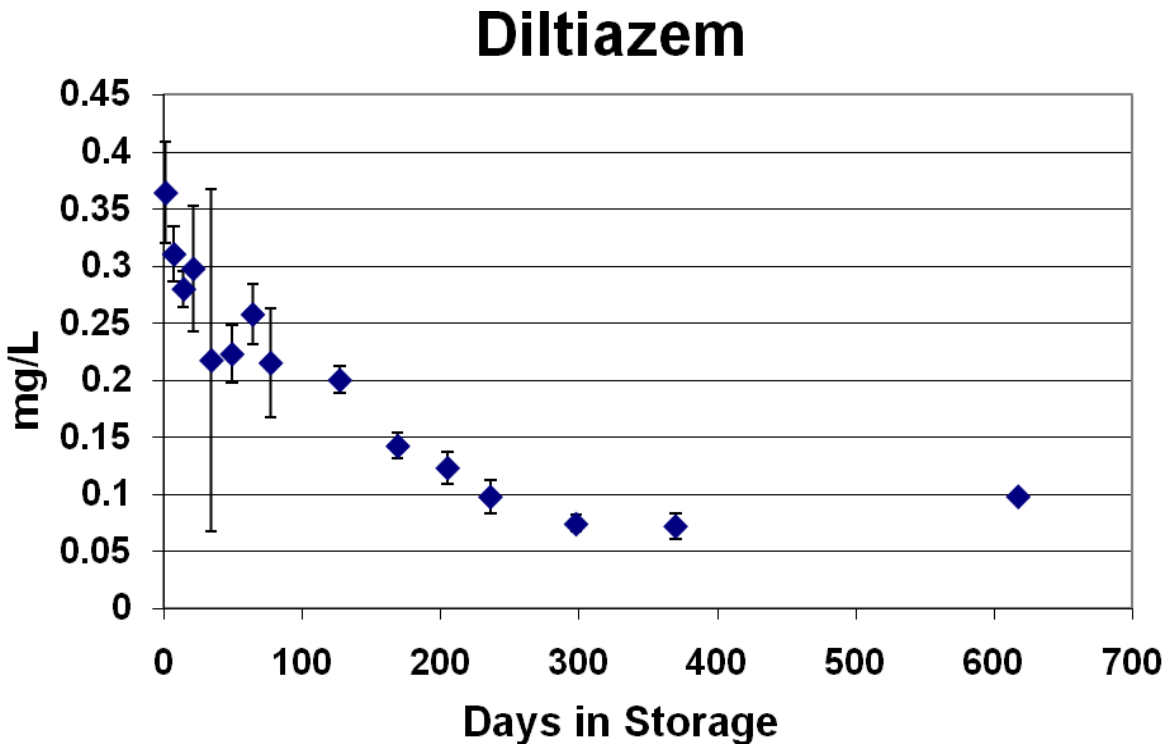

Figure 34. Loss of mesoridazine over study period.

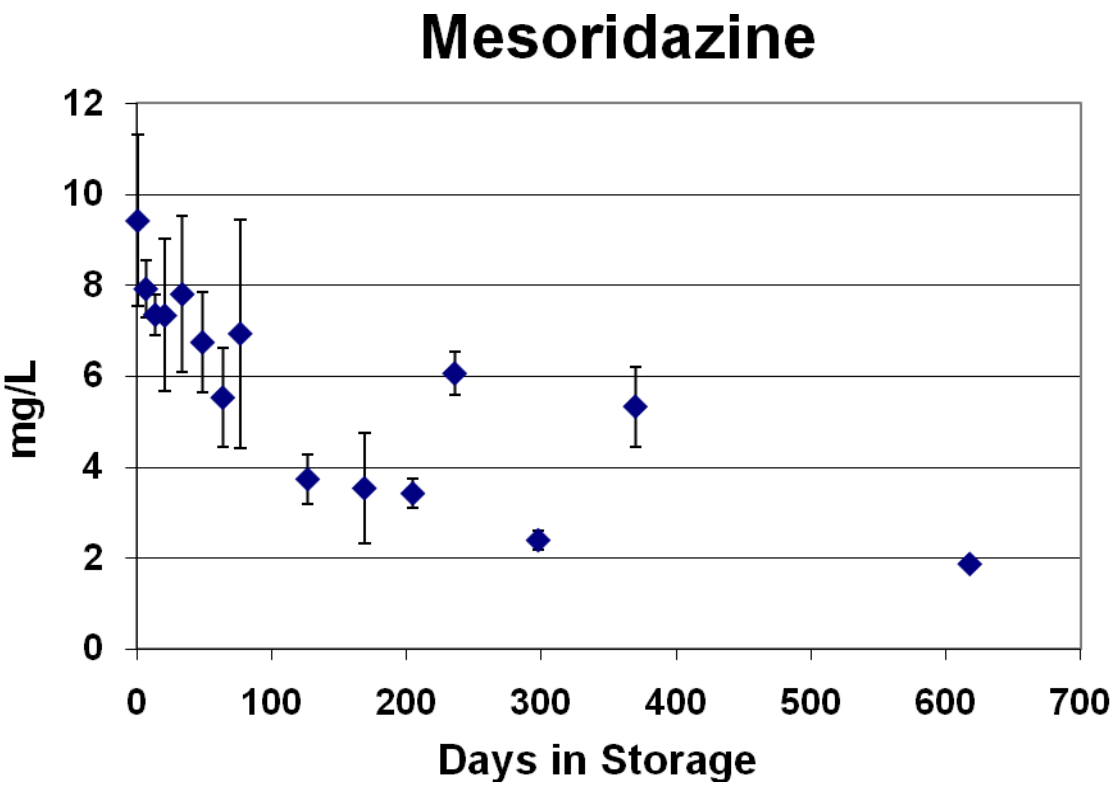

Figure 35. Ziprasidone stability over study period.

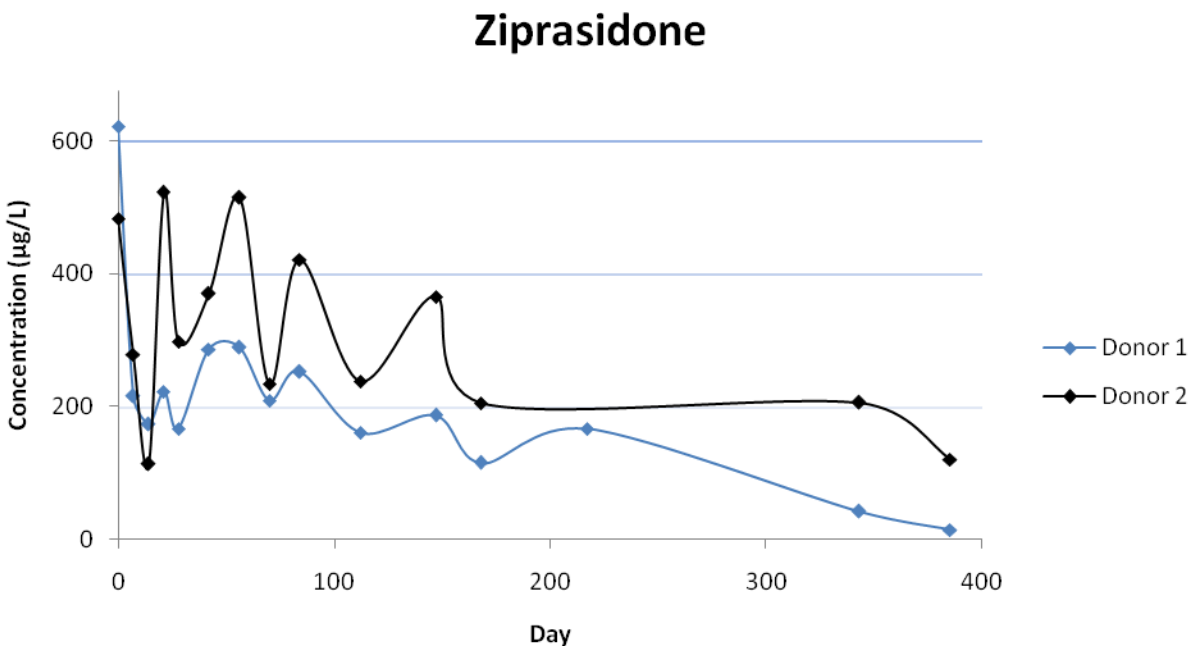

Figure 36. Methamphetamine stability over study period.

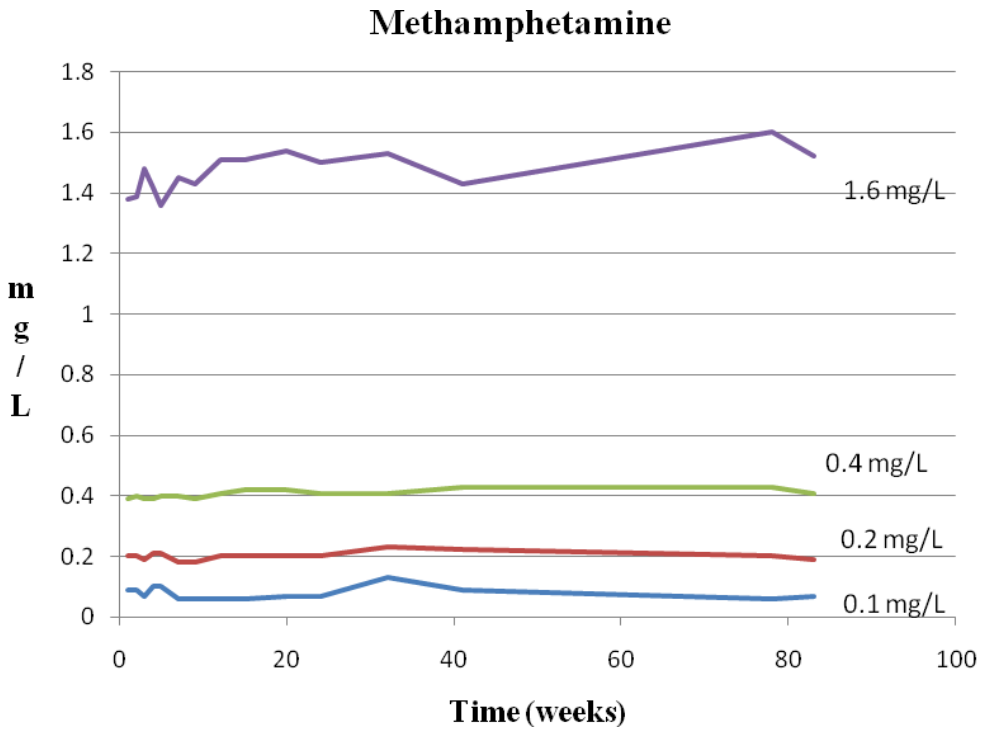

Figure 37. MDMA stability over study period.

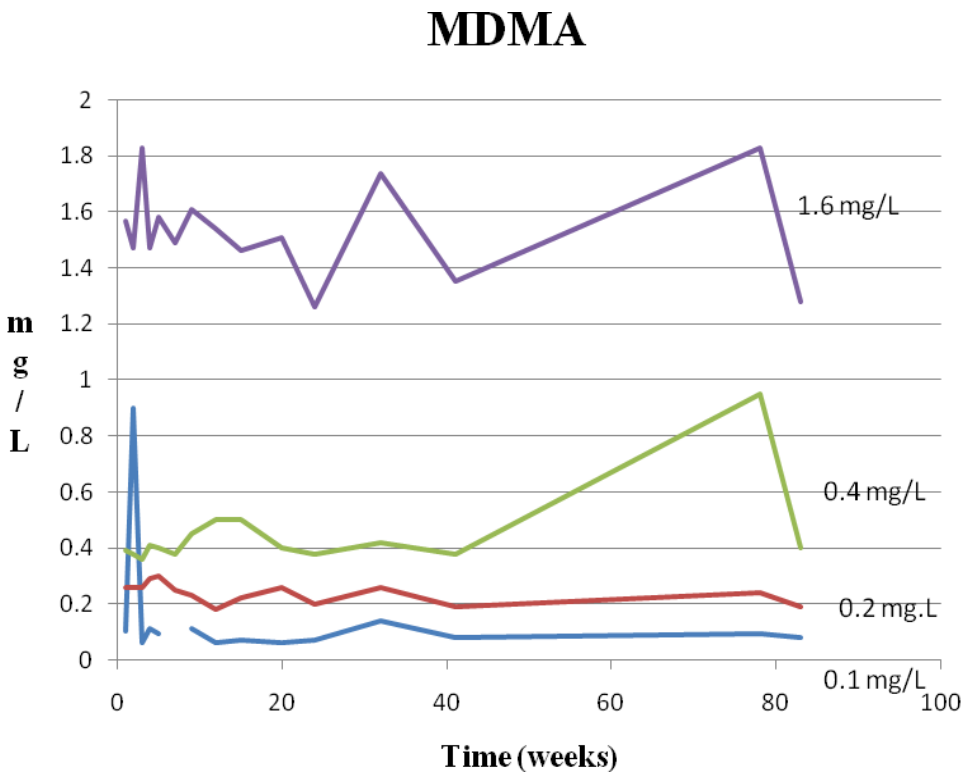

Table 17. MRM Transitions of drugs/analytes used for stability studies.

Drug Name	MRM Transition	Mix-Group
7-aminoclonazepam	286.1/121.2 amu	2-C
Acetaminophen	152.1/110.0 amu	5-G[2]
Alprazolam	309.1/205.0 amu	2-C
Amitriptyline	278.2/233.0 amu	4-D[2]
Benzoylecgonine	290.1/168.0 amu	5-E[2]
Benztropine	308.2/167.0 amu	4-D
Buprenorphine	468.3/55.1 amu	1-A
Bupropion	240.2/184.0 amu	2-C
Buspirone	386.2/122.0 amu	4-D[2]
Carbamazepine	237.1/194.0 amu	5-F
Carisoprodol	261.2/176.0 amu	5-G[2]
Chlordiazepoxide	300.1/227.0 amu	5-E[2]
Citalopram	325.2/109.0 amu	3-D[1]
Clonazepam	316.0/270.0 amu	1-B
Cocaethylene	318.2/196.0 amu	2-C
Cocaine	304.1/182.0 amu	3-D[1]
Cyclobenzaprine	276.2/215.0 amu	1-A
Dextromethorphan	272.2/128.0 amu	1-A
Diazepam	285.1/193.0 amu	2-C
Diltiazem	415.2/178.0 amu	3-D[1]
Diphenhydramine	256.2/167.0 amu	3-D[1]
Doxepin	280.2/107.0 amu	4-D[2]
EDDP	278.2/234.0 amu	1-A
EMDP	264.2/220.0 amu	2-C
Fentanyl	337.2/188.0 amu	1-A
Fluoxetine	310.1/44.0 amu	4-D[2]
Gabapentin	172.2/137.2 amu	5-F
Haloperidol	376.1/123.0 amu	1-B

Drug Name	MRM Transition	Mix-Group
Hydroxyzine	375.2/201.0 amu	2-C
Ketamine	238.1/125.0 amu	5-E[2]
Lamotrigine	256.0/211.0 amu	5-F
Lorazepam	321.0/275.0 amu	1-B
Maprotiline	278.2/191.0 amu	3- D[1]
Meperidine	248.2/220.0 amu	4-D[2]
Mepivacaine	247.2/198 amu	Internal Standard
Meprobamate	219.1/158.0 amu	4-G[1]
Mesoridazine	387.1/98.0 amu	5-F
Metaxalone	222.1/161.0 amu	5-F
Methadone	310.2/265.0 amu	3-D[1]
Metoprolol	268.2/116.0 amu	2-C
Midazolam	326.1/291.0 amu	4-D[2]
Mirtazepine	266.2/195.0 amu	3-D[1]
Nefazodone	470.2/274.0 amu	4-D[2]
Nordiazepam	271.1/140.0 amu	3-D[1]
Normeperidine	234.1/160.0 amu	3-D[1]
Norpropoxyphene	326.2/252.0 amu	5-E[2]
Nortriptyline	264.2/117.0 amu	3-D[1]
Olanzepine	313.1/256.0 amu	1-B
Oxcarbazepine	253.0/180.0 amu	4-E[1]
Paroxetine	330.1/70.0 amu	2-C
Pentazocine	286.2/218.0 amu	2-C
Promethazine	285.1/198.0 amu	1-B
Propoxyphene	340.2/58.0 amu	4-D[2]
Quetiapine	384.2/253.0 amu	5-E[2]
Scopolamine	304.1/138.0 amu	1-A
Sertraline	306.1/275.0 amu	3- D[1]
Temazepam	301.1/255.0 amu	5-E[2]
Thioridazine	371.2/98.0 amu	4-D[2]

Drug Name	MRM Transition	Mix-Group
Tizanidine	254.4/210.0 amu	1-A
Tramadol	264.2/58.0 amu	4-D[2]
Trazodone	372.2/176.0 amu	5-E[2]
Venlafaxine	278.2/58.0 amu	3-D[1]
Verapamil	455.3/165.0 amu	4-D[2]
Zolpidem	308.2/235.0 amu	3-D[1]
Zopiclone	389.1/245.0 amu	2-C

Table 18. Supplemental Drugs and MRM Transition

Drug Name	MRM Transition	MIX - Group
Mepivacaine	247.0/98 amu	
α-Hydroxyalprazolam	325.1/297 amu	
Carbamazepine-10,11-epoxide	253.1/180 amu	
10,11-Dihydro-10-hydroxycarbamazepine	255.1/194 amu	
Demethylcitalopram	311.2/109 amu	
Didemethylcitalopram	297.1/109 amu	
Zaleplon	306.2/236 amu	
Ziprasidone	413.1/194 amu	
Zolazepam	287.1/138 amu	

Table 19. LC gradient parameters for stability studies.

LC Program Table			
TIME (min)	**Flow (µL/min)**	**%A**	**%B**
0	200	95	5
1	200	95	5
17.9	200	5	95
18	200	95	5
20	200	95	5

Table 20. Mass Spectrometer parameters for stability studies.

SOURCE PARAMETERS			MS/MS PARAMETERS	
Source Mode	TurboSpray		MS Mode	MRM
Source Voltage	5500V		Q1	Unit
Curtain Gas	35 PSI		Q2	Unit
Nebulizer gas	50 PSI		CAD pressure	Medium
Drying Gas	55 PSI		CEM	~2400 V
Drying Gas	500°C		Scan Time	1.0 sec

Table 21. MIX 1 Containing Groups A&B

Level	Group A (mg/L)	Group B (mg/L)
1	0.001	0.02
2	0.01	0.05
3	0.05	0.15
4	0.1	0.5

Group A contained the following drugs**:** buprenorphine, cyclobenzaprine, 2-ethylidene-1,5-dimethyl3,3-diphenylpyrrolidine (EDDP), dextromethorphan, fentanyl, scopolamine, tizanidine

Group B contained the following drugs**:** clonazepam, haloperidol, lorazepam, olanzapine, promethazine

The calibration range for MIX 1 drugs was 0.0005 mg/L to 0.128 mg/L for group A, and 0.02 mg/L to 5.12 mg/L for mix B.

Table 22. MIX 2 Containing Group C

Level	Group C (mg/L)
1	0.02
2	0.05
3	0.15
4	0.50

Group C contained the following drugs: bupropion, 2-Ethyl-5-methyl-3,3-diphenylpyroline (EMDP), metoprolol, diazepam, 7-aminoclonazepam, pentazocine, alprazolam, cocaethylene, paroxetine, hydroxyzine, zopiclone

The calibration range for MIX 2 was 0.02 mg/L to 0.64 mg/L.

Table 23. MIX 3 Containing Group D[1]

Level	Group D[1] (mg/L)
1	0.05
2	0.01
3	0.3
4	0.50

Group D[1] contained the following drugs: normeperidine, diphenhydramine, nortriptyline, mirtazapine, nordiazepam, maprotiline, venlafaxine, cocaine, sertraline, zolpidem, methadone, citalopram, diltiazem

The calibration range for MIX 3 was 0.05 mg/L to 1.0 mg/L.

Table 24. MIX 4 Containing Group D[2], E[1], G[1]

Level	Group D[2] (mg/L)	Group E[1] (mg/L)	Group G[1] (mg/L)
1	0.1	0.25	5.0
2	0.2	0.5	10
3	0.4	1.0	20
4	1.6	4.0	50

Group D[2] contained the following drugs: meperidine, tramadol, amitriptyline, doxepin, benztropine, fluoxetine, midazolam, propoxyphene, thioridazine, buspirone, verapamil, nefazodone

Group E[1] contained the following drug: oxcarbazepine

Group G[1] contained the following drug: meprobamate

The calibration range for MIX 4 was 0.1 mg/L to 3.2 mg/L for group D[2], 0.25mg/L to 8 mg/L for group E[1], and 5 mg/L to 75 mg/L for group G[1].

Table 25. MIX 5 Containing Groups E^2, F, G^2

Level	Group E^2 (mg/L)	Group F (mg/L)	Group G^2 (mg/L)
1	0.25	1.5	5.0
2	0.5	3.0	10.0
3	1.0	10.0	20.0
4	4.0	40.0	50.0

Group E^2 contained the following drugs: ketamine, benzoylecgonine, chlordiazepoxide, temazepam, norpropoxyphene, trazodone, quetiapine

Group F contained the following drugs: gabapentin, metaxalone, carbamazepine, lamotrigine, mesoridazine

Group G^2 contained the following drugs: acetaminophen, carisoprodol

The calibration range for MIX 5 was 0.25 mg/L to 8 mg/L for group E^2, 1.5 mg/L to 48 mg/L for group F, and 5 mg/L to 75 mg/L for group G^2.

Table 26. Mixtures for Amphetamine Studies.

	MIX 8	MIX 9
Level	**C**	**D**
1	0.05	0.10
2	0.01	0.20
3	0.3	0.40
4	0.50	1.6

All drug concentrations are given in mg/L. The group identifier is provided as the column header.

Level 1 is a low/sub therapeutic dose, Level 2 is therapeutic dose, Level 3 is a high therapeutic/toxic dose and Level 4 is overdose.

Mix 8 Group C contained the following drugs**:** amphetamine, metheylenedioxy-methamphetamine (MDA), with a calibration range 0.05 mg/L-1.0 mg/L

Mix 9 Group D contained the following drugs**:** methamphetamine, methylenedioxymethamphetamine (MDMA), with a calibration range 0.10 mg/L-3.2 mg/L.

Table 27. MIX 6 containing Groups A^2, E^3, F^2 - Supplemental Stability Study

	MIX 6		
Level	**A^2**	**E^3**	**F^2**
1	0.01	0.25	1.5
2	0.02	0.5	3.0
3	0.05	1.0	6.0
4	0.10	4.0	NA

All drug concentrations are given in mg/L. The group identifier is provided as the column header.

Level 1 is low/sub therapeutic, Level 2 is therapeutic, Level 3 is high therapeutic/toxic and Level 4 is overdose

Level 1 and 2 concentrations in Group A^2 differ from those used in the Stability Study for these drugs.

Group A^2 contained the following drugs: Alpha-hydroxy alprazolam, dimethylcitalopram. The calibration range was 0.0005 mg/L to 0.128 mg/L.

Group E^3 contained the following drug: Zolazepam. The calibration range was 0.25 mg/L to 8.0 mg/L.

Group F^2 contained the following drug: 10,11-Dihydro-10-hydroxycarbamazepine (Note: the level 4 sample was not prepared due to a limited supply of the drug standard) The calibration range was 0.25 mg/L to 8.0 mg/L.

Table 28. MIX 7 containing Groups A^3, B^3, F^3 - Supplemental Stability Study

MIX 7		
A^3	**B^3**	**F^3**
0.01	0.02	1.5
0.02	0.05	3.0
0.05	0.15	6.0
0.10	0.50	24

Group A^3 contained the following drugs: Didemethylcitalopram, zaleplon. The calibration range was 0.0005 mg/L to 0.128 mg/L.

Group A^3 contained the following drug: Ziprasidone. The calibration range was 0.02 mg/L to 0.64 mg/L.

Group E3 contained the following drug: Carbamazepine-10,11-epoxide. The calibration range was 1.5 mg/L to 48 mg/L.

Table 29. Testing Interval for Stability Study

Test Number	Date	Elapsed Time (days) Since Last Test	Total Elapsed Time (days) Since Prep
0	9/13/2007	0	Preparation Day
1	9/20/2007	6	7
2	9/27/2007	7	14
3	10/04/2007	14	21
4	10/17/2007	13	34
5	11/01/2007	15	49
6	11/16/2007	15	64
7	11/29/2007	13	77
8	01/18/2008	50	127
9	02/29/2008	42	169
10	04/05/2008	36	205
11	05/16/2008	31	236
12	07/17/2008	62	298
13	09/27/2008	72	370
14	05/22/2009	248	618

Table 30. Testing Interval for Supplemental Stability Study

Test Number	Date	Elapsed Time (days) Since Last Test	Total Elapsed Time (days) Since Prep
0	3/25/08	0	Preparation Day
1	4/1/08	7	7
2	4/8/08	7	14
3	4/15/08	7	21
4	4/22/08	7	28
5	5/6/08	14	42
6	5/20/08	14	56
7	6/30/08	14	70
8	6/17/08	14	84

Table 31. Testing Interval for Amphetamine Stability Study

Test Number	Date	Elapsed Time (weeks) Since Last Test
1	3/11/2008	0
2	3/18/2008	1
3	3/25/2008	2
4	4/1/2008	3
5	4/15/2008	5
6	4/29/2008	7
7	5/13/2008	9
8	5/27/2008	11
9	6/23/2008	15
10	7/29/2008	20
11	8/26/2008	24
12	10/21/2008	31
13	12/17/2008	40
14	9/1/2009	77
15	10/8/2009	82

Table 32. Stability Drugs Exhibiting Significant Losses of Concentration

Analyte	% Change Since Day One		
	34 days	370 days	618 days
cocaine	-17%	-86%	-75%
cocaethylene	-20%	-54%	-76%
benzoylecgonine	6%	-34%	-27%
clonazepam	-17%	-94%	-85%
mesoridazine	-17%	-43%	-80%
bupropion	-34%	-84%	-51%
diltiazem	-40%	-80%	-73%
zopiclone	-52%*	-98%	ND

* zopiclone loss at 49 days

Table 33. Supplemental Stability Study Drugs Exhibiting Significant Losses of Concentration

Analyte	% Change Since Day One		
	28 days	112 days	385 days
Ziprasidone (A)	-73%	-74%	-98%
Ziprasidone (B)	-38%	-51%	-75%

Literature Cited

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Chapter 6. Summary and Conclusions

This research project set out to accomplish three different objectives and all three were achieved. The first goal was the investigation and development of new or enhanced methods for the analysis of drugs using an LC/MS/MS instrument platform. The main goal of these method development efforts was to determine if additional drug classes could be successfully analyzed on the LC/MS/MS instrument following a simple one to two hour sample preparation step. If this analytical approach were successful, time consuming and costly sample preparation steps could be avoided, thus enhancing the overall effectiveness of forensic toxicology laboratories.

The first method development study involved THC and THC metabolites. The goal of this study was to improve the ability of forensic toxicology laboratories to detect major THC metabolites and the THC parent compound following the simple drug extraction protocol. Another key element was to improve the sensitivity of the method so that lower concentrations of THC and THC compounds could be positively identified and quantitated. The research presented in this report demonstrated that such a method was possible and practical using laboratory prepared specimens. However, when actual casework blood samples were analyzed, the method failed to recover sufficient quantities of the drug analytes or internal standards. This was presumably due to matrix effects of the whole blood samples received as part of routine casework, since similar effects were not observed from the control samples. Although the results of this study are very promising, significant additional development work and validations using samples that have been previously analyzed will be needed prior to implementation of this THC method into routine casework.

The second method development study involved opioid compounds and opioid metabolites. Again, the goal of this particular study was to improve the ability of forensic toxicology laboratories to detect opioid compounds and analytes on the Applied Biosystems QTrap® LC/MS/MS following the simple extraction procedure. During a previous NIJ sponsored research study into the use of this instrument platform (Herrin, McCurdy & Wall, 2005), analysis of opioid compounds was unsuccessful due to ion suppression of the signal. During this study alternative chromatography methods were investigated to determine if the opioid compounds could be eluted earlier or later, thus avoiding the region of ion suppression observed in the previous project. Chromatography results obtained using a Phenomenex Synergi column achieved this objective, allowing adequate separation of the various analytes and sufficient specificity. Additional experiments undertaken during this opioid study included determination of limit of detection (LOD), limit of quantitation (LOQ), interference studies to recognize and minimize any potential misidentification of analytes, and concordance studies with samples previously analyzed using gas chromatography/mass spectrometry (GC/MS). The LOD and LOQ for opioid analytes commonly encountered in the GBI Division of Forensic Sciences toxicology section were determined using the method developed here and have been provided earlier in this report. The concordance study demonstrated that the method developed is viable for implementation into routine casework, however because this method utilizes a different LC column and mobile phase solvents, additional factors such as instrument availability and the costs associated with implementing several different methods have to be considered.

The third method development study was to determine if barbiturate and acidic drugs could be

successfully analyzed on the QTrap® instrument. Different instrument parameters and chromatography conditions were evaluated, but none produced results suitable in quality or reproducibility as compared to more traditional GC/MS methods. The major difficulty in analysis of these compounds was the lack of sufficient detail in the mass spectra generated by the QTrap® instrument. In most instances the barbiturate and acidic drugs produced only a single fragment ion peak, and in many cases this fragment peak was the same between different compounds. Although it is possible to make possible identifications using a combination of elution time or relative retention time from the chromatography phase of the analysis plus a single ion peak, this is not an optimal situation, especially when several different compounds exhibit the same ion peak. For this reason, further development of this method was suspended and there are no immediate plans to continue investigations into this analytical approach for barbiturates or acidic drugs.

The second goal of this research project was the investigation of drug stability after extended storage in refrigerated liquid blood samples. This line of research was prompted by the observations made during the prior R&D project (Herrin, McCurdy & Wall, 2005). While conducting the concordance studies in that project several samples were encountered in which the drug concentrations appeared to have dropped since the original analysis was conducted. To clarify and better understand this phenomenon, samples were prepared and stored under controlled conditions, then periodically analyzed over a 20 month period. These analyses confirmed that nine drugs significantly degraded under these conditions. Although the degradation of cocaine has been well documented (Isenschmid, 1989), the rapid degradation of mesoridazine, bupropion, and diltiazem observed in the blood samples was not as expected. The stability of samples containing amphetamine compounds was also studied. These compounds have special relevance when consideration is made of the increased utilization of drugs such as methamphetamine and ecstasy over the last several years. None of the amphetamine compounds exhibited any degradation during the study period.

The third major goal of this research project was to disseminate the findings of the methods developed during the 2003 research project (2003-IJ-CX-K007) on this same instrument platform. The National Institute of Justice funds a numerous research projects within forensic science, but the value of many of those projects goes unrealized unless forensic laboratories actually take the time and effort to implement the new methods and techniques into routine casework. One such mechanism to facilitate the implementation of new methods is through training workshops that familiarize forensic scientists with the concepts and practical applications of the methods developed as a result of a NIJ research and development award. In this project, the GBI Division of Forensic Sciences developed a week long workshop on the practical applications of using the LC/MS/MS QTrap® instrument in forensic toxicology. Attendees to the workshops got hands on experience with the extraction method used in our laboratory and initial familiarization with the instrument and the data analysis software. A total of 34 forensic scientists attended the workshops at no cost to their parent agencies. The feedback regarding the workshop content and knowledge transfer was overwhelmingly positive from the attendees. Future funding of similar workshops would be a very effective tool to improve the dissemination of R&D project outcomes, especially for projects involving method development. The benefits of the workshop at a working forensic laboratory include the ability for attendees to gain knowledge regarding the technique, building of professional relationships, and informal exchanges of information concerning casework trends, and alternative analytical approaches to take in unusual cases.

This research project was very successful, with the development of new methods for analysis of THC, THC metabolites, and opioids on an LC/MS/MS instrument platform following a simple one to two hour sample preparation step. By implementing these methods, laboratories have the potential to reduce labor time for sample preparation by up to 60% over more conventional methods such as solid phase or liquid-liquid extraction methods. The analytical methods developed during this project require further validation prior to implementation into routine casework but they have the potential to substantially reduce the labor associated with these types of analysis. Opioid compounds are involved in a significant number of postmortem cases each year and any improvements in the analytical method will be a welcome addition to the techniques available since it could result in faster turnaround times of results to the medical examiner or coroner who is responsible for establishing cause and manner of death. In many cases the main cause of delay in issuance of a death certificate is the availability of postmortem toxicology results.

This project also determined that some drugs present in biological specimens stored at normal refrigeration temperatures do degrade or decompose over time. Having this knowledge is crucial when interpreting the results of testing that occurs months or even years after the sample is collected. There are many cases where the results of initial toxicology testing results are challenged or additional testing is required to resolve an issue arising from civil litigation. The knowledge that the later testing may produce results inconsistent with the original analysis allows the toxicologist to make an informed decision as to the validity and reliability of the conclusions reached in a particular case. Knowing that certain drugs degrade fairly rapidly in storage also has significant implications for forensic laboratories experiencing large backlogs or where testing may be delayed for other reasons. Forensic laboratories can utilize the information from this study to modify sample storage policies and analytical testing schema to ensure accurate and reliable results reflective of actual drug content are obtained in all cases.

Finally, through the use of the training workshops conducted as part of this project and presentations at national toxicology meetings, the forensic toxicology community has been exposed to the potential of using LC/MS/MS instrumentation to improve the scope and timeliness of toxicology analysis. This project produced new knowledge, aided in the development of new methods, and disseminated information in a very effective fashion.

Literature Cited

Herrin, G., McCurdy, H.H., and Wall, W.H. (2005) Investigation of an LC–MS–MS (QTrap®) Method for the Rapid Screening and Identification of Drugs in Postmortem Toxicology Whole Blood Samples**.** *J. Anal. Toxicol.* **29**: 599-606.

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Appendix A. Workshop Training Materials

Overview of LC/MS/MS training

1. Welcome, Introduction and goals
2. Why LC- triple quadrupole MS?
3. GBI Forensic Toxicology Applications, case flow, benefits to GBI
4. Theory
5. Hardware introduction
6. Software introduction
7. Tune, resolution optimization and test mix
8. Batch setup and inject test mix
9. Compound infusions (quantitative optimizations)
10. GBI LC/MS/MS methods and QC
11. Acquisition method and method development
12. Library
13. Blood extractions and qualitative analysis
14. Inject quantitative standards
15. Introduction to quantitations
16. Quantitation exercise
17. Validation studies review
18. Validation exercises
19. Cleaning, maintenance, and troubleshooting
20. Review
21. Certificate presentations

Section 1. Introduction

1. Staff Introductions
2. Student introductions (experience with LC, MS, LC/MS/MS, etc)
3. Security, restrooms, class hours, informal, breaks, questions
4. Why LC-triple quad? (module 1-2)
5. Goals of workshop
6. Introduction to LCMS – goal is not to train you to use the instrument but to show what the system can do.
7. Benefits to forensic toxicology laboratory
8. Useful information and hands-on experience
9. Use this week of experience to gain knowledge to determine if this instrument would be suited to your operations.
10. Possibly help you provide justification for purchase of LC-triple quad instrument
11. Integration into FT, applications, benefits to GBI Toxicology, case flow
12. Instrument specifications

Section 2. Why LC-Triple Quad?

1. Does not entirely replace other instruments (GC/MS and EIA)
2. Greatly reduced sample preparation time and expense
3. More suited for "dirty" samples
4. Faster throughput and results
5. Less use of solvents
6. Safer
7. Lab tech can extract the samples
8. Less problems with sample consumption
9. Can eliminate some EIA testing
10. Ability to target specific drug panel screens based on case needs
11. Extracts more stable during analysis time (solvent evaporation)
12. Able to detect co-eluting drugs with no interference
13. Similar to GC/MS for quantitative reliability

Section 3. Hardware Introduction

1. LC Autosampler – vials, screw caps, septa, needle, plumbing, sample tray, slot 99, liquids
2. LC requirements – no PO4, frit, solvents A and B and wash solution and usage
3. Blood extracts – reconstitution solvent – quantitative versus qualitative, solvent and "trash" dead zone
4. Column and tubing
5. TurboIon spray
6. Syringe pump
7. Curtain plate, spray pattern, cleaning, gases
8. Orifice plate, skimmer, Q0, Q1, Q2, and Q3 (fixed and ramped)- review MRM uses Q1 and Q3 fixed; Q2 is CAD for fragmentation. For IDA experiment Q3 is ramped (LIT)
9. Vacuum system – turbo system is differentially pumped
10. Gas generator – produces nitrogen and zero grade air
11. Waste gas and liquids

Section 4. Software Introduction

1. Analyst software
2. File structure, “API Instrument Project” folder
3. *.dam are method files, .
4. *.wiff are data files,
5. *.dab are batch files
6. Hardware configuration setup
7. Overview of main menu screen
8. queue, start, stop, ready, standby, centroid, info, Explorer, etc
9. LC software

Section 5. Tuning and Calibration

1. Curtain plate cleaning
2. *Exercise*: Perform curtain plate cleaning
3. PPG
4. Hardware setup
5. Infusion of PPG and requirements, see SOP
6. Manual tuning
7. *Exercise:* Perform PPG infusion and calibration, check for suitability
8. Resolution optimization
9. Exercise: Perform PPG infusion and Resolution optimization, re-run PPG calibration check, check for suitability
10. Batch setup
11. Test mix, see SOP
12. Components and requirements
13. *Exercise*: Perform 3 test mix injections, check for suitability, compare chromatograms

Section 6. Quantitative Optimization

1. Used to determine method parameters for compound detection by MRM
2. Review Validation Study 1 (Determination of Optimal Collision Energy for Drugs**)**
3. Infusions – hardware setup usually the same as PPG infusions
4. Exercise: Using codeine, midazolam and nortriptyline, dilute one drug to 5 mcg/mL with mobile phase, infuse, perform quantitative optimization. Dilute if necessary. Repeat with the other 2 drugs. Obtain the parameters. Compare to those used in the current instrument screening method.
5. Review the "Mass Spectrometer Parameters" chart

Section 7. GBI Toxicology LC – triple quad SOP and QC

1. Review Q Trap Operation SOP
2. Review Q Trap Calibration SOP
3. Review Q Trap Maintenance SOP
4. Review Q Trap Acceptable Work Product SOP
5. Review portion of Comprehensive Quantitation of Drugs Using Precipitation SOP
6. Brief review of Blood Enzyme Immunoassay Analysis SOP

Section 8. Library

1. Somewhat instrument dependent
2. Identifications
    a. different than single quad GC/MS, fragments produced are from Q1 selected ion only
    b. problems: examples of too few ions (see SOP), quantitations – other drug metabolite with same parent ion and daughter ion.
3. Overlapping or co-eluting compounds with same mass result in a combined spectrum.
4. Library spectra ratio of product ions are very dependent on collision energy. The library spectra are obtained by averaging 3 spectra taken at 3 different CES settings: 10, 20, and 50EV (20 +/- 30). Object is to retain a little of the precursor ion and produce as many fragment ions as possible leaning toward retention of the highest mass ions since they have greater identification value.
5. *Exercise*: Using a test mix run, perform library searches on all found peaks of interest.
6. Library retrieval (list with constraints)

Section 9. Sample Preparation and Qualitative Analysis

1. "Pure" drugs for infusions
2. Urine samples, diluted 1:10, limitations, not validated
3. *Exercise*: dilute provided urine samples 1:10 and analyze. Provide a list of the drugs found. See Validation Study 16 (Analysis of Urine)
4. Blood samples
5. GBI method for blood samples (acetone precipitation)
6. *Exercise*: View or review BLEIA extraction and reconstitution. Transfer to sample vials, inject samples into LC-MS/MS. Provide TIC, EIC for each drug, and library comparison of unknown mass spectrum.

Section 10. Quantitative Analysis

1. Compare quantitative method to qualitative method
2. Identify a quantitative batch run on the computer or prepare and inject a series of at least 10 standards ranging from 0.005 to 5 mg/L. Also inject the standards after diluting by adding 1 mL of buffer to a 100 mcl aliquot of each.
3. *Exercise:* Set up a quantitative method using one of the standards and then process the run
    a. build a new method using one standard as a representative sample
    b. fill in the internal standard (if used), and transitions for each analyte
    c. check the integration using the integration tab
    d. check the calibration tab
    e. save the method
    f. click the Quantitation Wizard
    g. move the desired files to include list
    h. select the method
    i. when the table appears, set the table settings
    j. set the sample type if needed
    k. fill in the calibrators concentrations
    l. click on the upper gray area for options, e.g. graphing and queries
    m. obtain the results for the controls (unknowns)

Section 11. Validation Studies Review

1. Reproducibility Study (3)
2. Carry-over Study (4)
3. Spiked Blind Proficiency Test Study (5)
4. Dwell Optimization Study (6)
5. Same Mass Co-elution Study (7)
6. Limit of Identification Study (8)
7. Limits of Identification Chart
8. Mixed Drug Study (9)
9. Batch Size Study (10)
10. Enzyme Immunoassay Extract Stability Study (11)
11. Extract Suitability for Quantitation Study (12)
12. System Performance Acceptability Study (13)
13. Calibrator Suitability for Quantitation Study (14)
14. Brief Pesticide Detection Study (15)

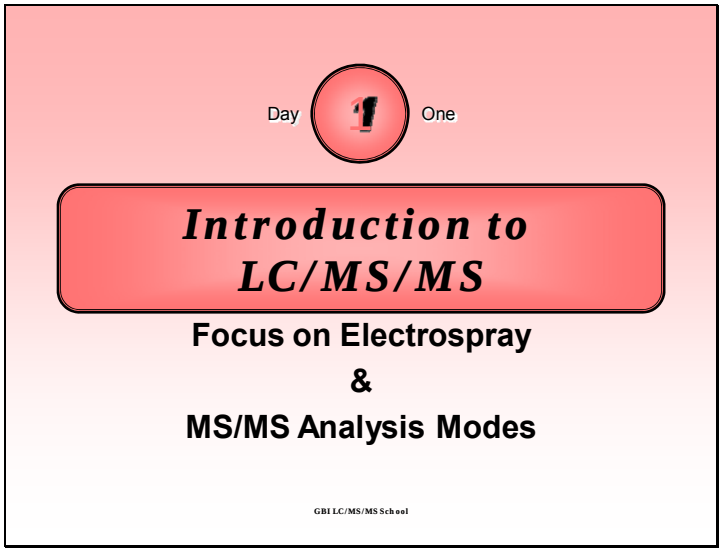
Section 12. Unknowns and projects

1. Obtain 2 unknowns per student. Inject and present a qualitative report of the analytes found.
2. Using the Qualitative screening method, modify it to only screen for the drugs in the test mix. Inject the test mix two times with each method and compare the results, e.g. retention times, scans across a peak, peak areas, peak heights, mass spectra, EPI TIC, noise, background, etc.
3. Using a codeine, midazolam, and nortriptyline standard, create 3 methods whereby one has a CE of 10, the next uses 30, and the last uses 50. Inject the standard using the 10, 20, 50, and the method using a CES of 20 +/- 30. Compare the results.

Appendix B. Workshop Training Presentations

Day 1

Slide 1



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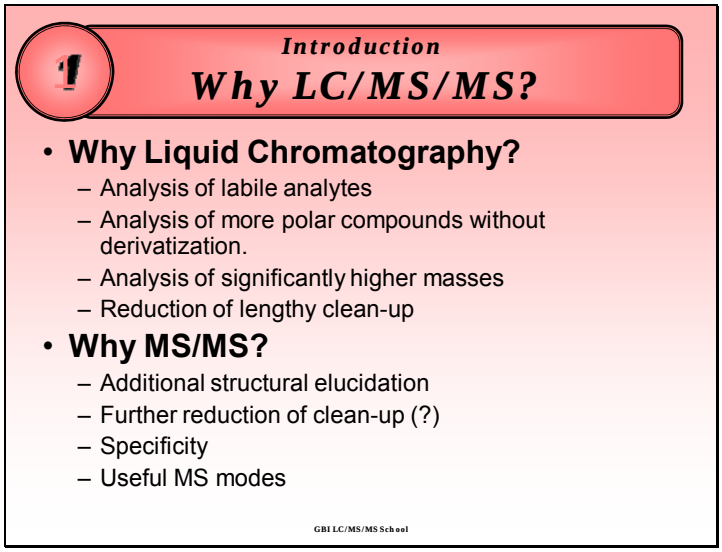

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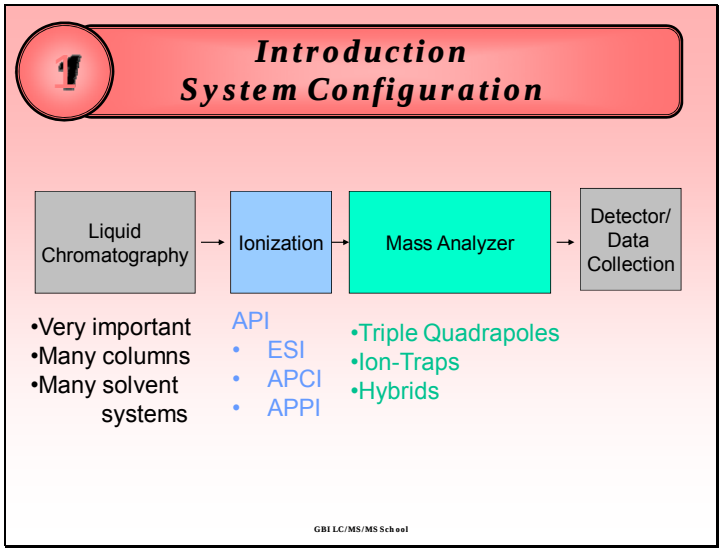

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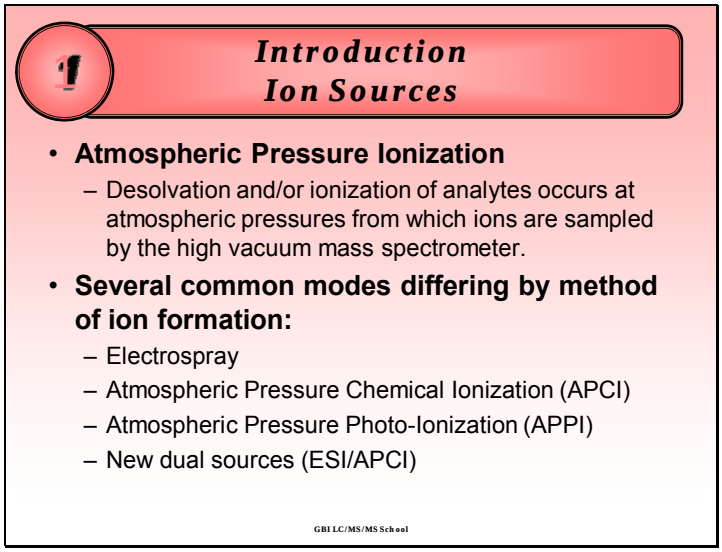

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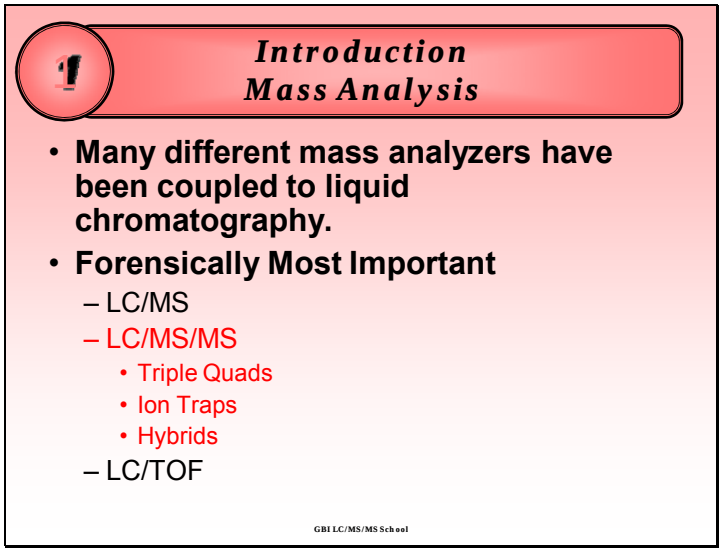

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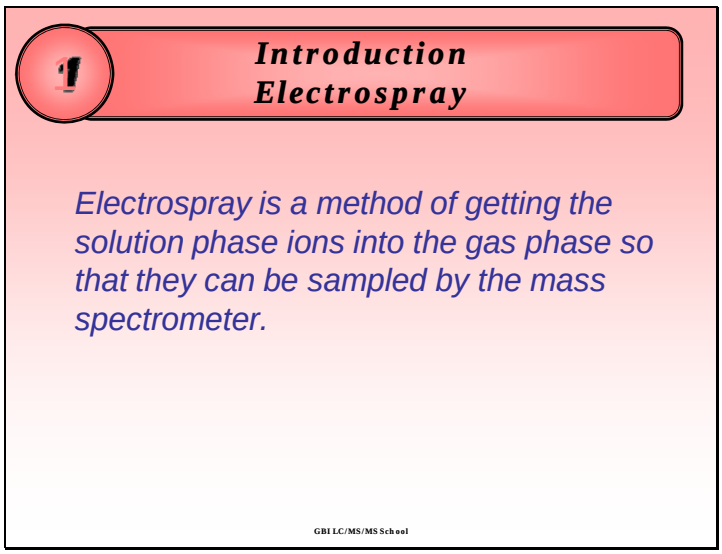
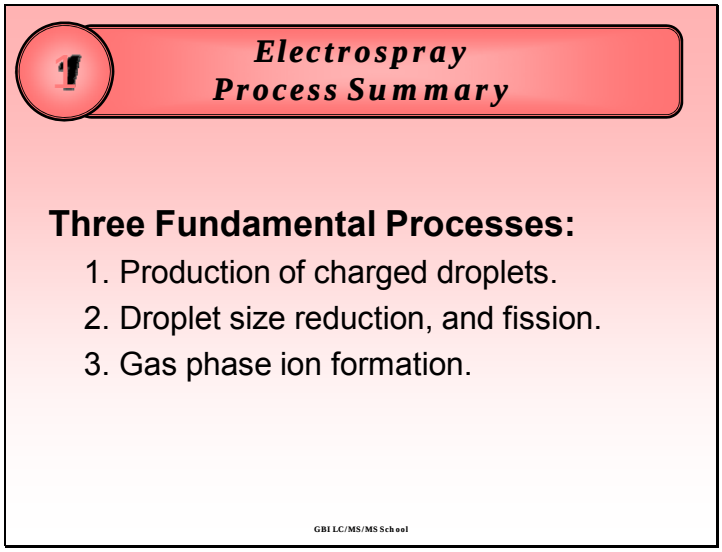


The LC eluent is nebulized in a strong electric field forming small charged droplets. As those droplets move toward the orifice opening of the MS they reduce in size. As they grow smaller columbic forces cause them to "explode" resulting in very small droplets. Ultimately bare gas phase ions are released from these very small droplets

Slide 7



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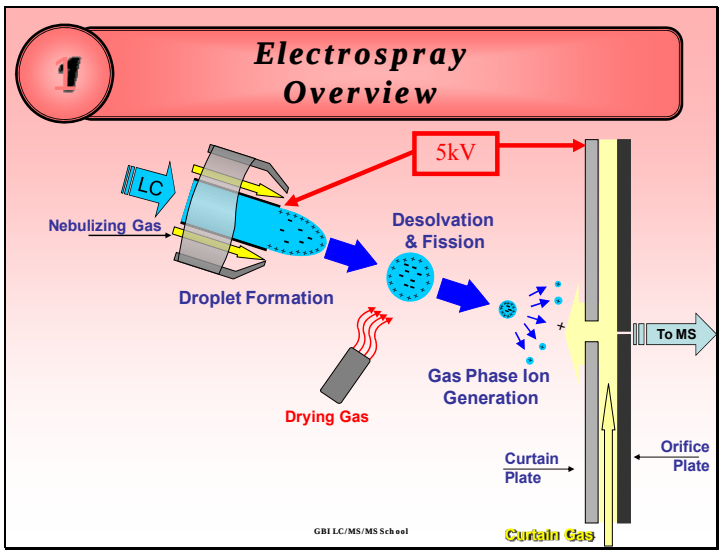

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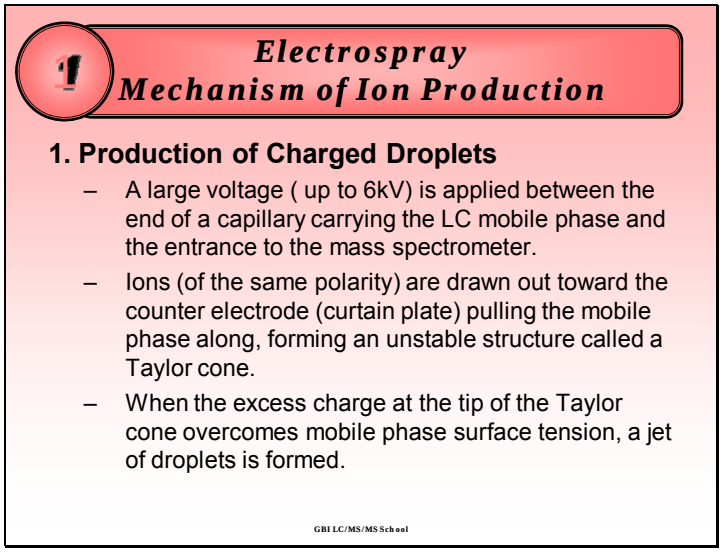

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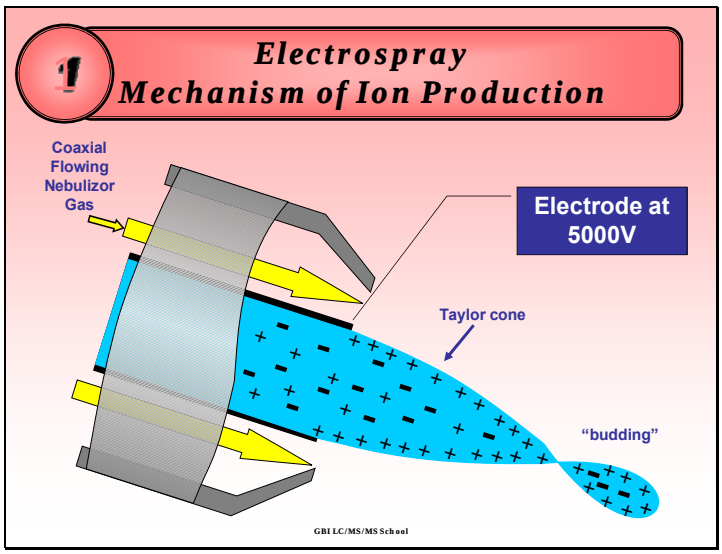


Slide 11

Electrospray Mechanism of Ion Production

- **The Droplets are …**
  - Typically less than 1000 nm in size, though size is proportional to flow rate. (faster = bigger)
  - enriched in ions of the same polarity as the potential gradient. (Positive mode = Positive ions)
    - In positive mode ions are $[M+H]^+$, $[M+nH]^{n+}$ and $[M+Na^+]^+$
    - In negative mode ions are $[M-H]^-$, $[M-nH]^{n-}$ and $[M+I^-]^-$
- **Ionization is…**
  - More efficient at lower flow rates, with smaller droplets.
  - More efficient with higher analyte concentration (where the mobile phase is the major diluent.)
- **Electrospray is concentration dependent!**

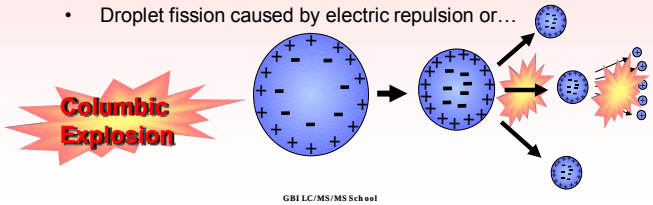
GBI LC/MS/MS School

Slide 12

Electrospray Mechanism of Ion Production

2. Droplet size reduction and fission.

- Ionization efficiency is related to droplet surface area.
  - Smaller droplets >> more gas phase ions >> better sensitivity.
- Droplet size reduction occurs by the continual repetition of two processes:
  - Desolvation (evaporation of neutral solvent and volatile buffers)
  - Droplet fission caused by electric repulsion or…



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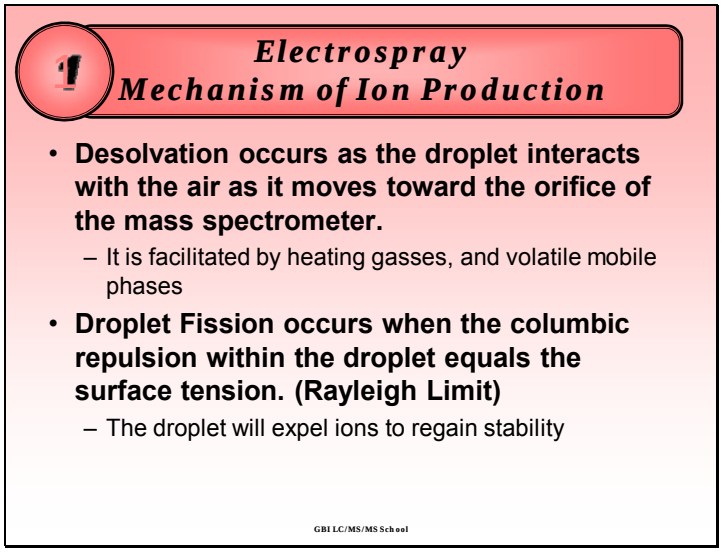
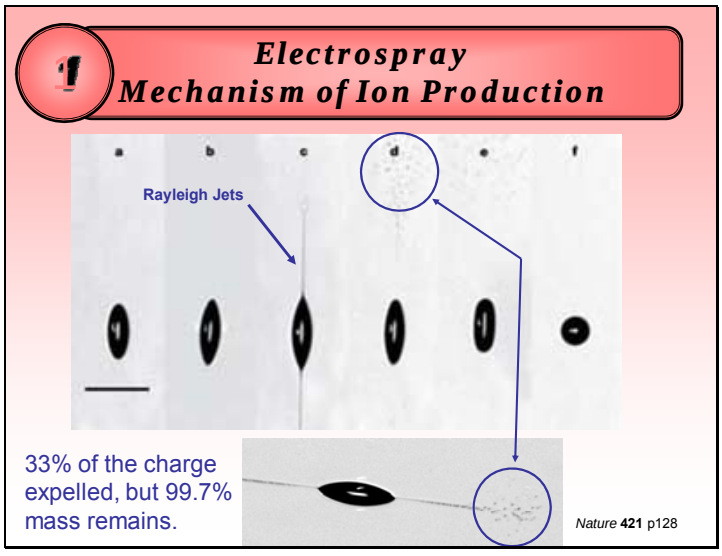


It is facilitated by:

- using a volatile mobile phase (H_20, MeOH)
- Using volatile buffers (formates vs. phosphates)
- Passing the droplets through a stream of heated gas.

Slide 14



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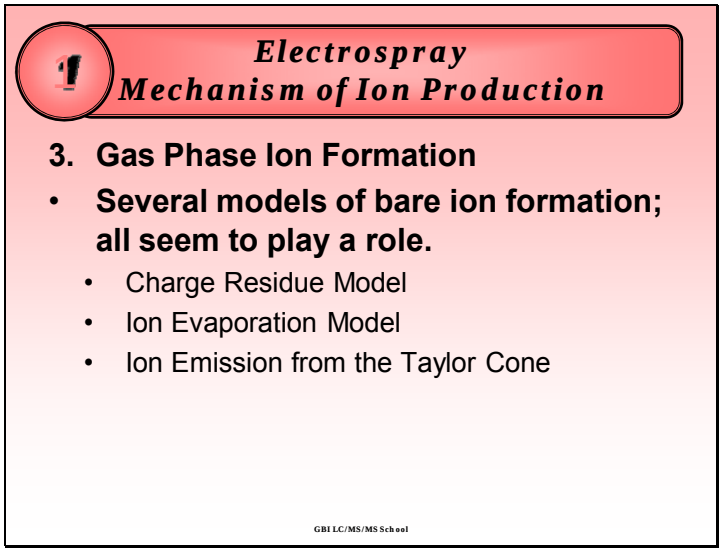

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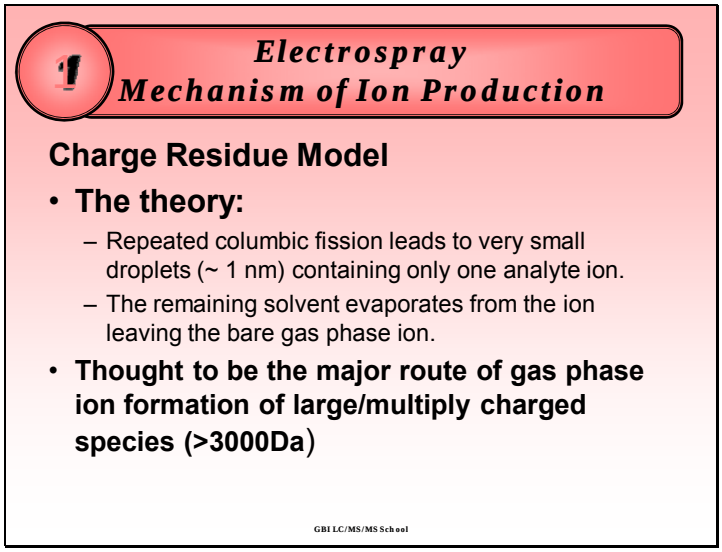

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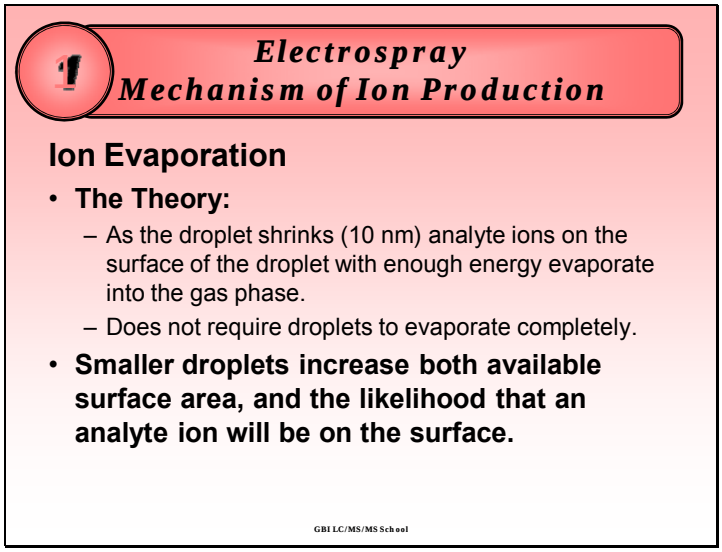

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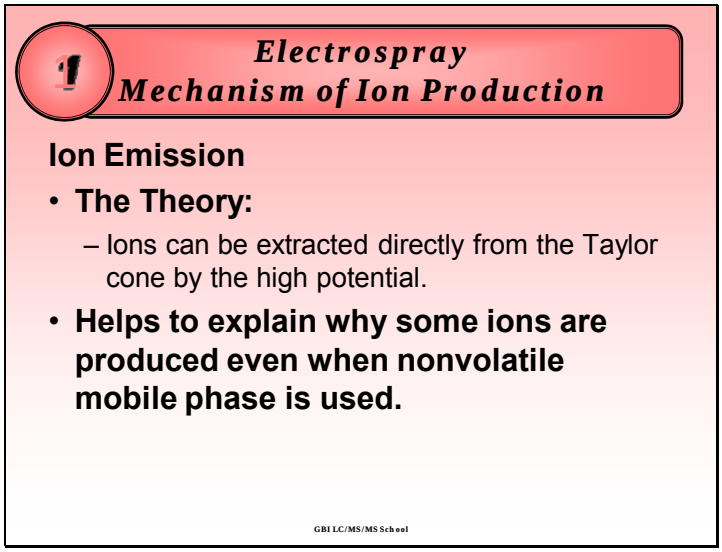

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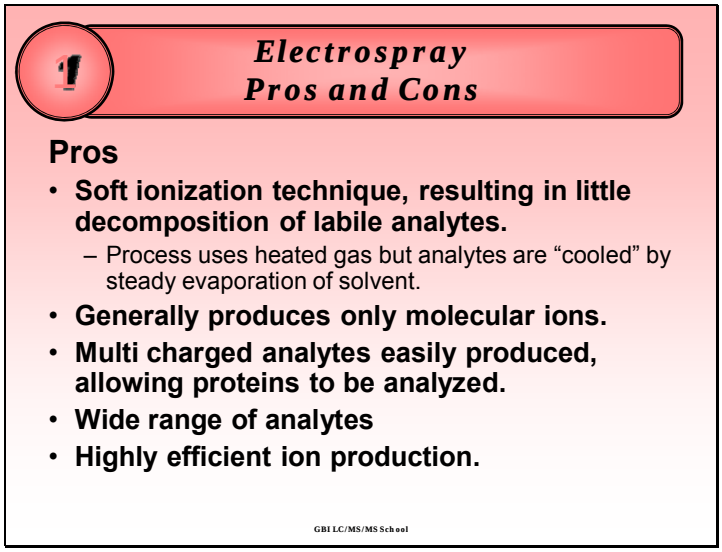

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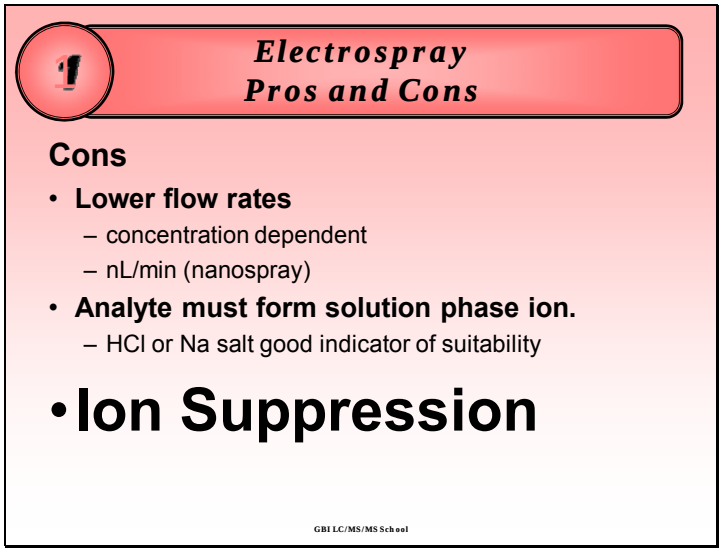

Slide 21

Electrospray Ion Suppression

- **Thought largely to result from inefficient droplet formation.**
- **More prominent early in an LC run, but can occur at anytime.**
- **Causes:**
  - Nonvolatile buffers or salts (phosphates)
  - Nonvolatile materials in mobile phase (i.e. biological junk)
  - Ion pairing
  - Reported that higher molecular weight analyte ions can suppress smaller analytes.
- **Underscores the need for good chromatography**

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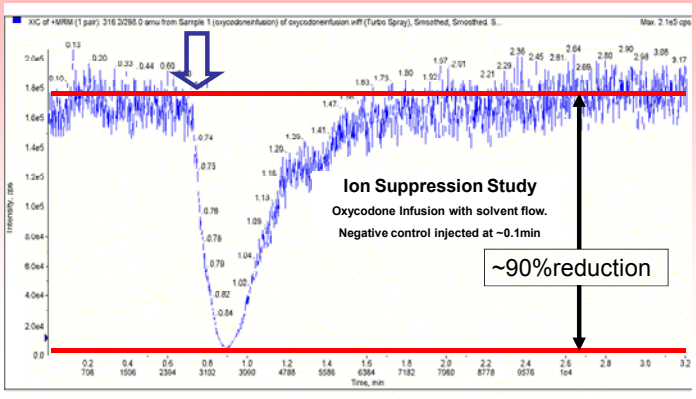
Slide 22

Electrospray Ion Suppression



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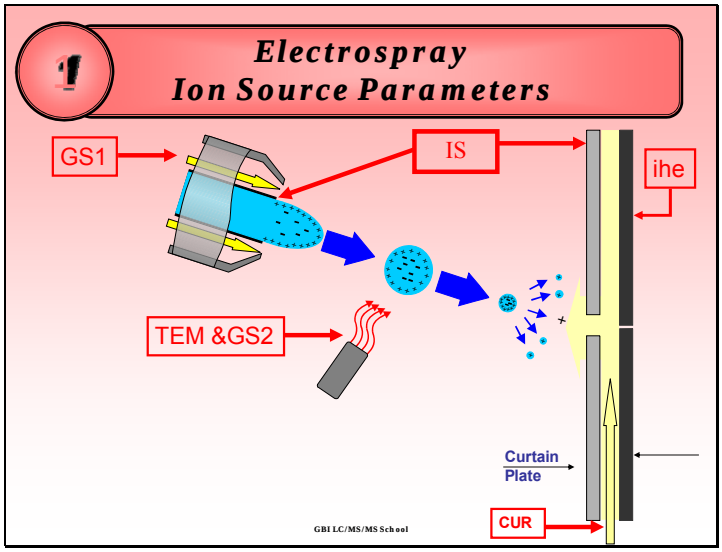

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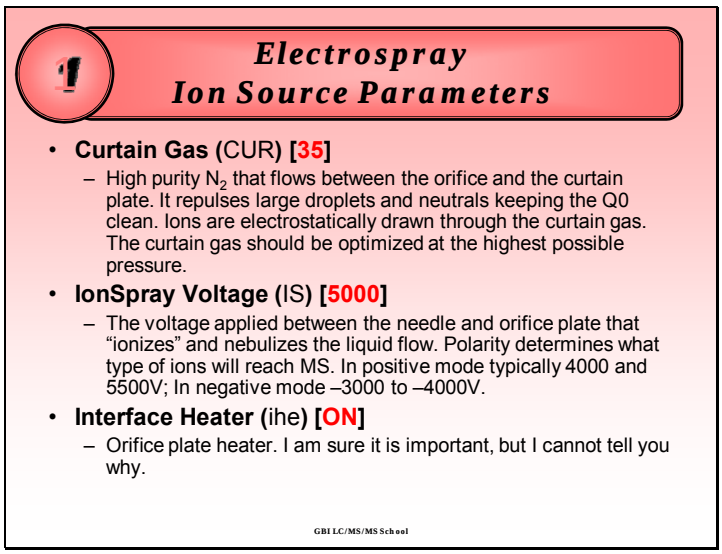

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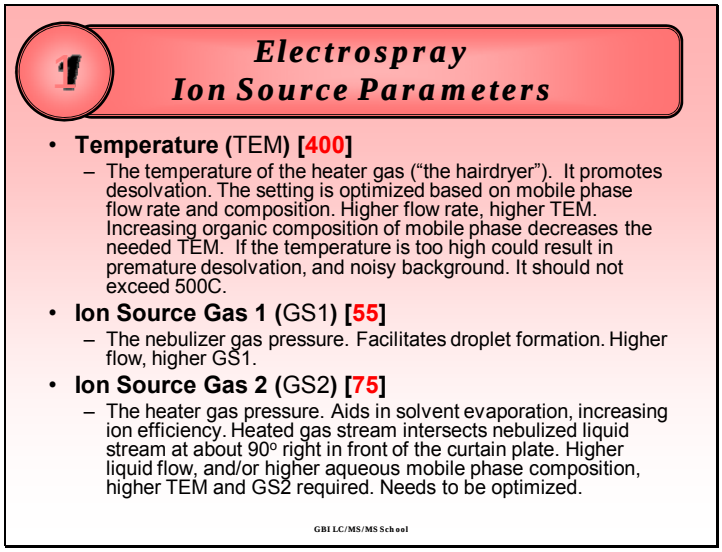

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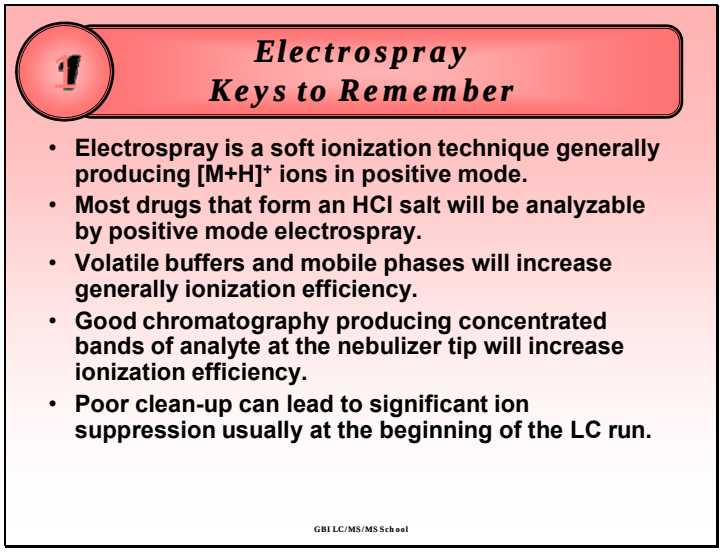

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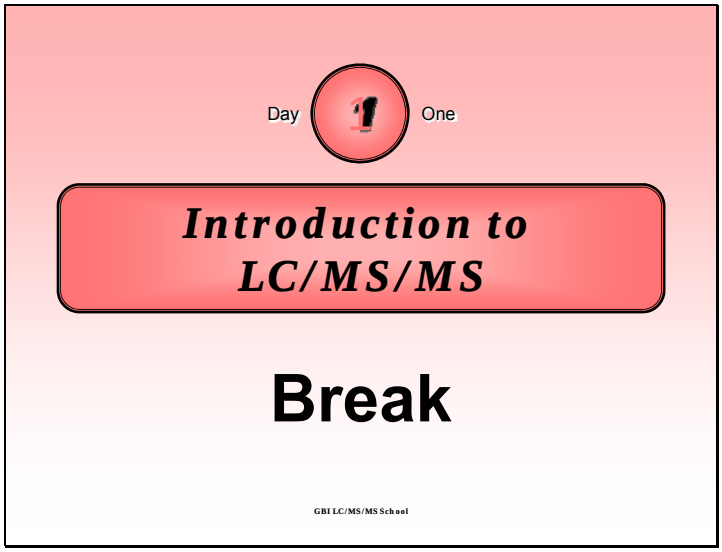

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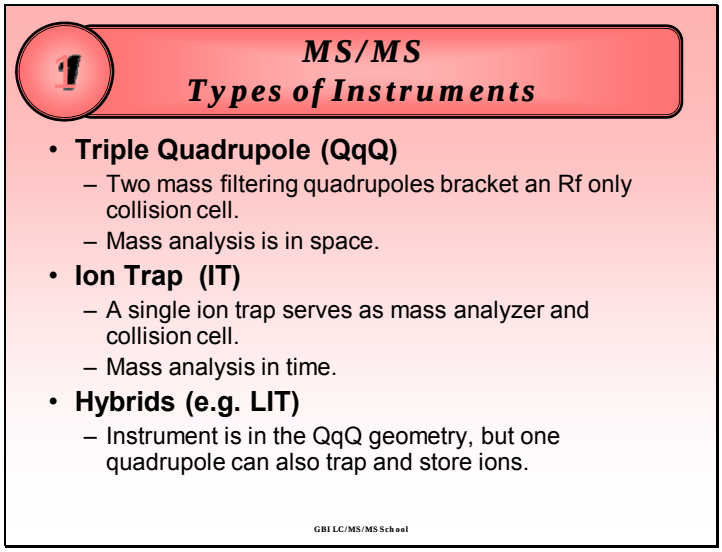

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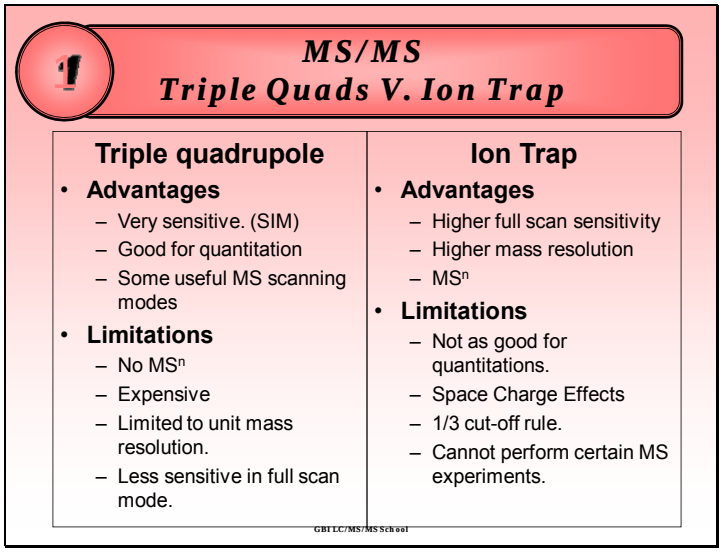

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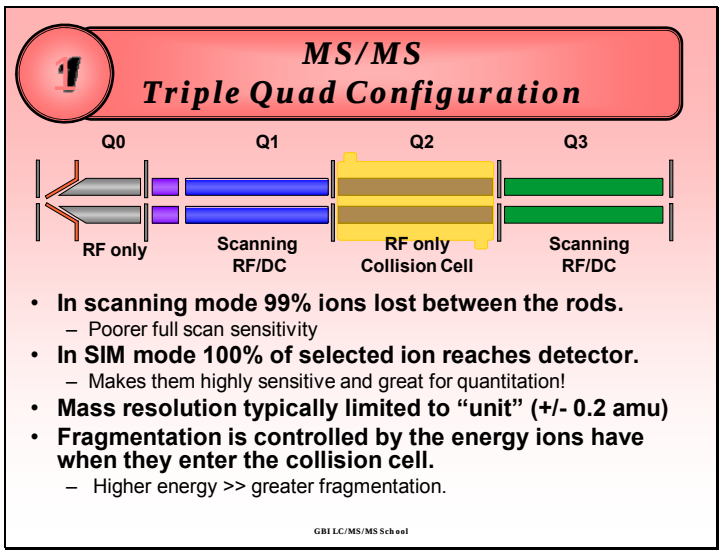

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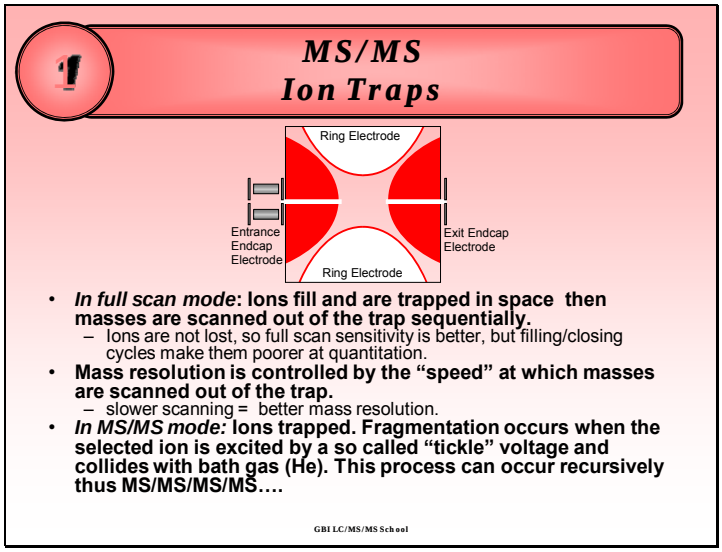

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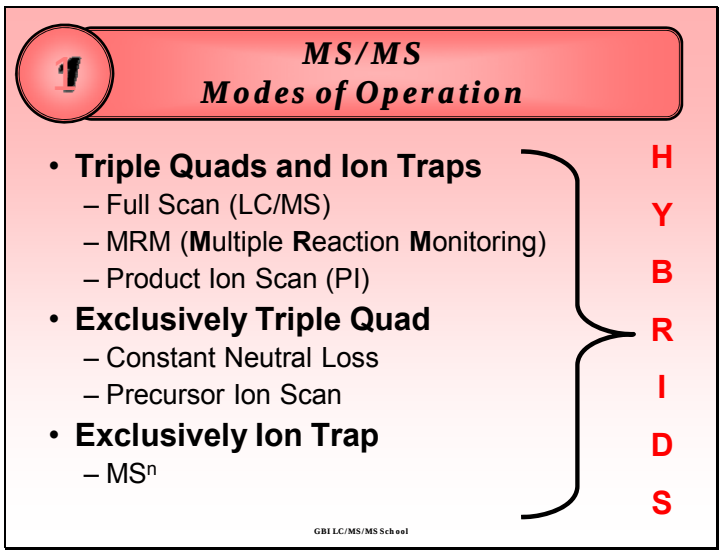

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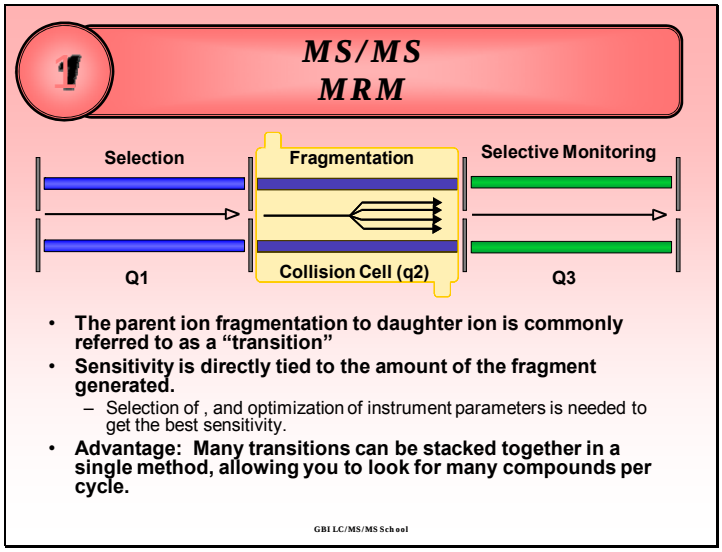

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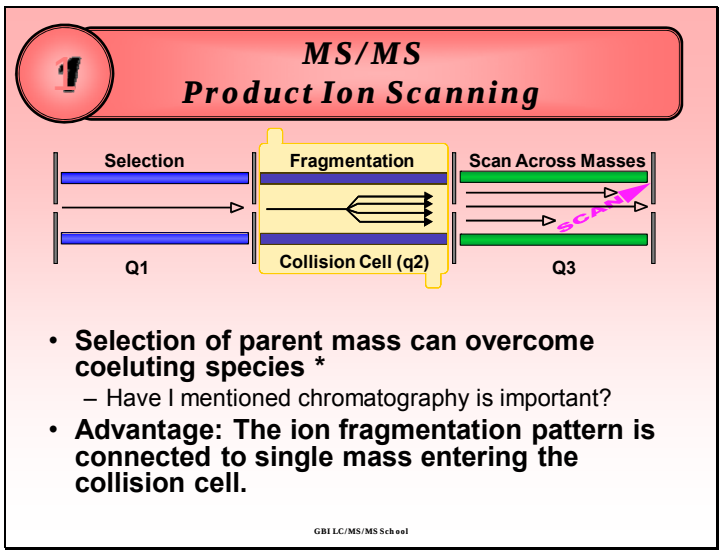

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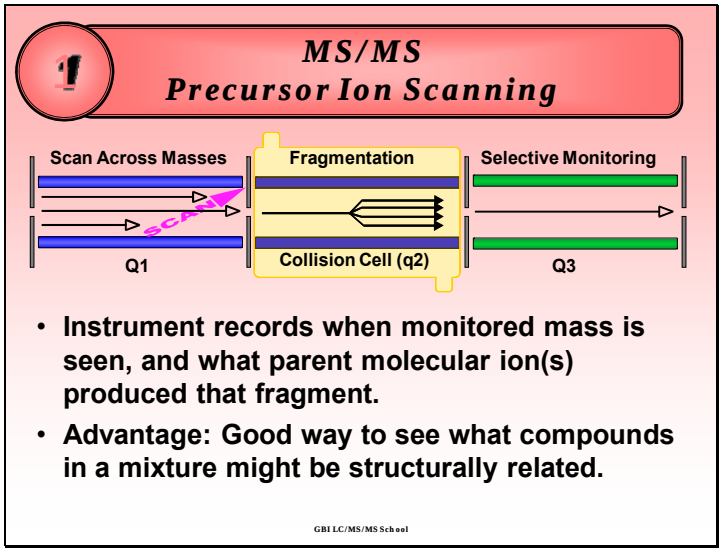

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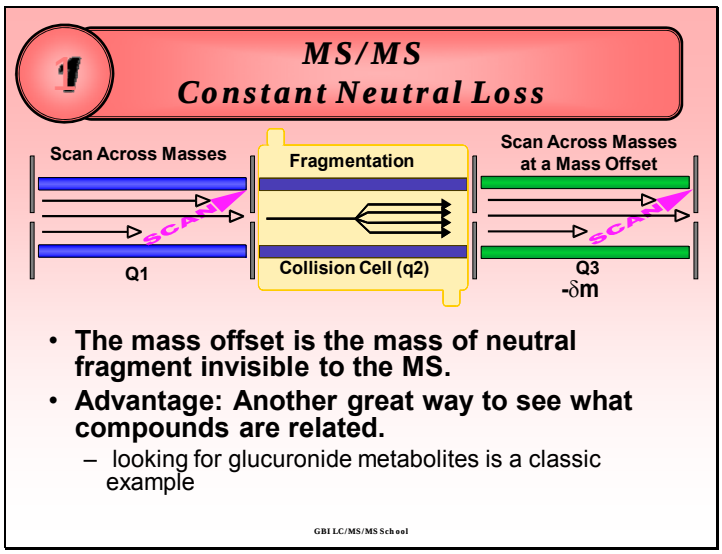

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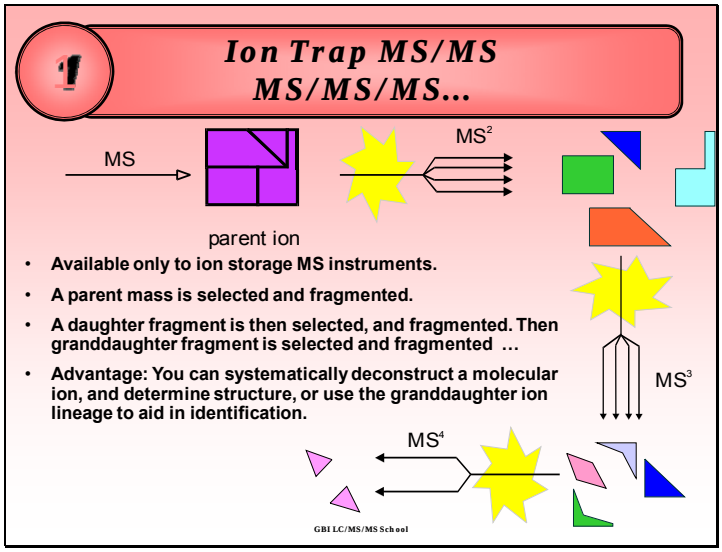

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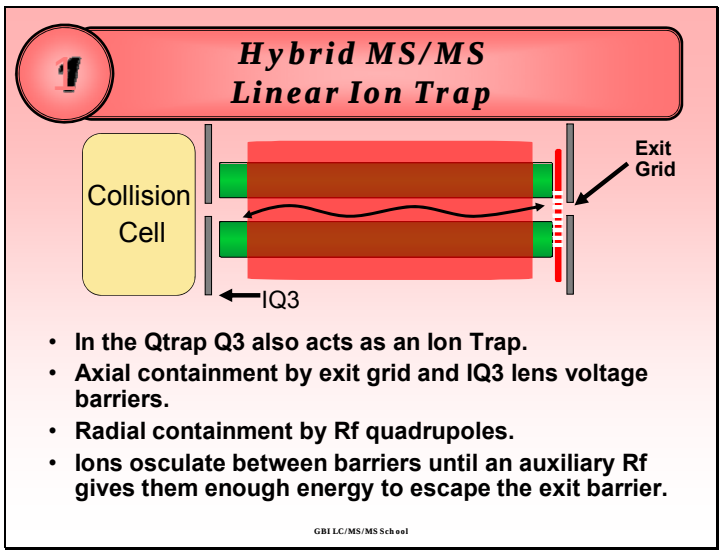

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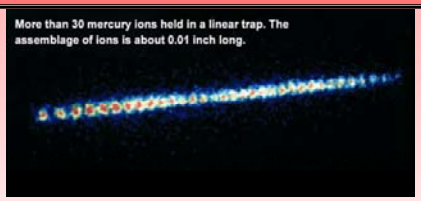
Hybrid MS/MS LIT Advantages



- **Has a larger "volume" so it can be filled with more ions before exhibiting space charge effects.**
- **Ions are formed outside the trap, so it is not limited by the 1/3 rule.**
- **Can perform MS/MS/MS experiments by selecting an ion and fragmenting it using the spillover collision gas. (1/3 rule applies here…)**

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MS/MS IDA

- **Information Dependent Acquisition**
  - Allows on-the-fly software switching between MS modes.
- **For Example**
  - Experiment 1: MRM
    - Survey Scan of 100+ drugs
  - IDA (Decision Maker)
    - When a transition is above a threshold…
    - And is the most intense transition…
    - And is not on an ***exclusion list***…
    - Then trigger a second experiment
  - Experiment 2: Product Ion Scan
    - Full scan mass spectrum for compound identification.

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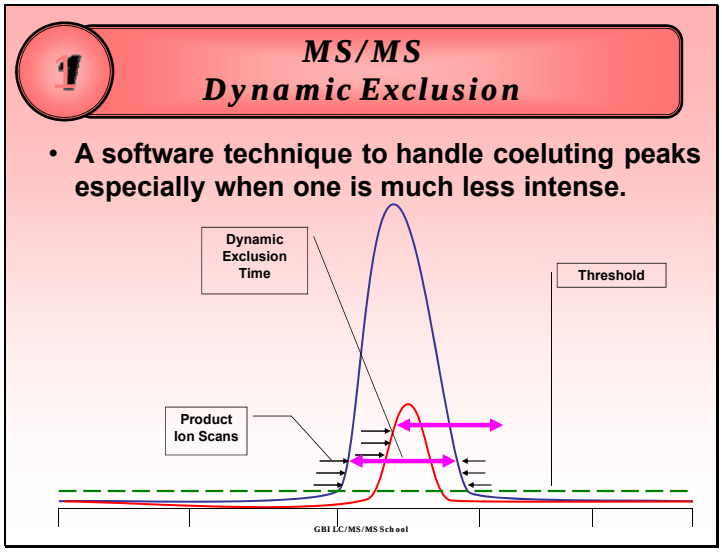

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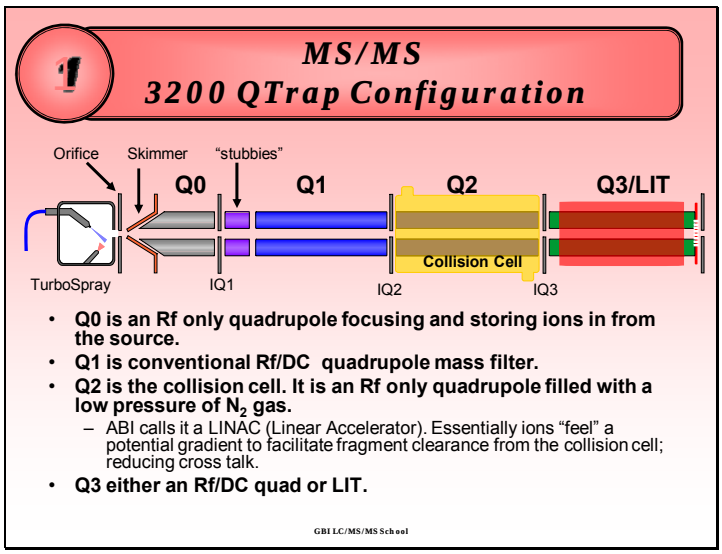

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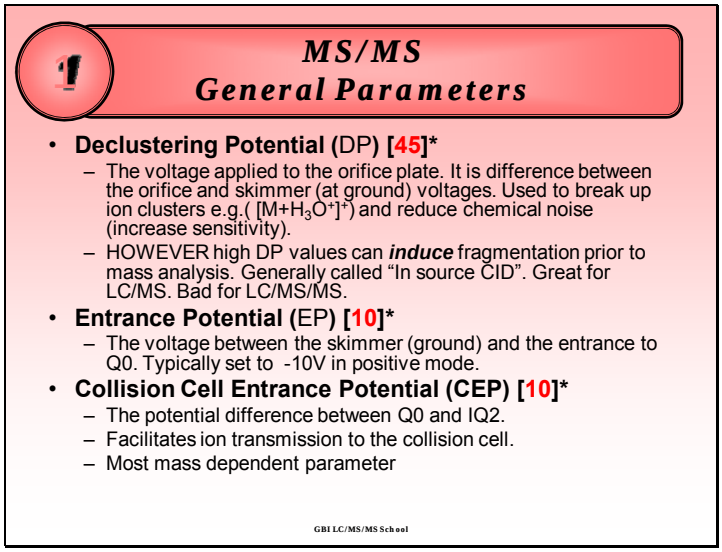

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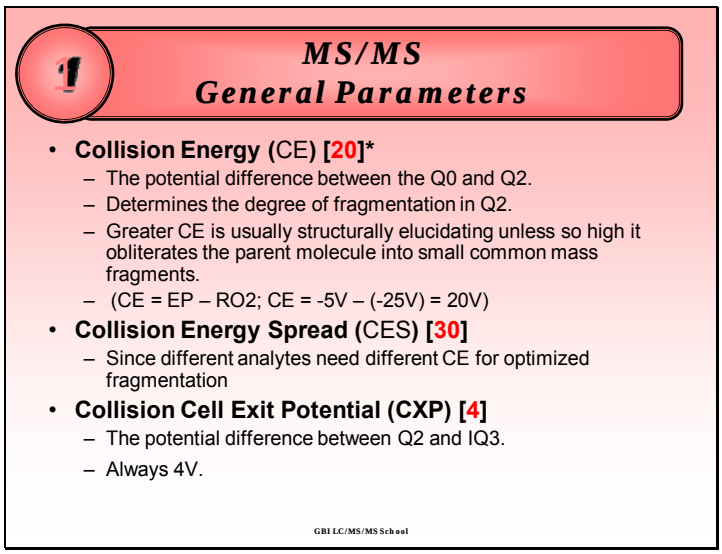

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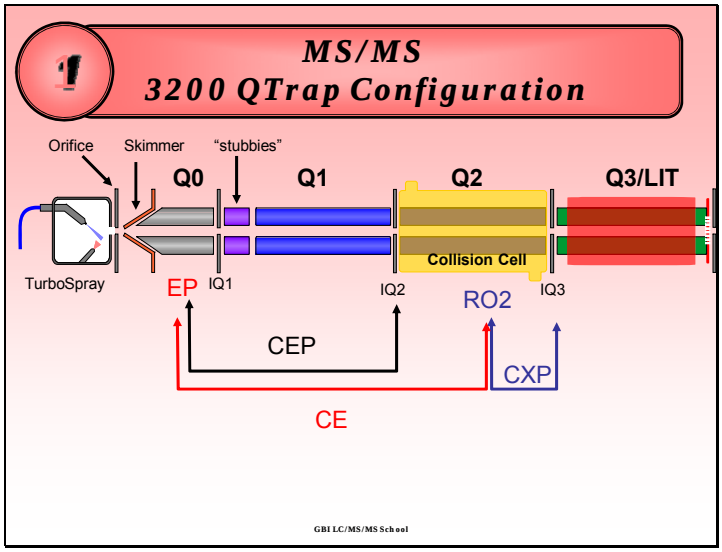

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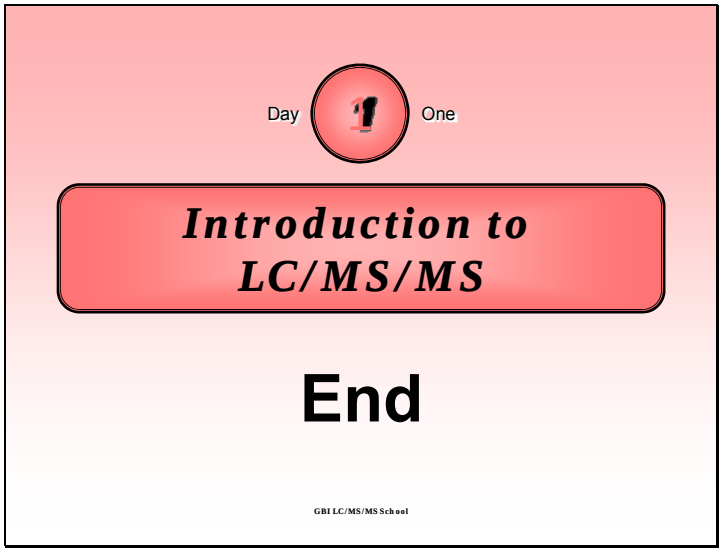
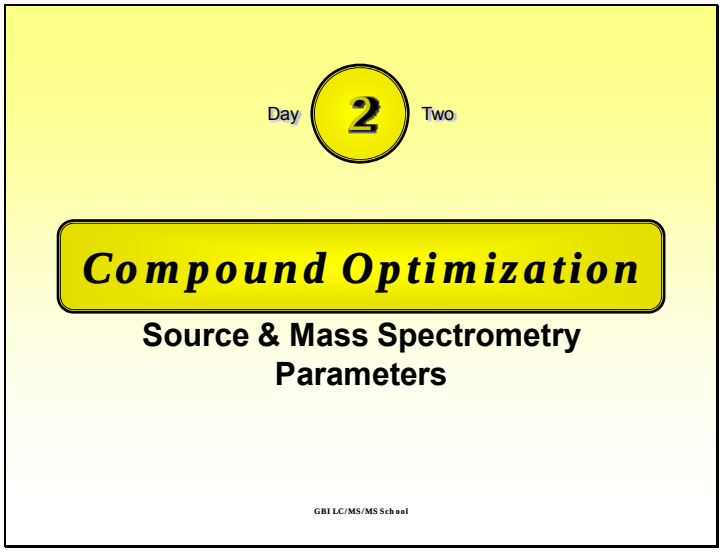


Day 2

Slide 1



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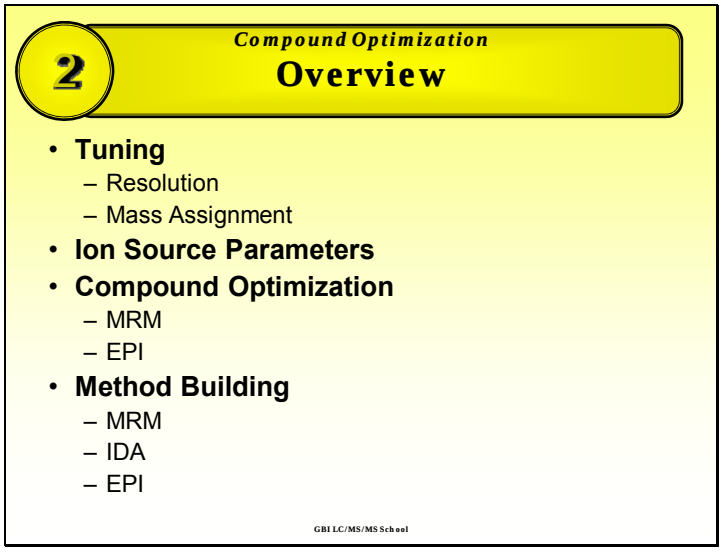

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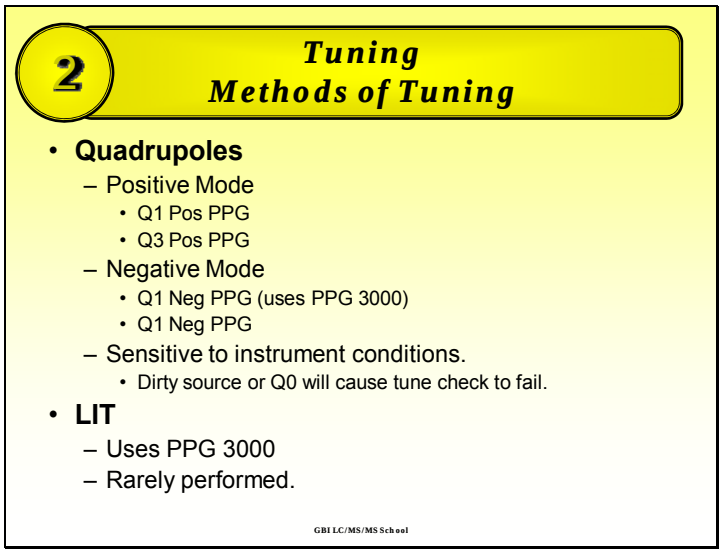

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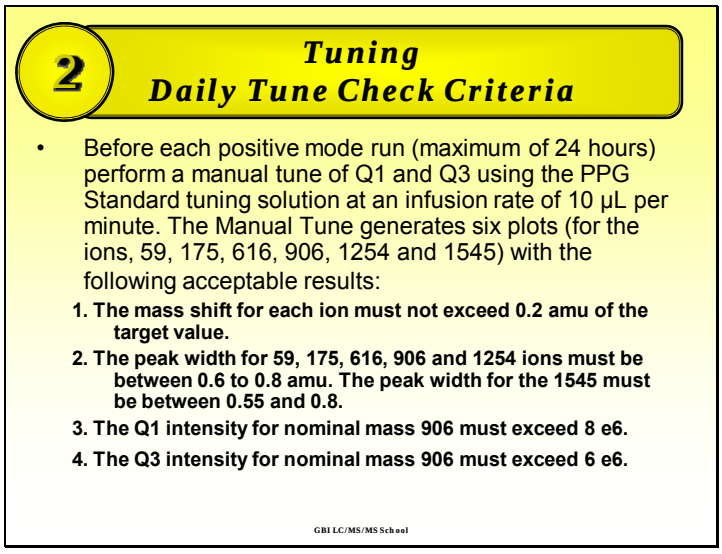

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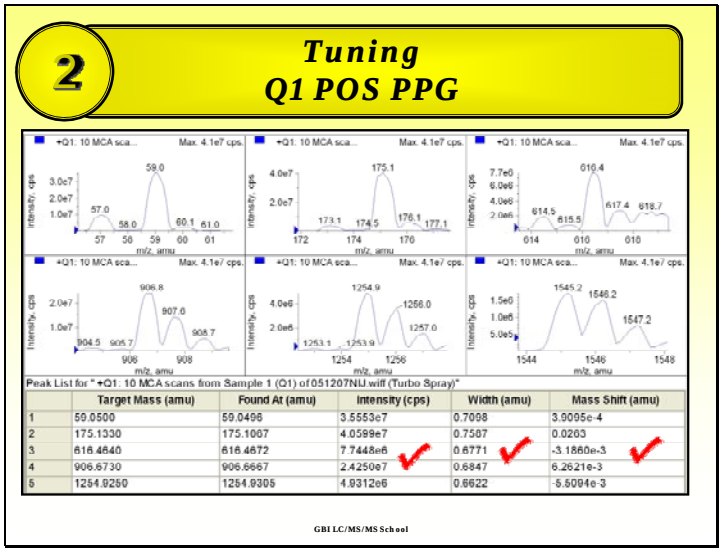

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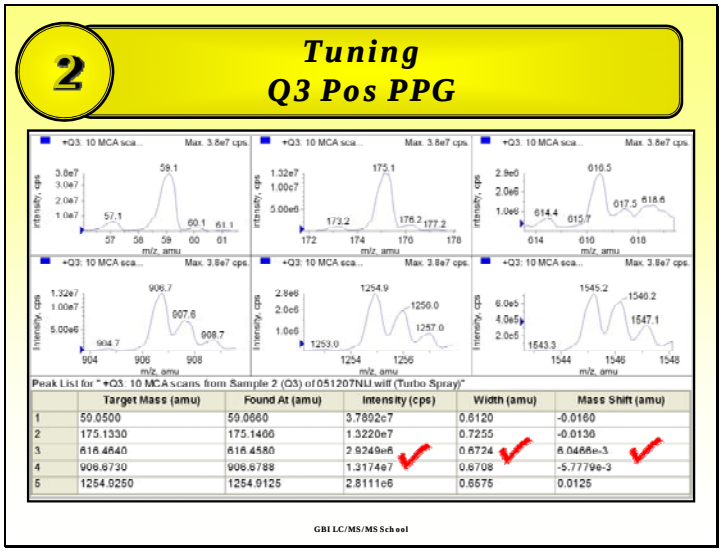

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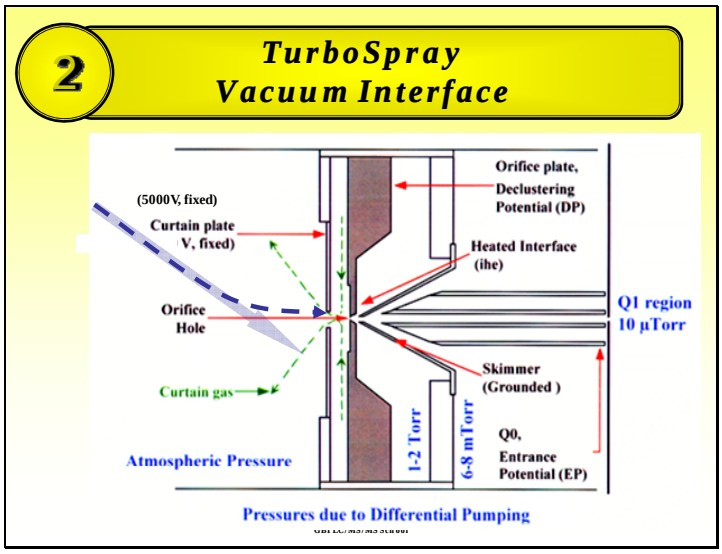

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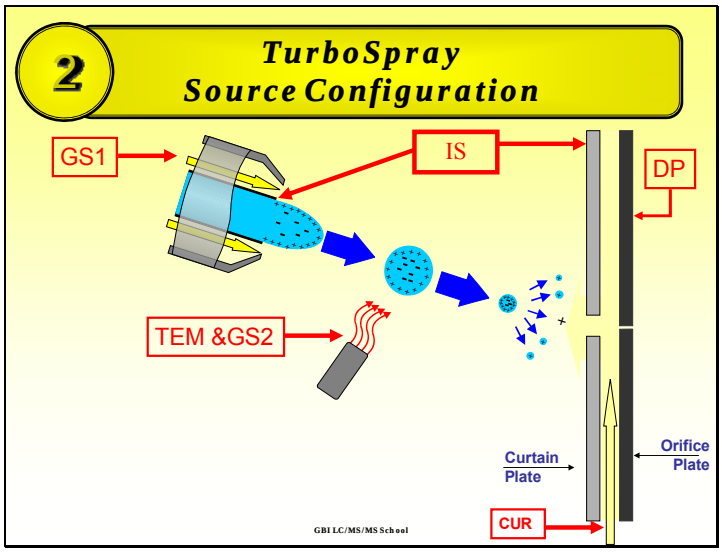

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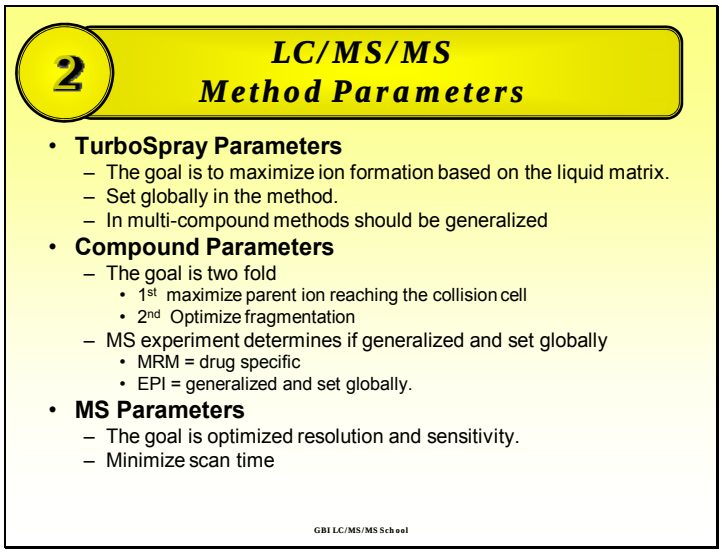

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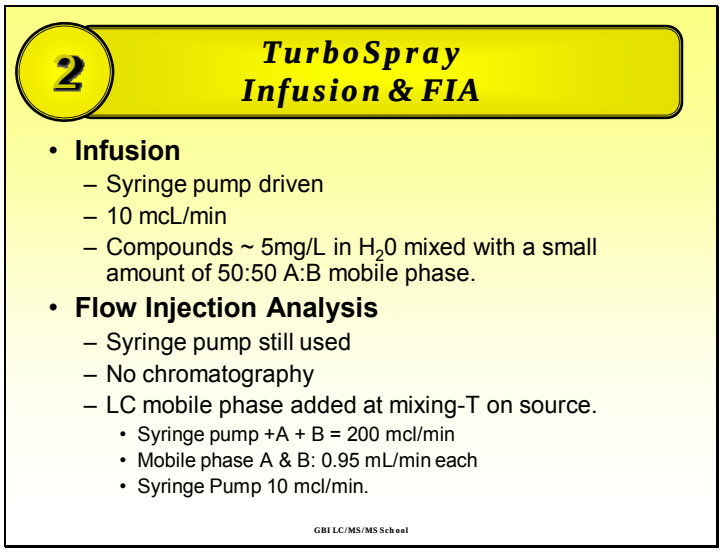

Slide 11

2 TurboSpray Parameters

- **Ion Spray Voltage [IS]**
- **Nebulizer Gas [GS1]**
- **Heater [TEM]**
  - The "hairdryers"
- **Heater Gas [GS2]**
- **Curtain Gas [CUR]**
- **Interface Heater [ihe]**
  - On or Off…we leave it on.

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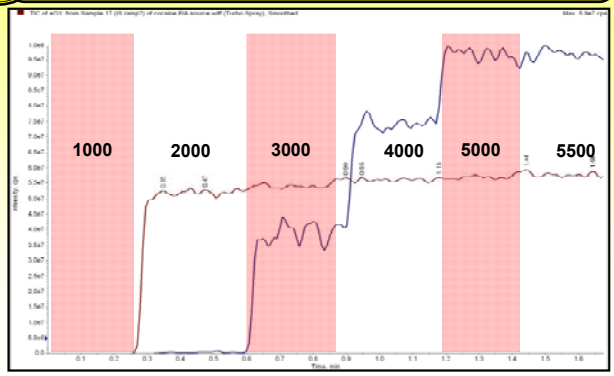
2 Turbospray Ion Spray Voltage



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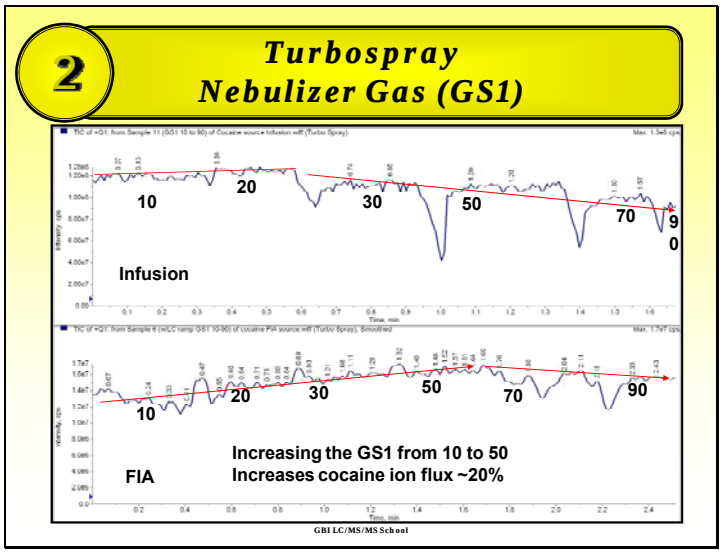

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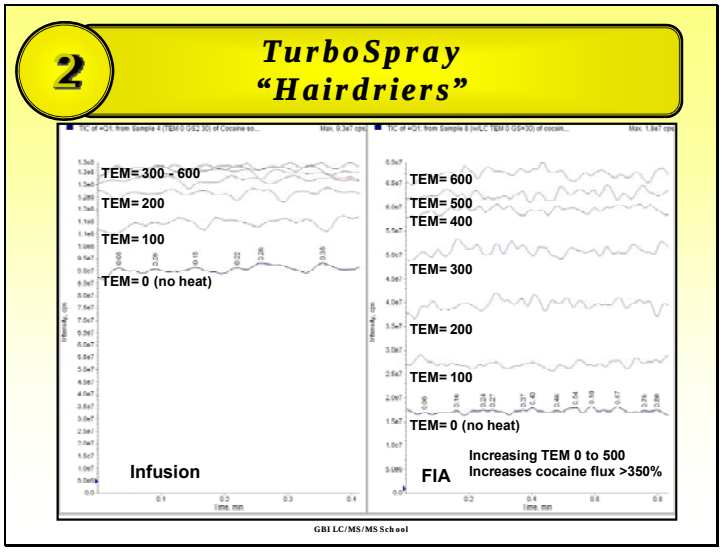

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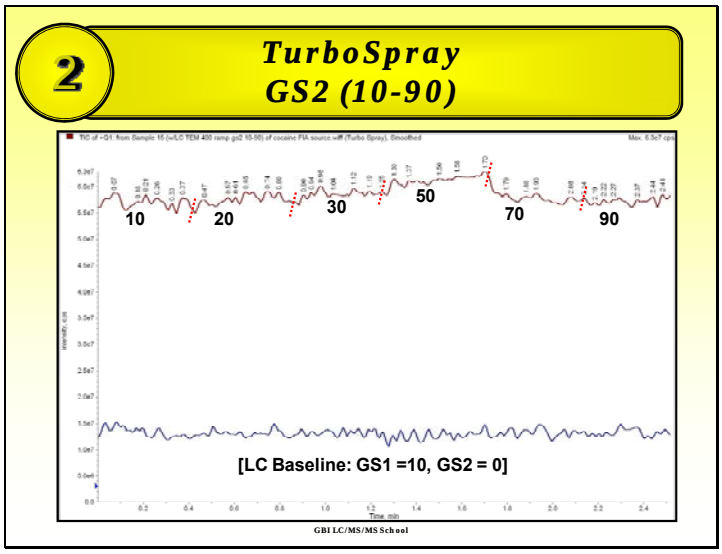

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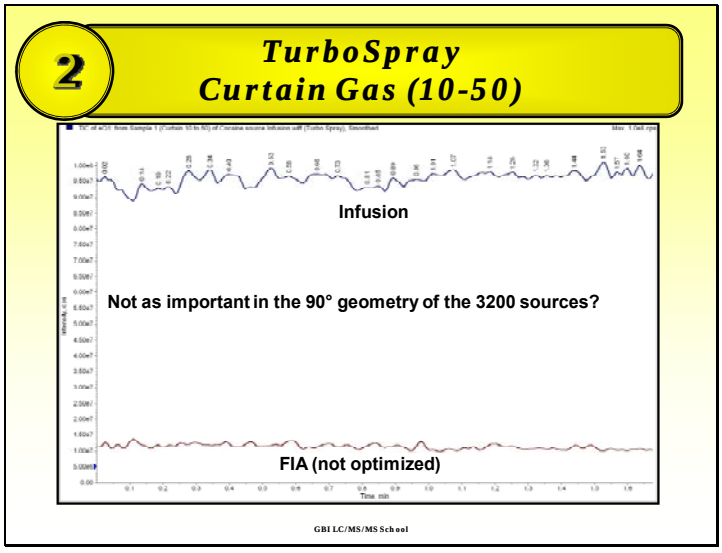

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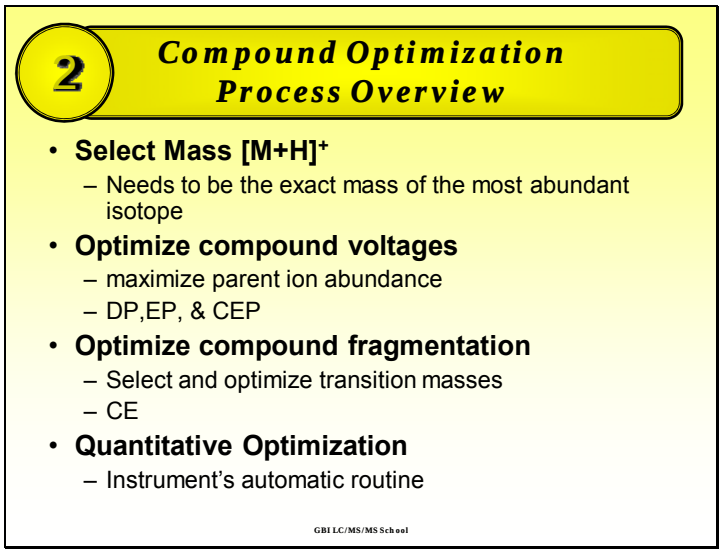

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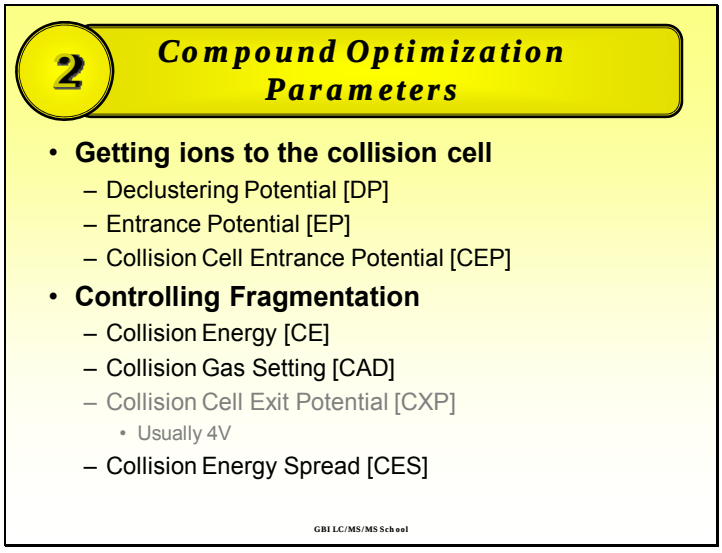

Slide 19

2 Compound Optimization Declustering Potential

- **"The potential applied to the orifice plate (OR) has the greatest effect on the amount of declustering in the orifice region of the interface."**
- **"The declustering potential (DP) is the difference between the orifice and ground. The higher the potential difference, the greater the amount of declustering."**
- **"The working range of DP is typically 0 to 100 V, although it may be set higher."**
- **Decluster what?**
  - Example clusters include: $[M+H_3O]^+$, $[M+Na]^+$, $[M+H+CH_3OH]^+$

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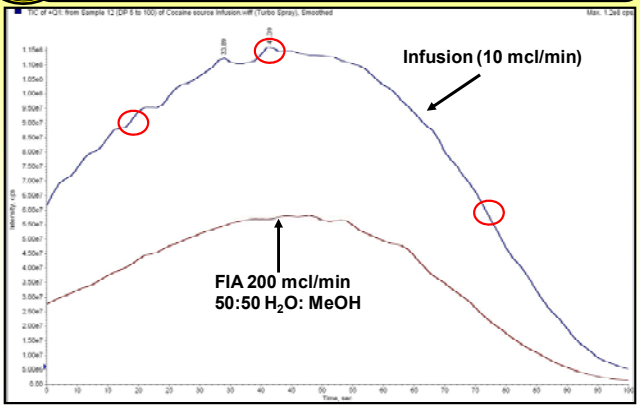
2 Compound Optimization Cocaine DP Ramp 5.0 to 100



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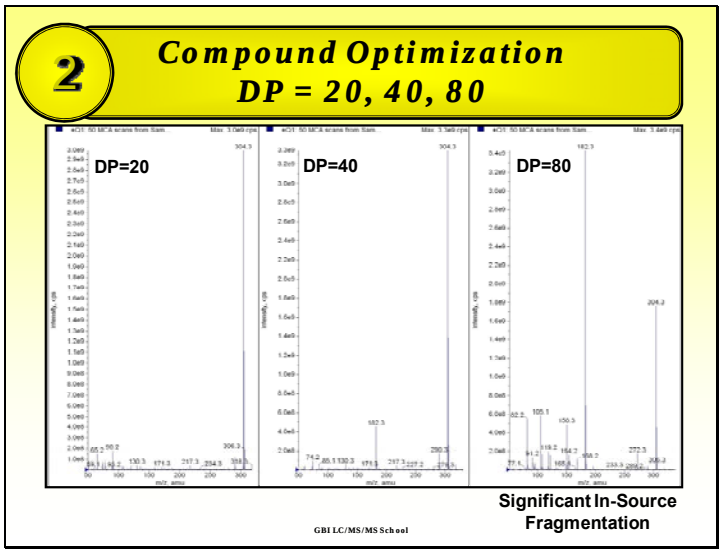

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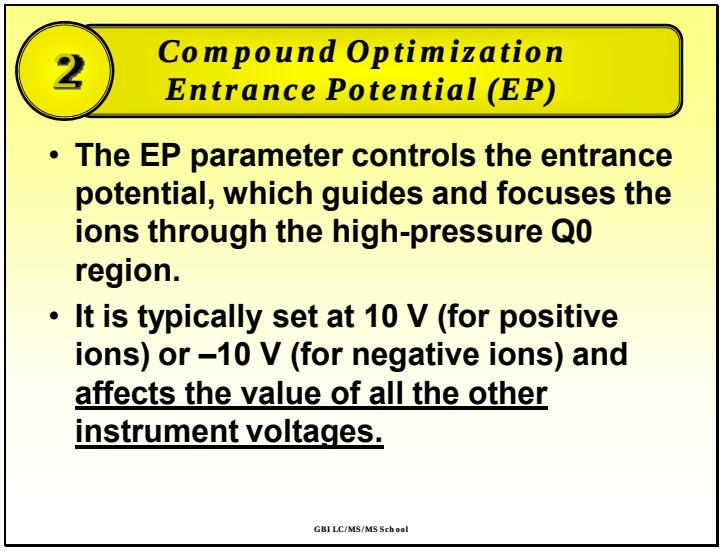

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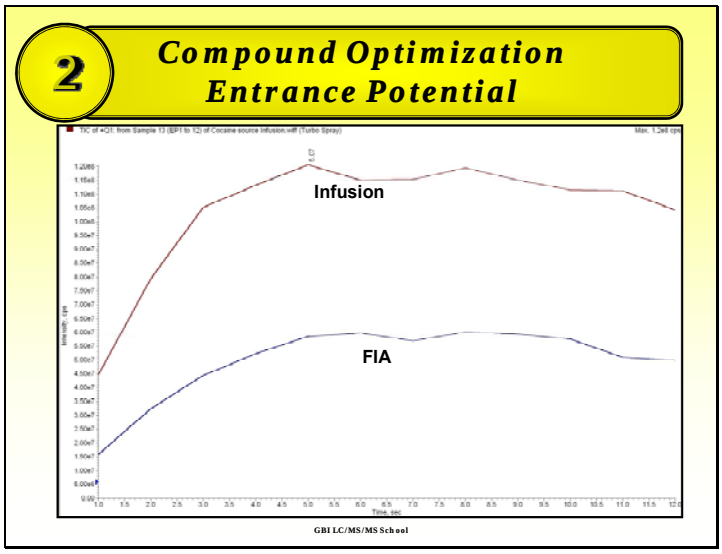

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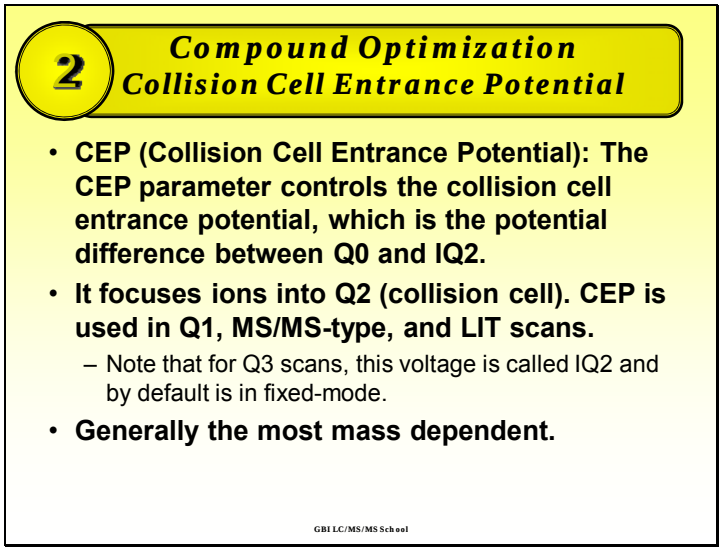

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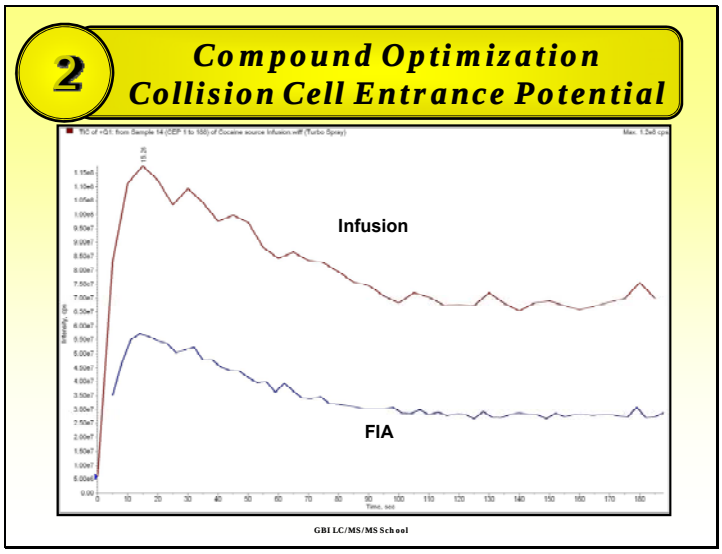

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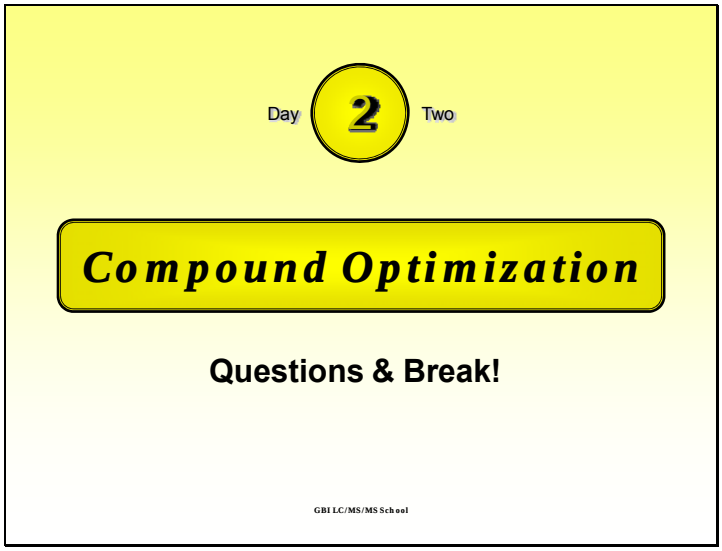

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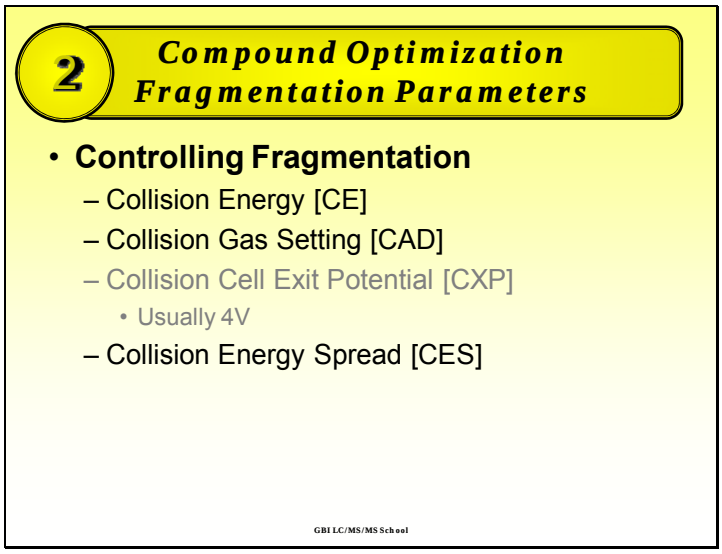

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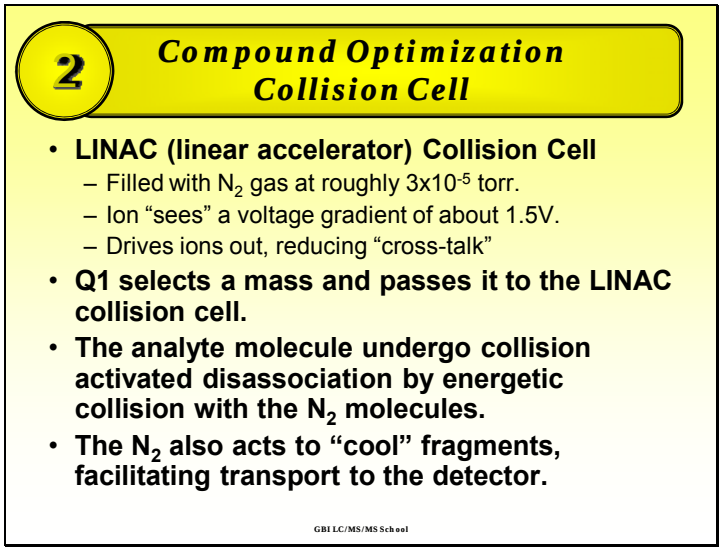

Slide 29

2 Compound Optimization Collision Enery (CE)

- **The energy of that collision is controlled by the Collision Energy (CE) setting.**
  - CE is a voltage difference between the Q0 and Q2 (EP – RO2).
  - CE can be optimized for each drug.
    - (Quantitation Optimization)
  - Higher CE results in greater fragmentation of the parent molecule.
- **Consider dextromethorphan…**

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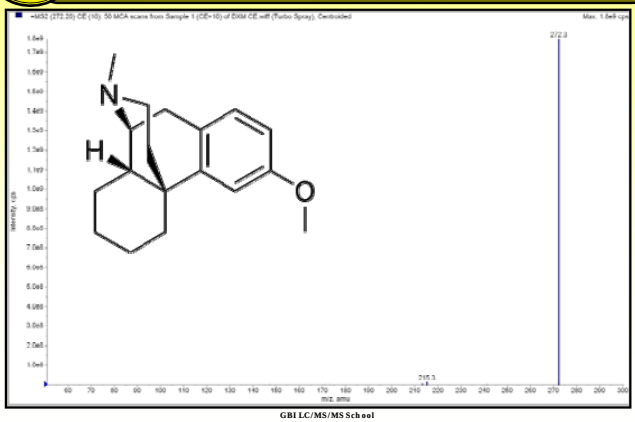
2 Compound Optimization DXM: CE =10



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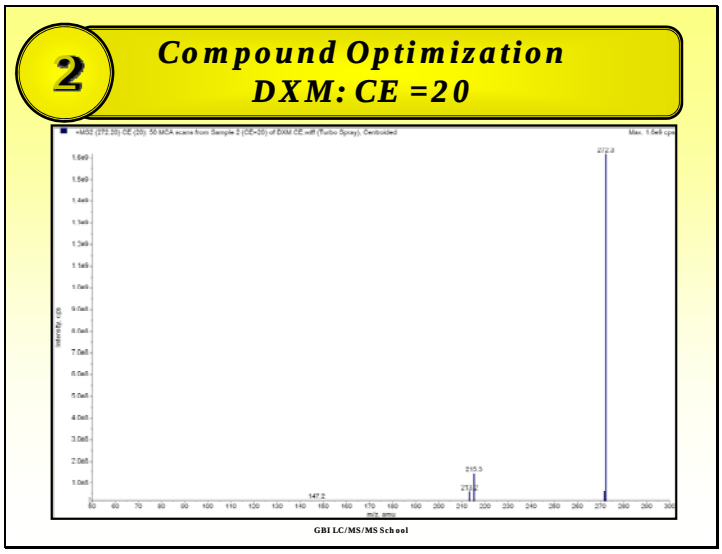

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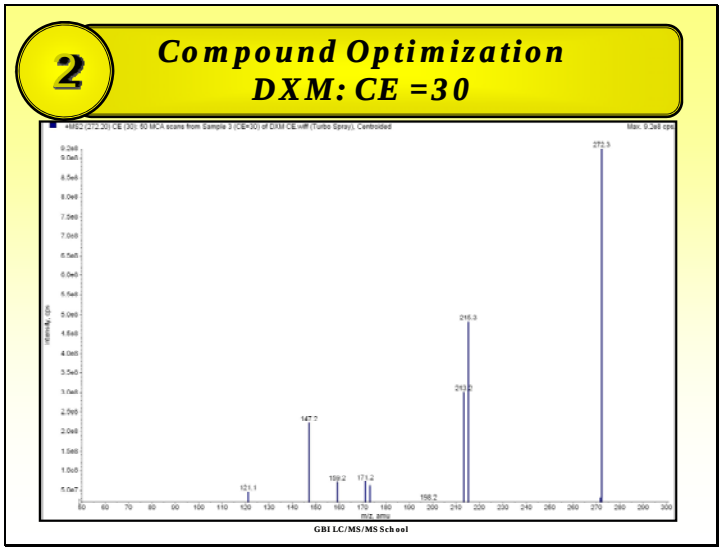

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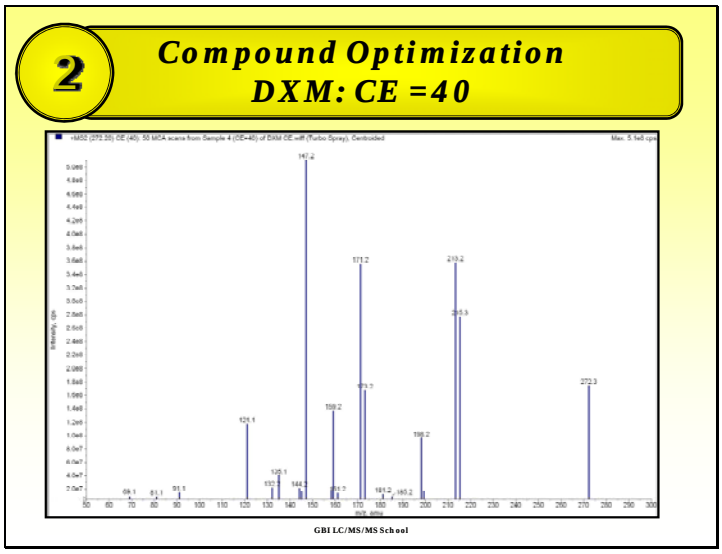

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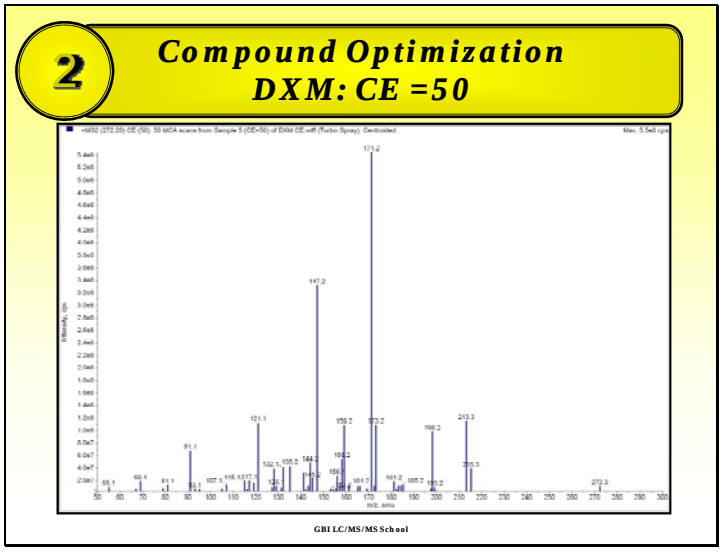

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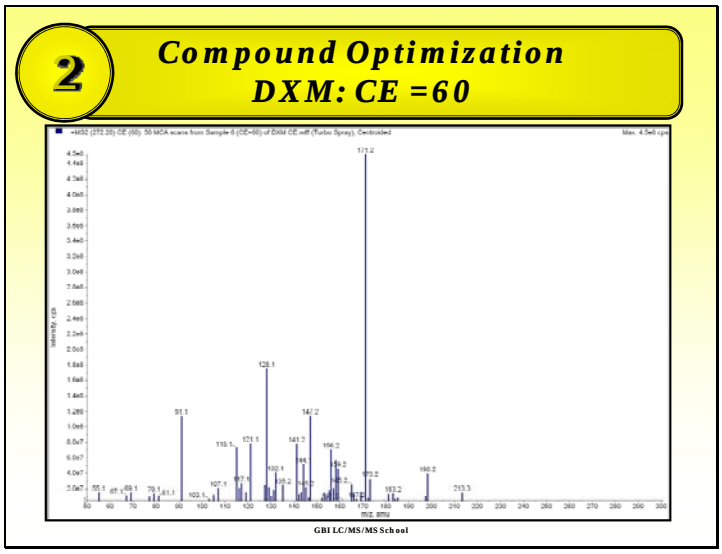

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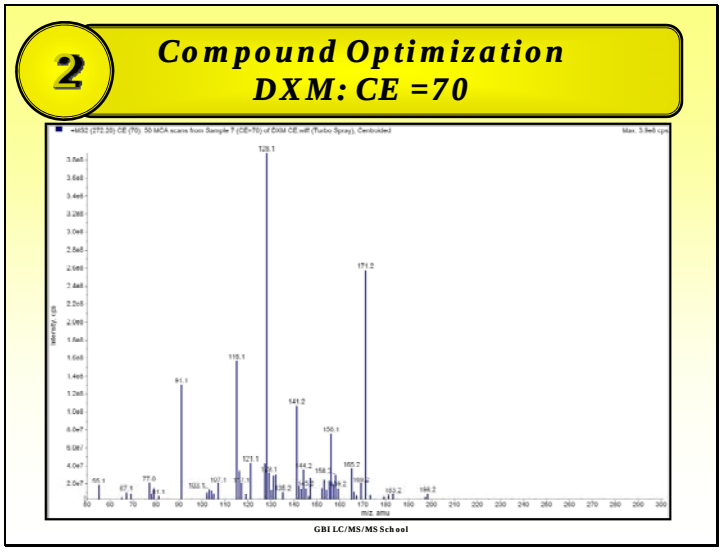

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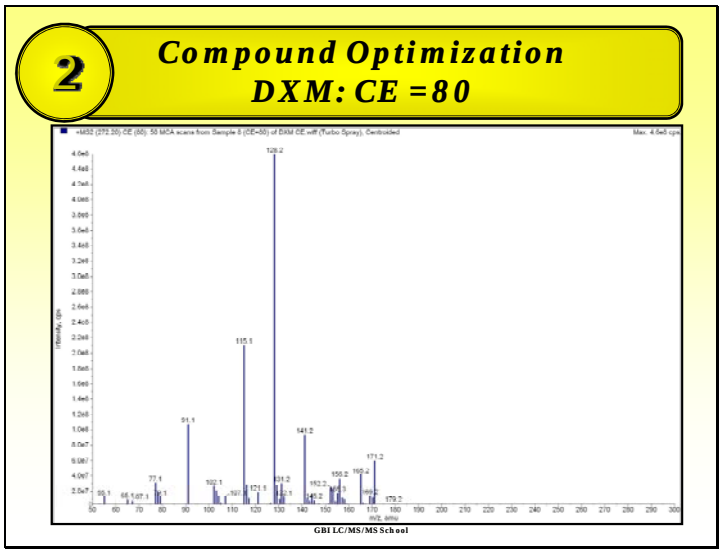

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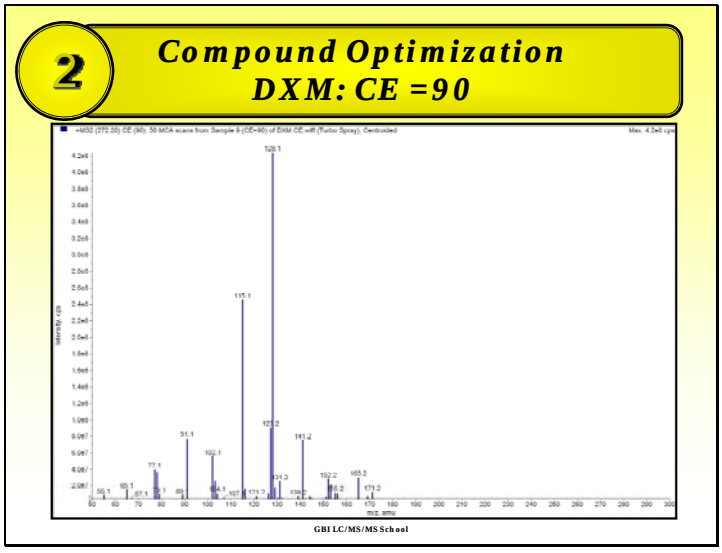

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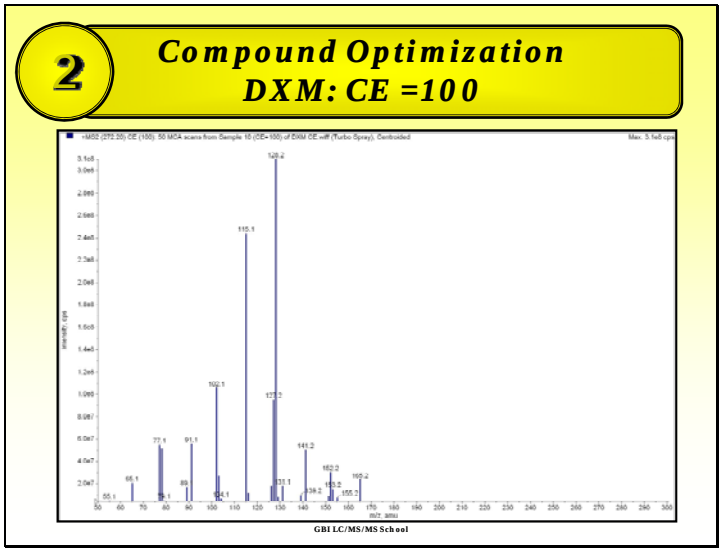

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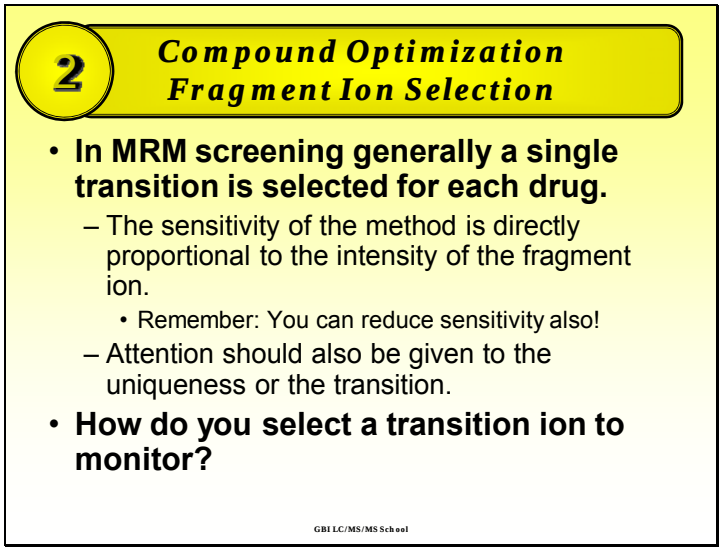

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2 Compound Optimization Fragment Ion Selection

- **Quantitative Optimization Continued**
  - Starts with compound optimization
    - DP, EP, & CEP
- **Fragment parent mass at many different collision energies.**
- **Pick the four most intense ions**
  - Avoid loss of H_2O
    - (fragments δm >19 amu parent)
- **Determine the collision energy that produces the maximum amount of each transition ion.**

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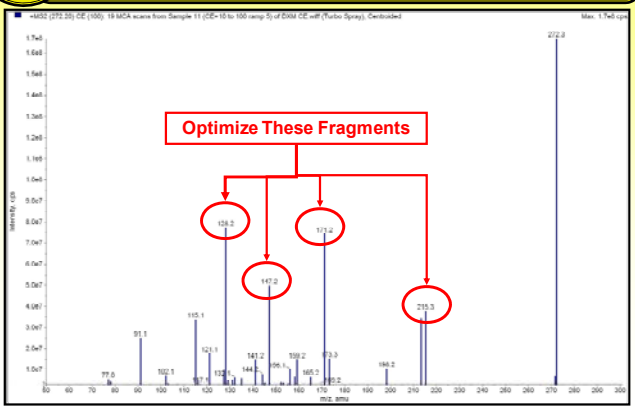
2 Compound Optimization CE =10 to 100 [summed]



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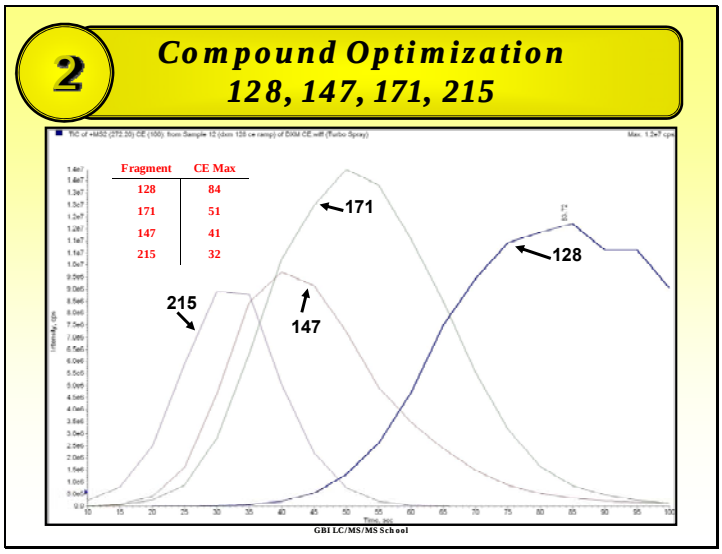

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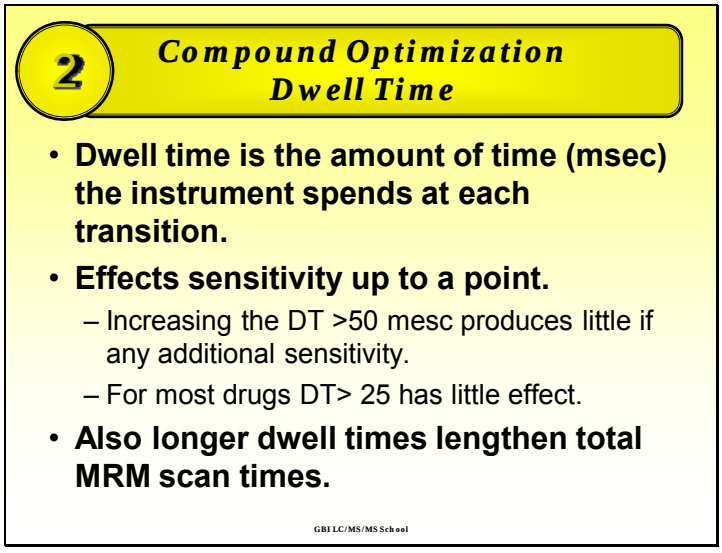

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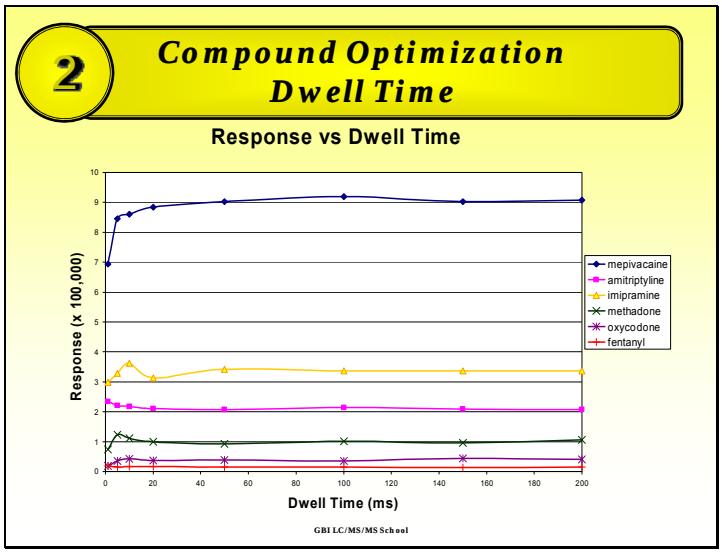

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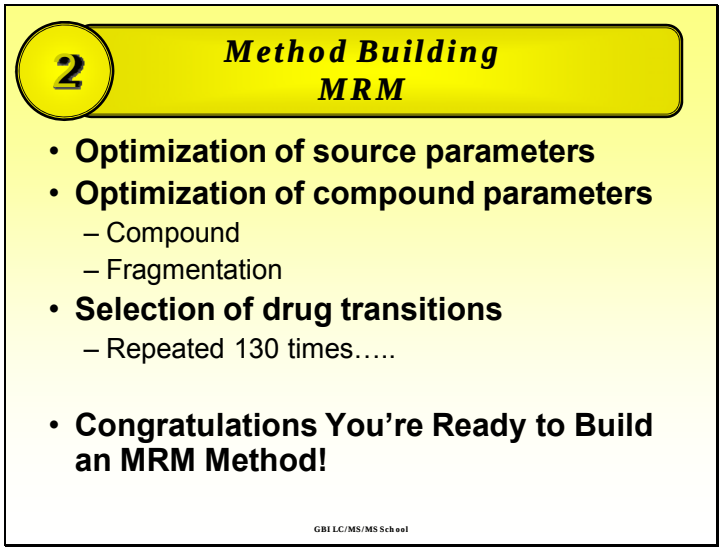

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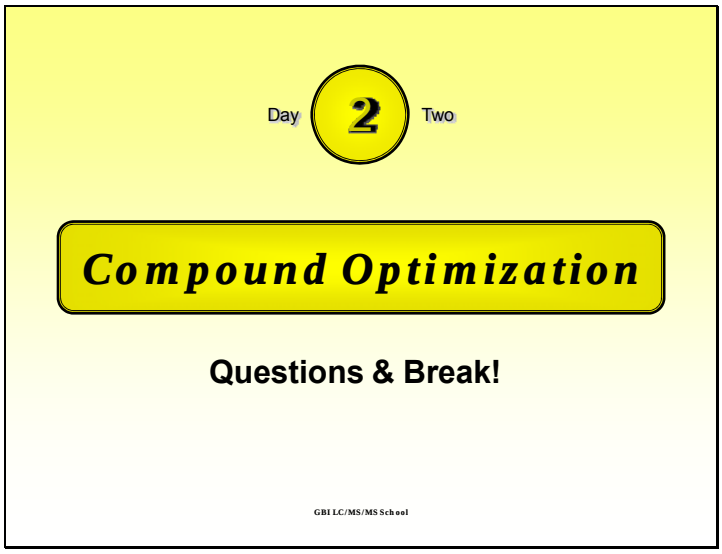

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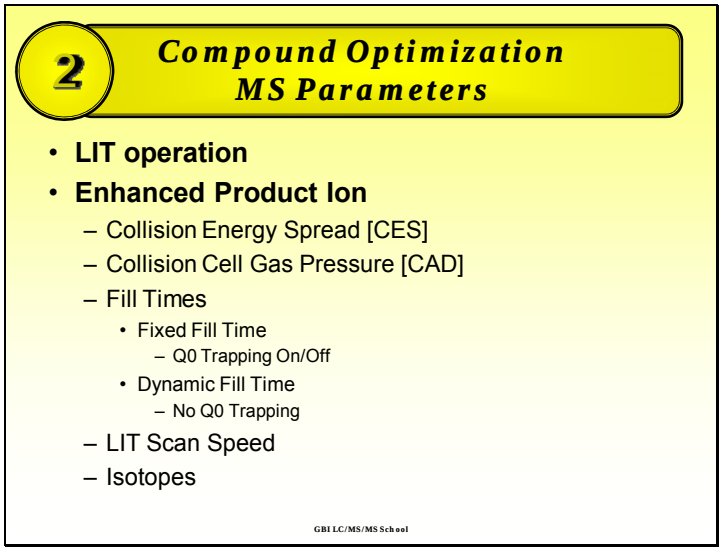

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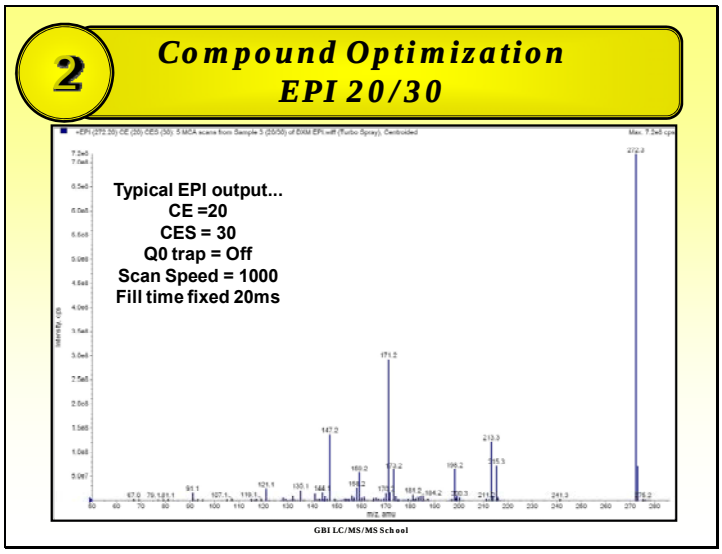

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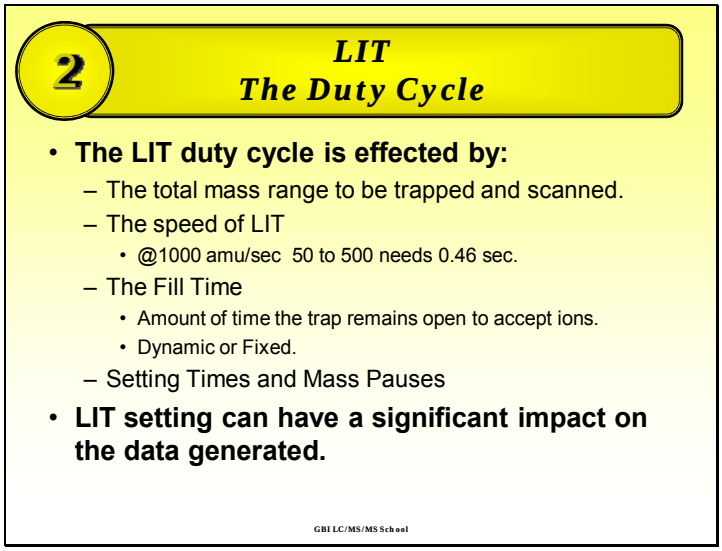

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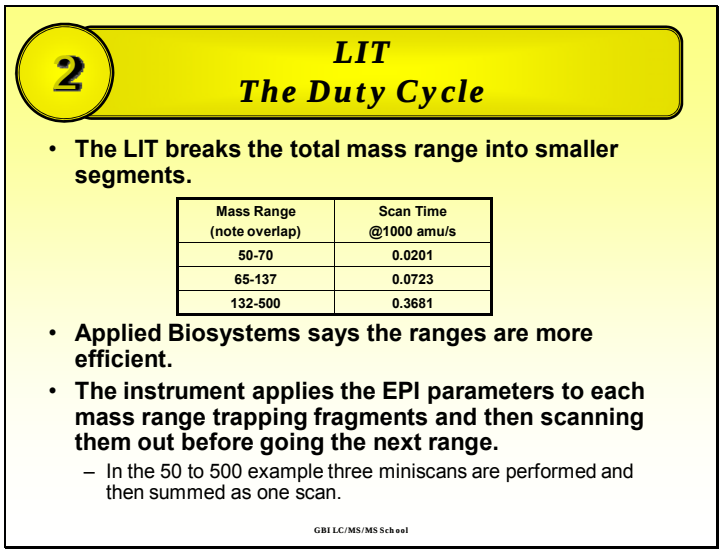

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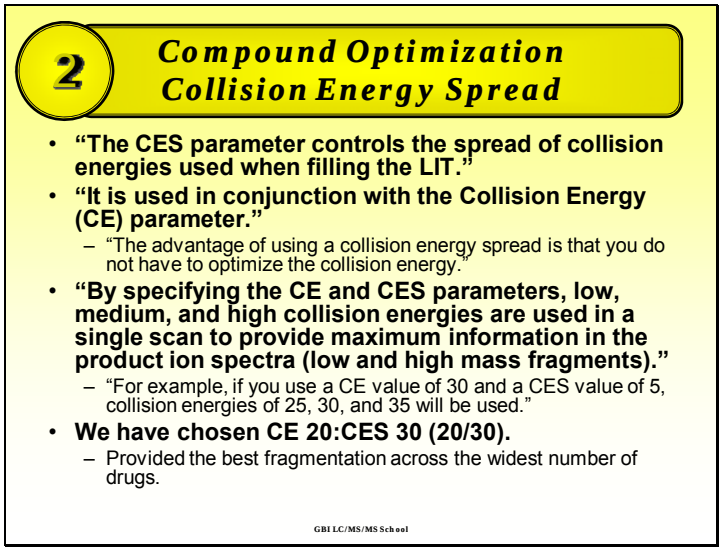

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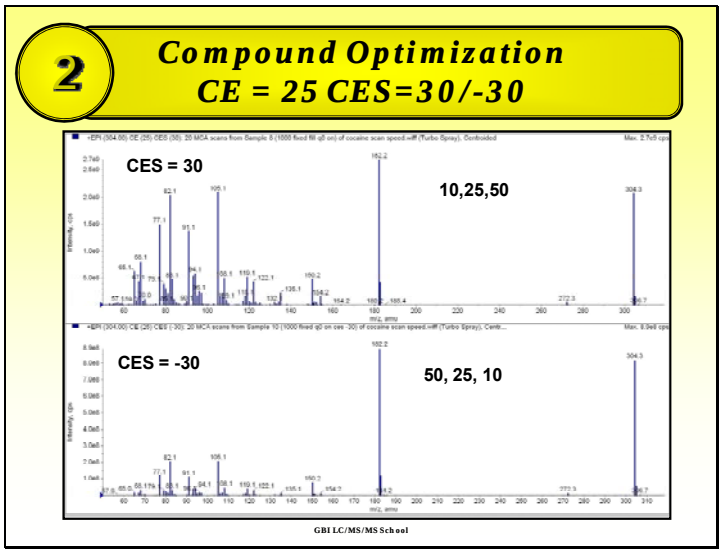

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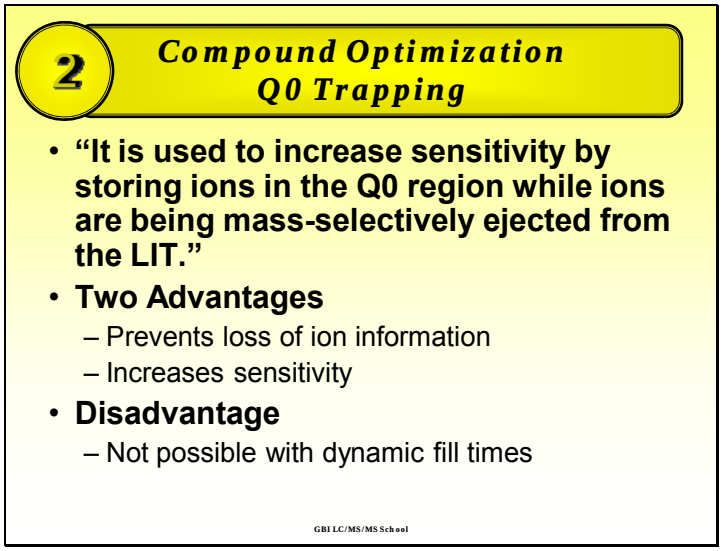

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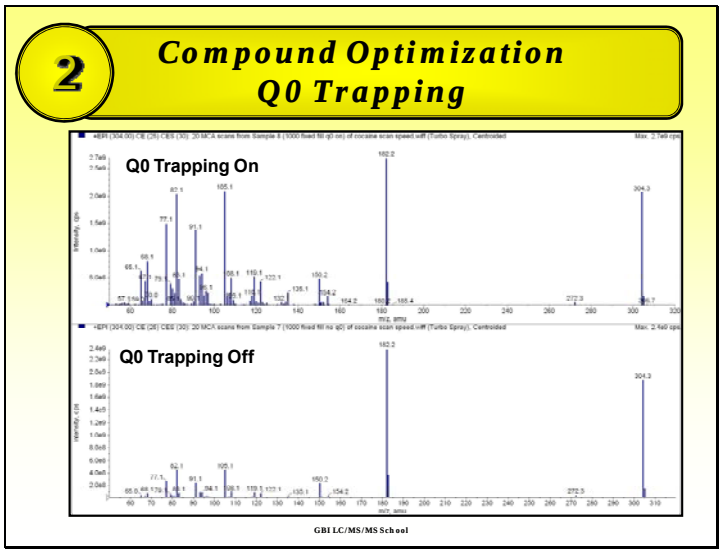

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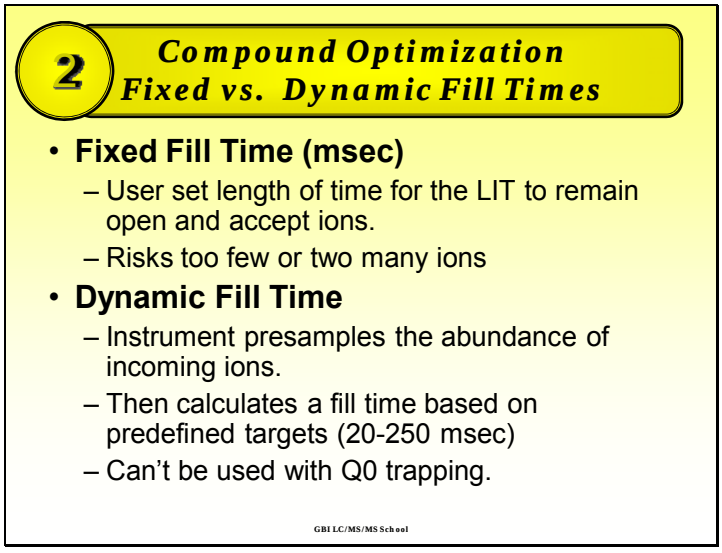

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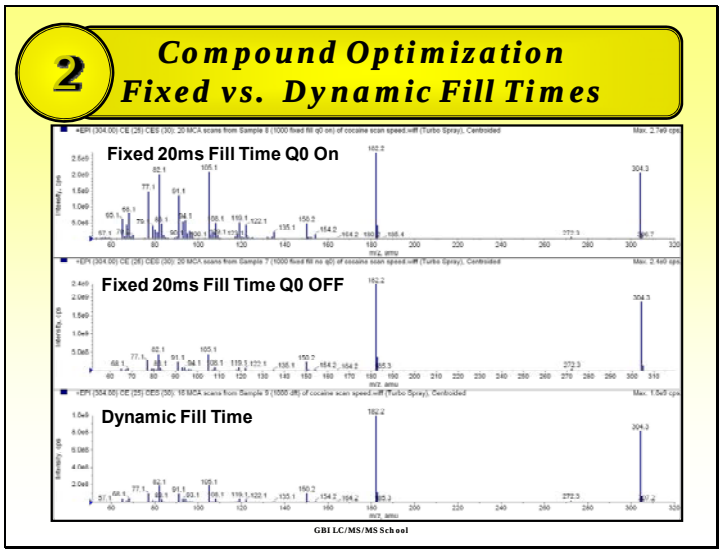

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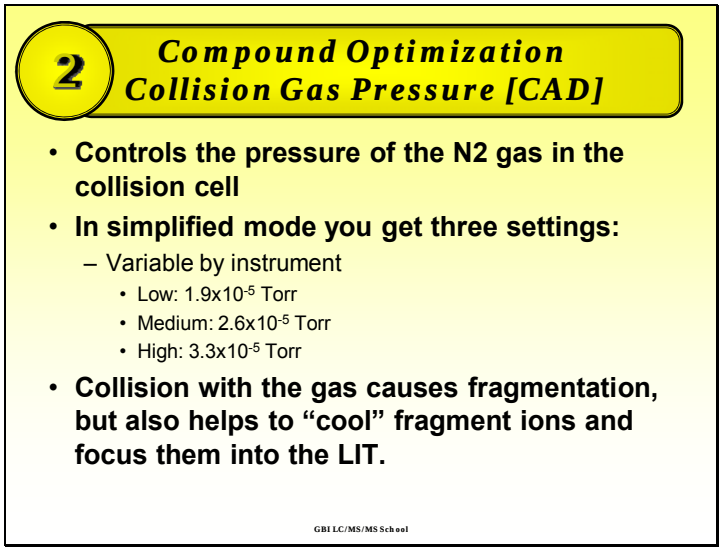

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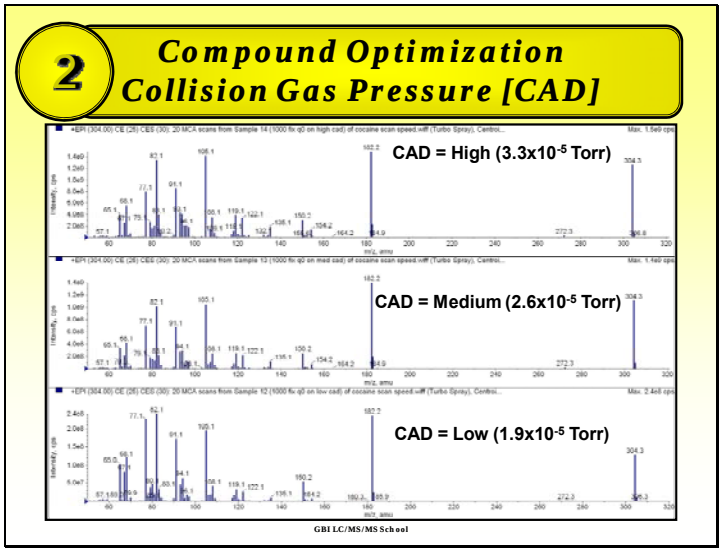

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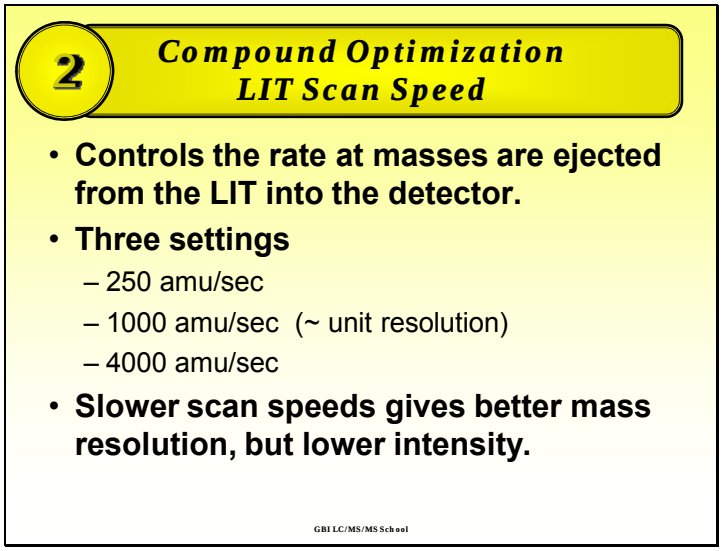

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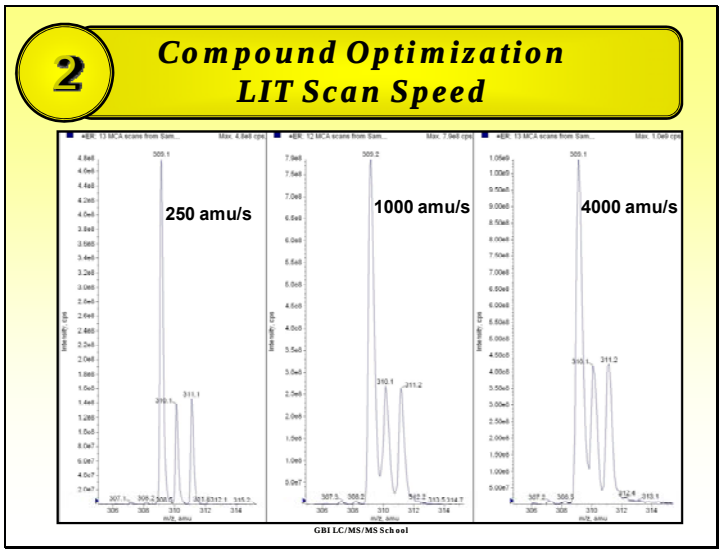

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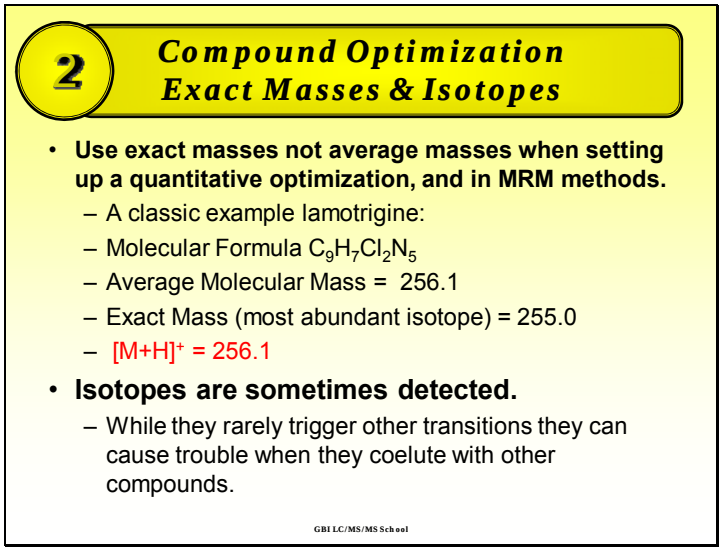

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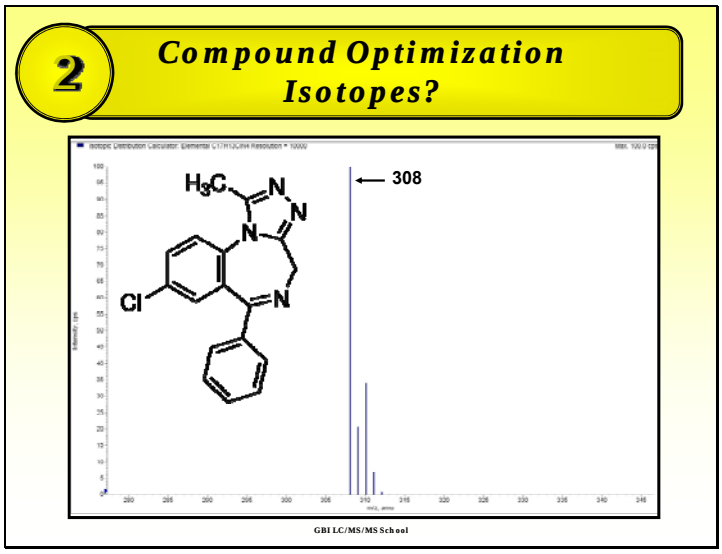
Slide 63



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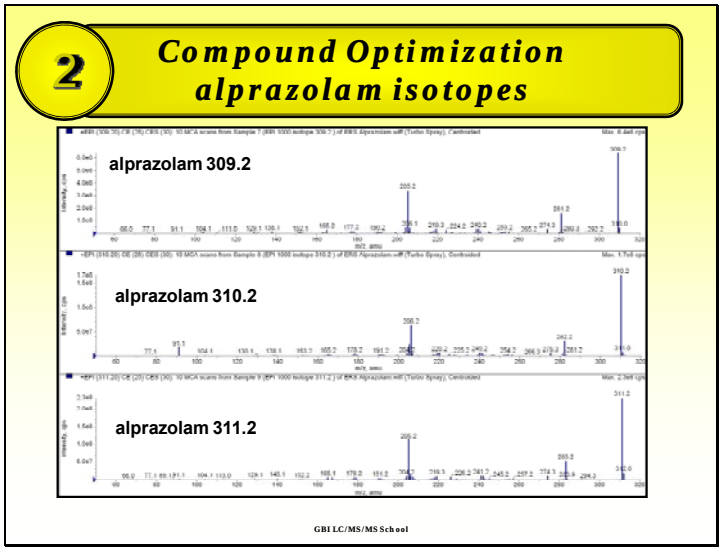

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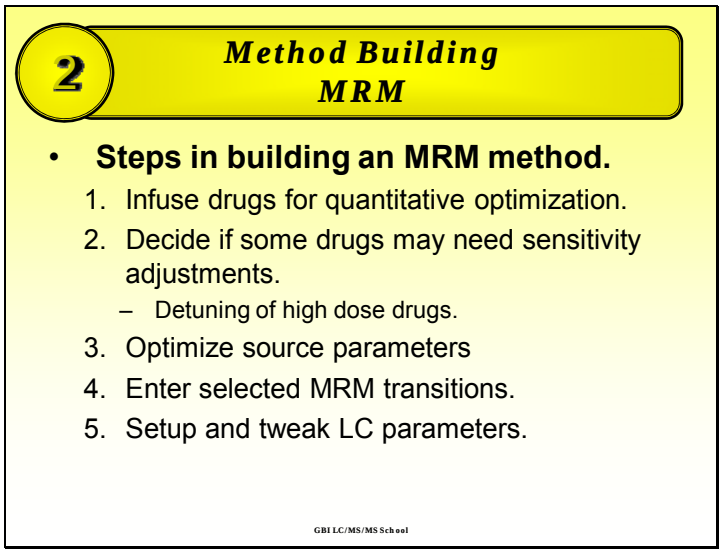

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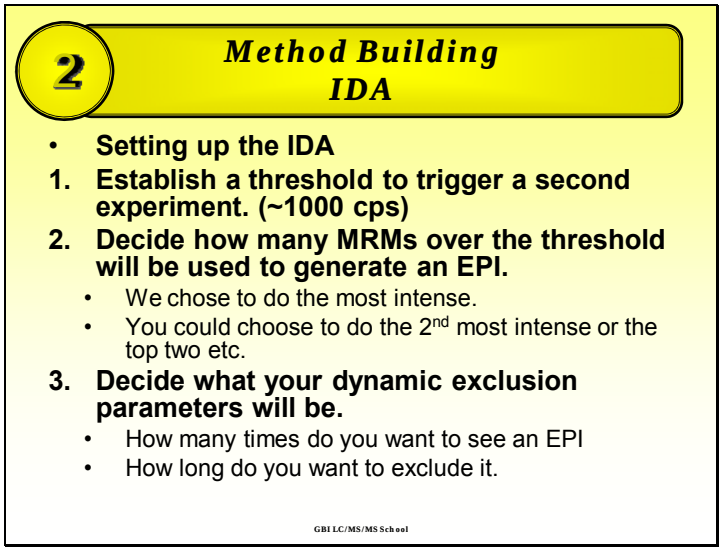

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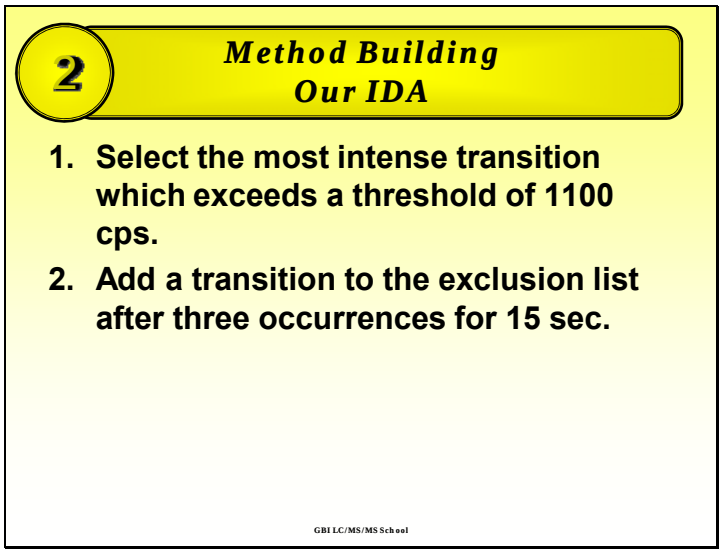

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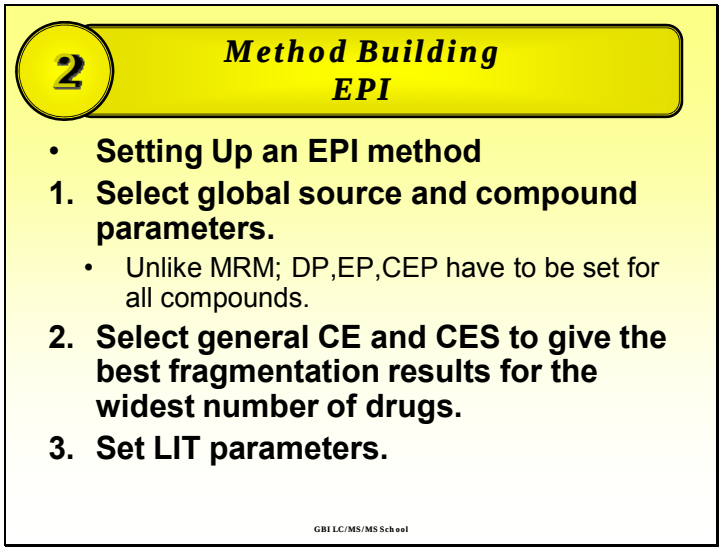

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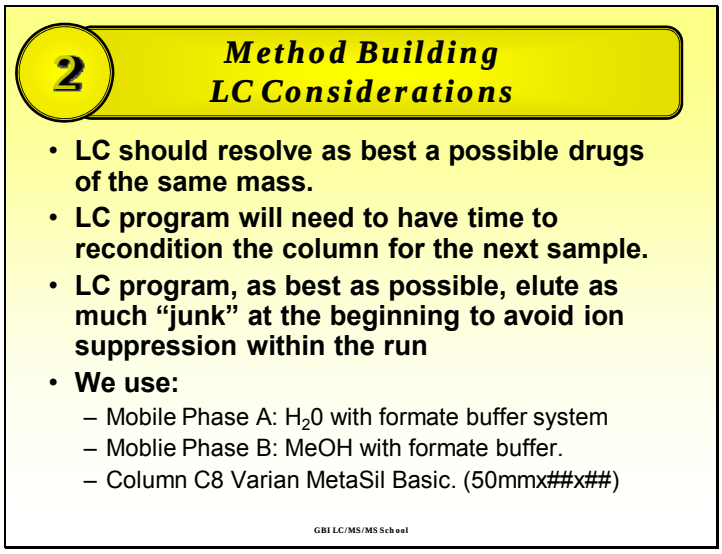

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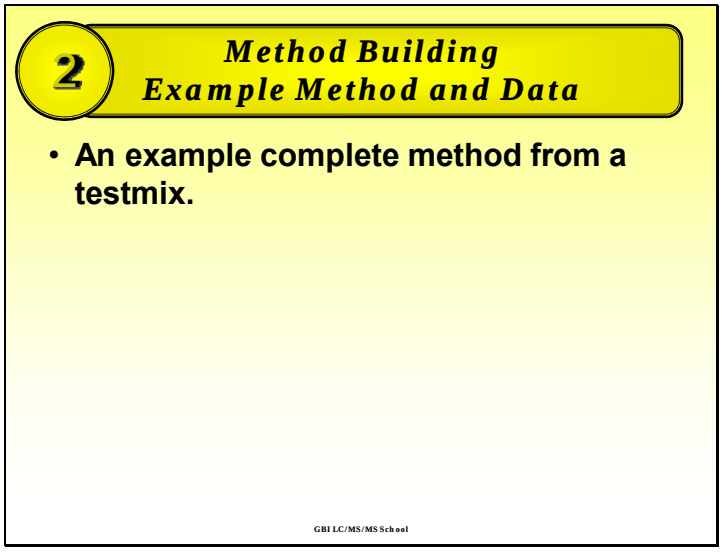

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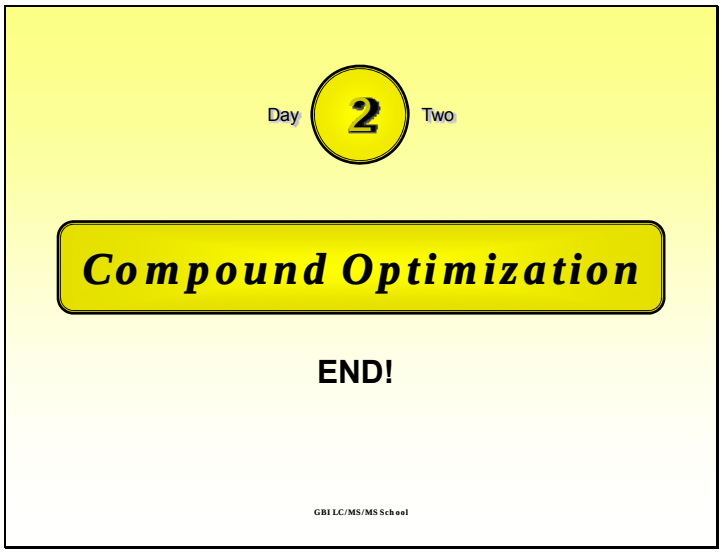
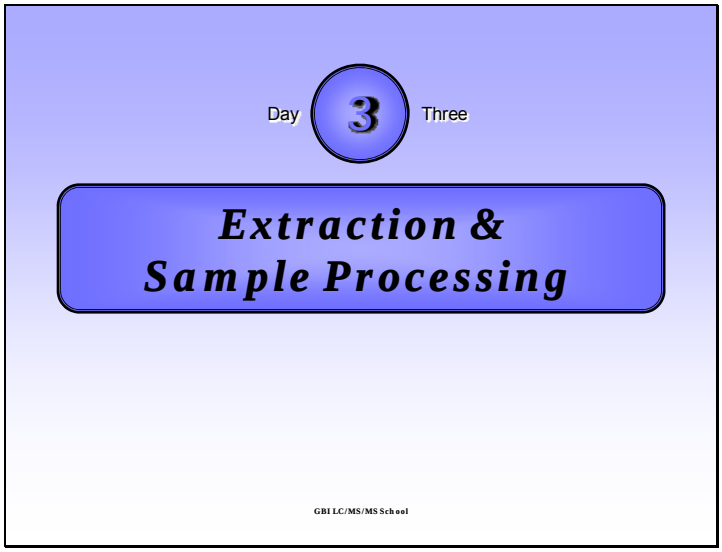


Day 3

Slide 1



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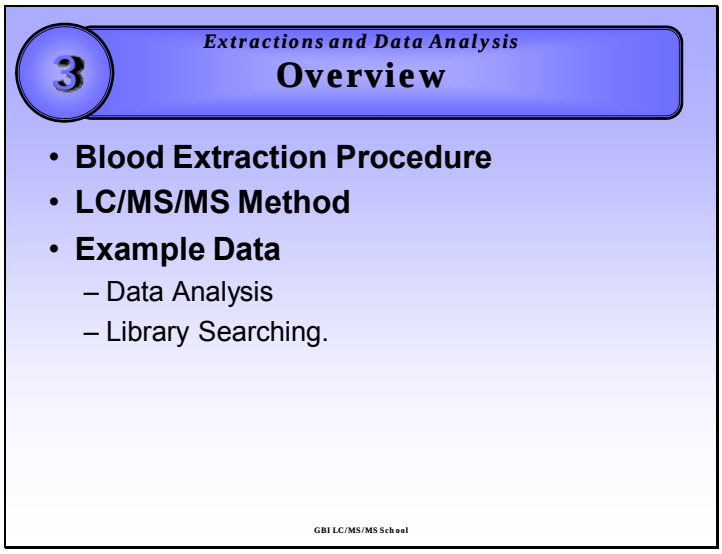

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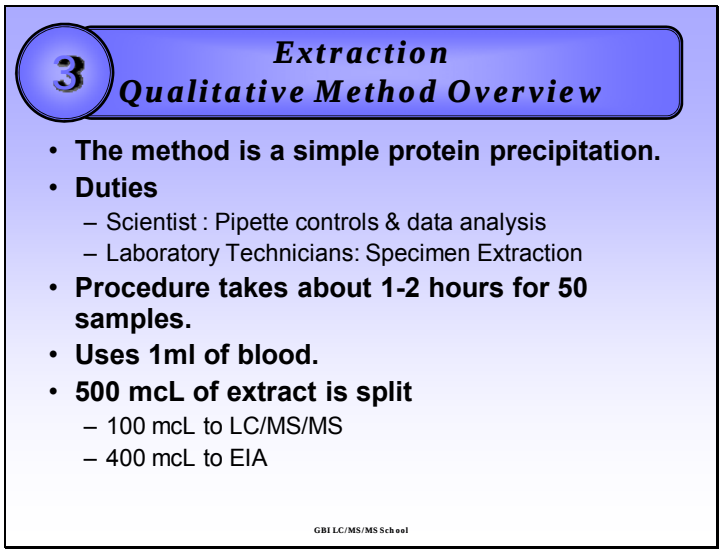

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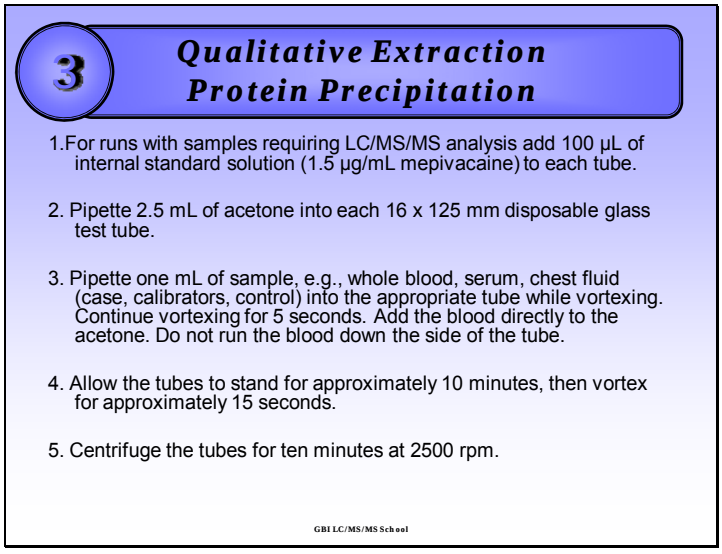

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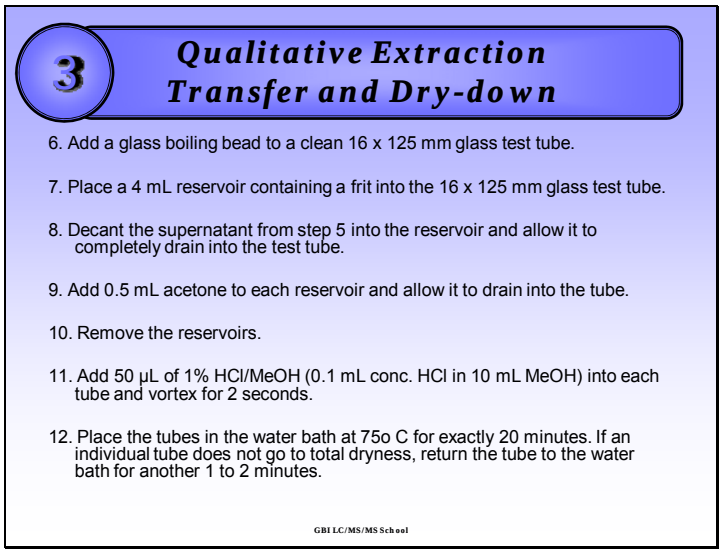

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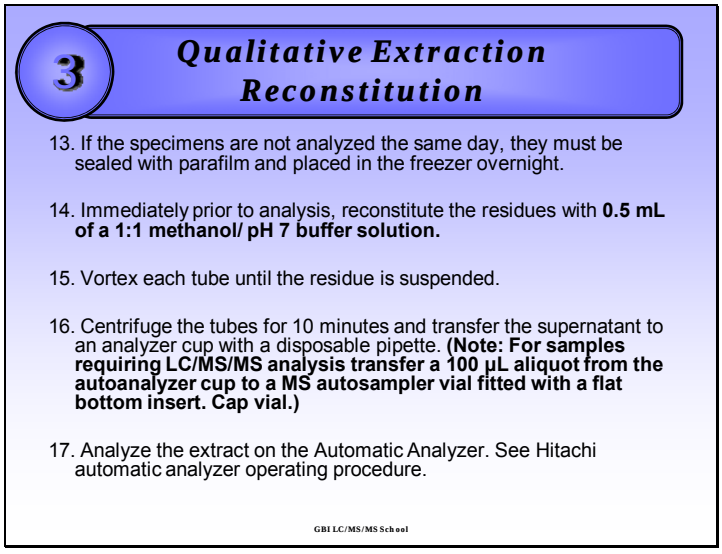

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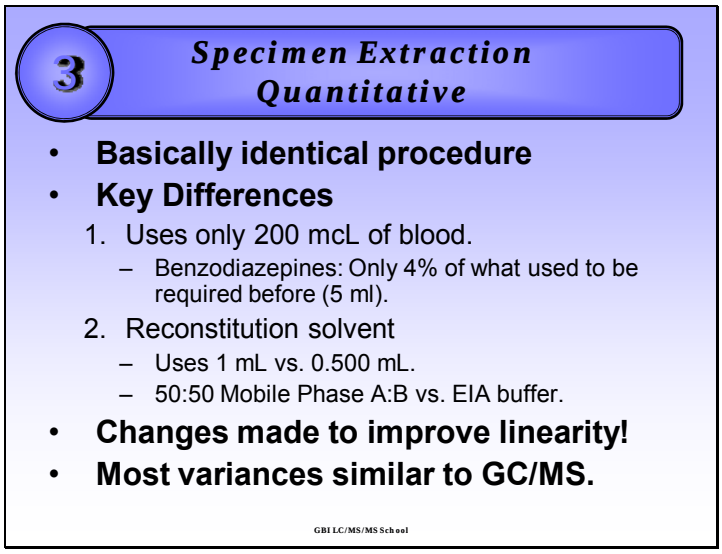

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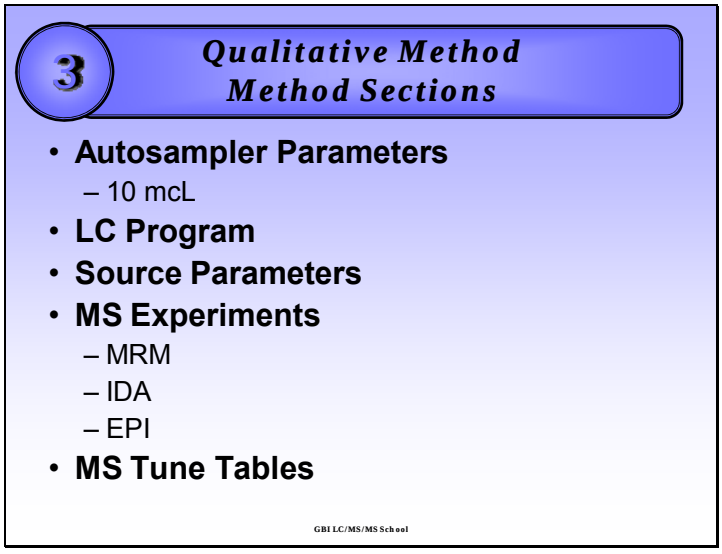

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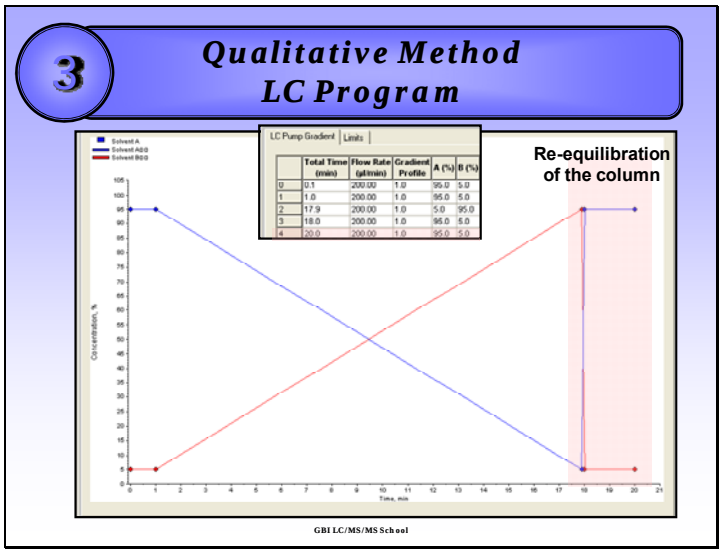

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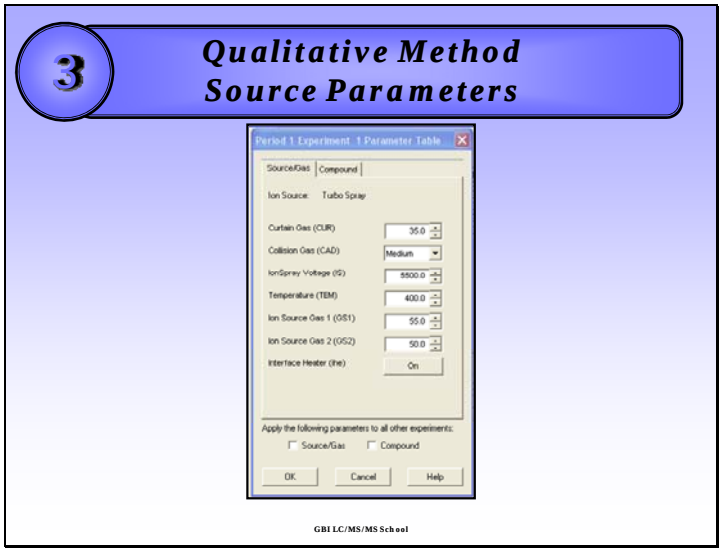

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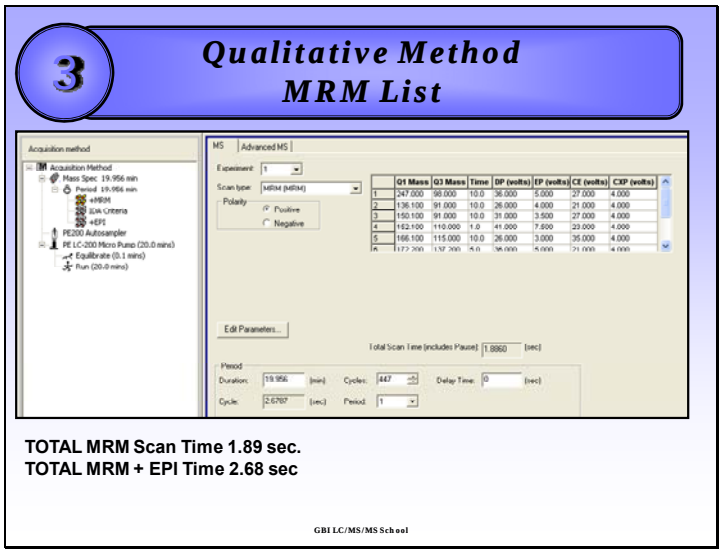

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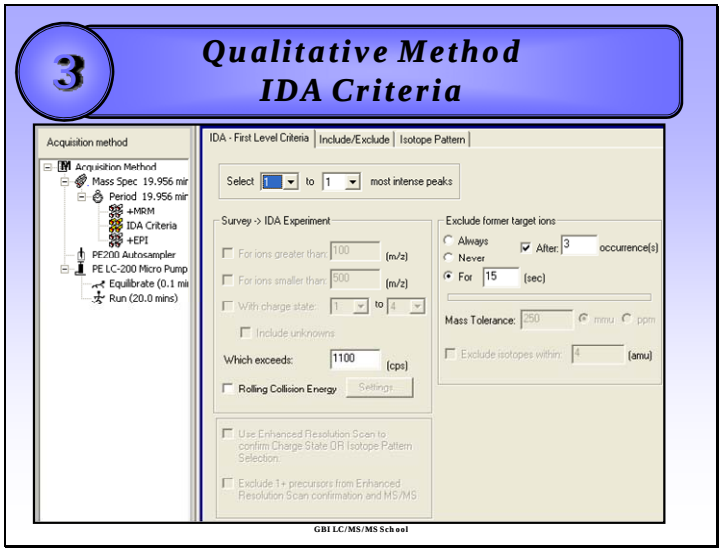

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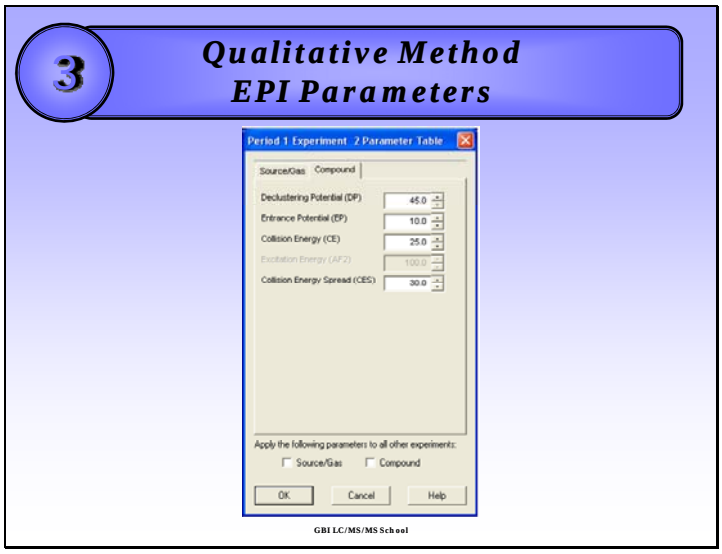

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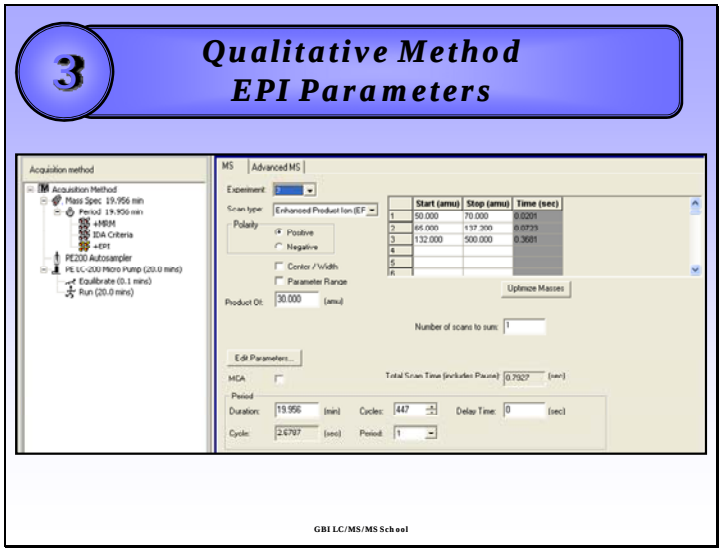

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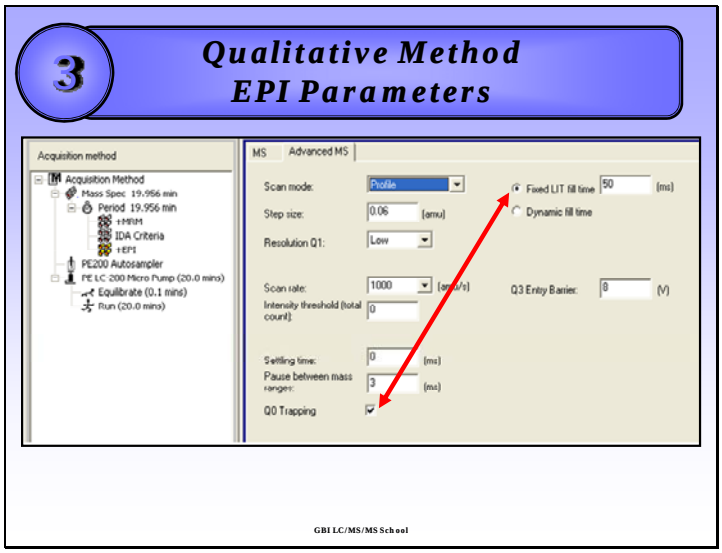

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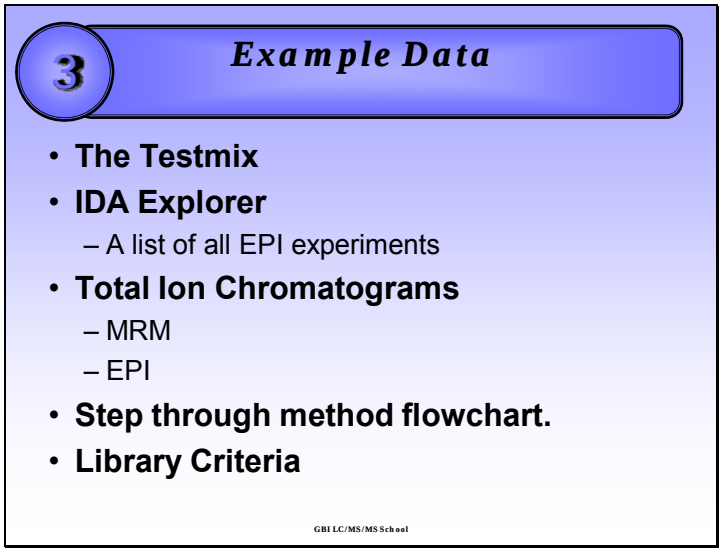

Slide 17

3 Example Data The Testmix

- A test mixture must be injected before each run (maximum of 24 hours). The test mix is an aqueous solution containing:
- 0.2 mg/L of oxycodone (ion suppression)
- 0.01 mg/L of fentanyl (low dose)
- 1.0 mg/L of piroxicam (column trouble)
- 0.2 mg/L of imipramine (resolution)
- 0.4 mg/L of amitriptyline (resolution)
- 0.04 mg/L of methadone (peak masking)
- 0.3 mg/L of mepivacaine (retention time shifts)

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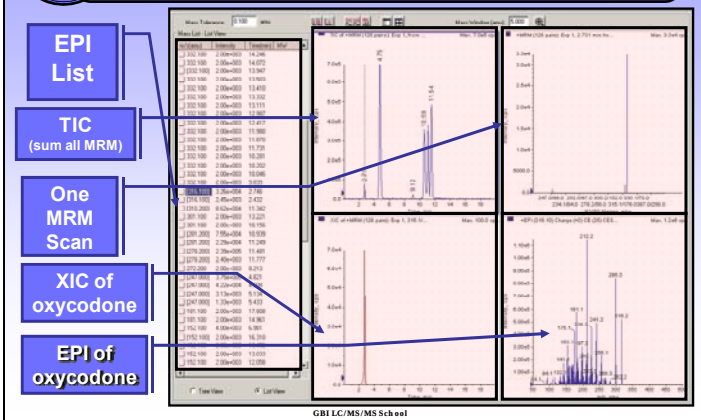
3 Example Data IDA Explorer



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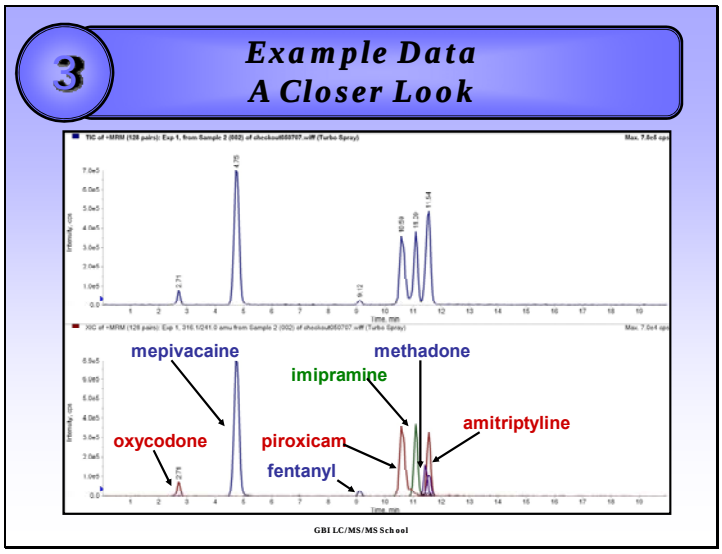
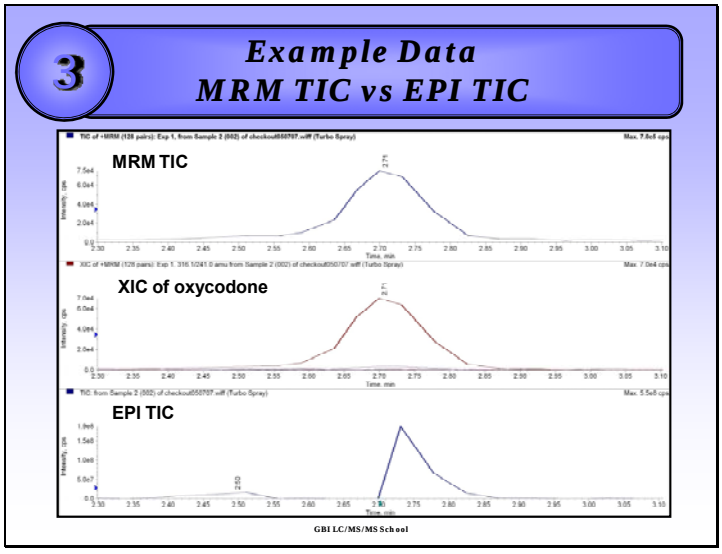
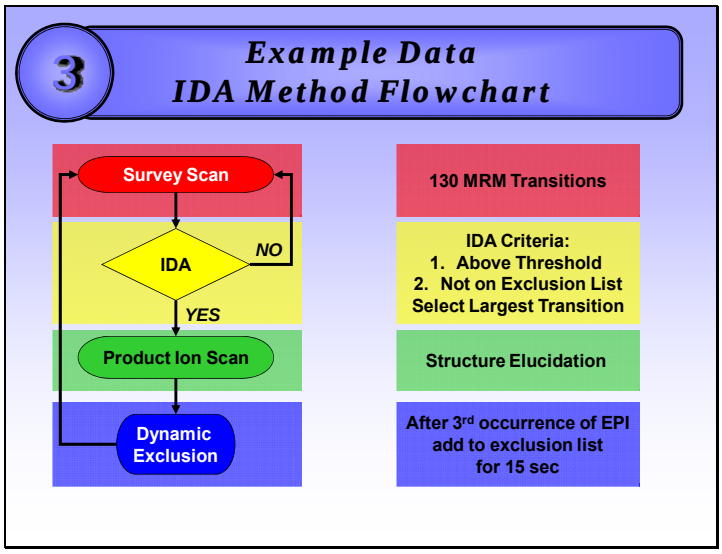


Slide 20



Slide 21



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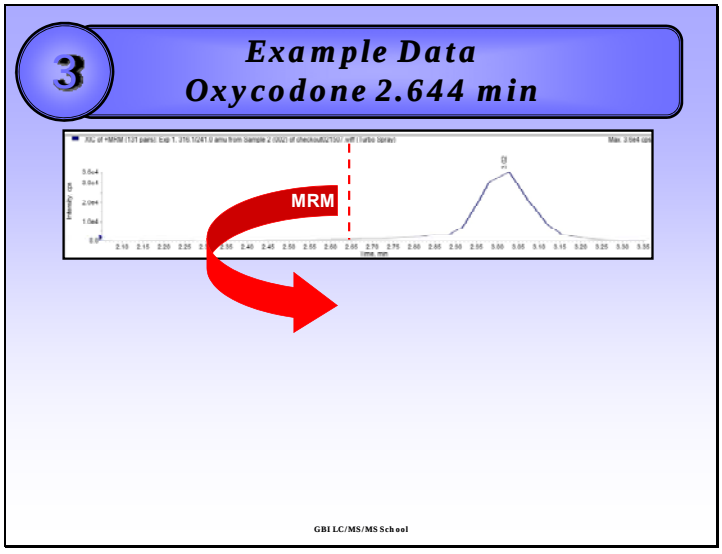

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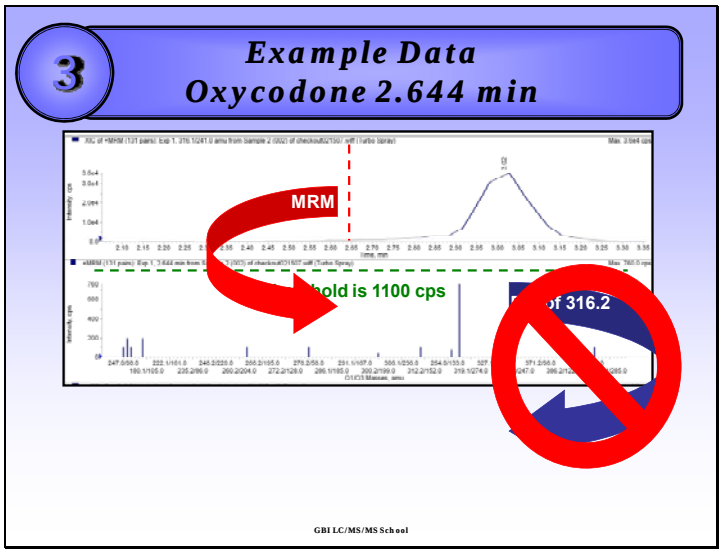

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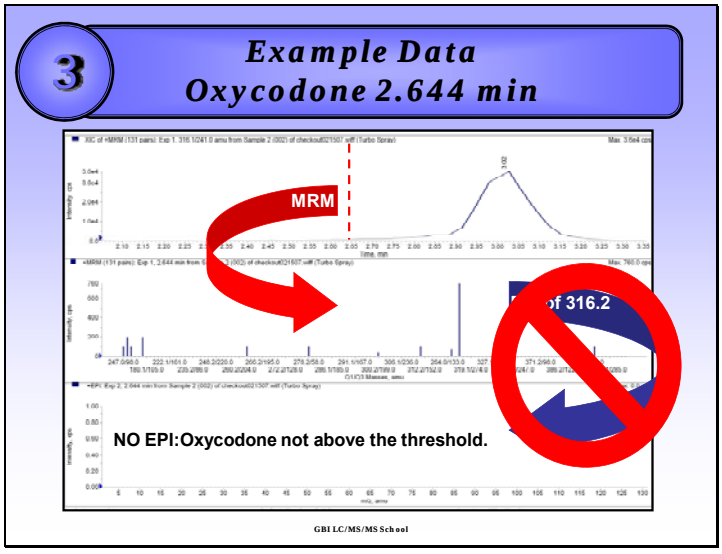

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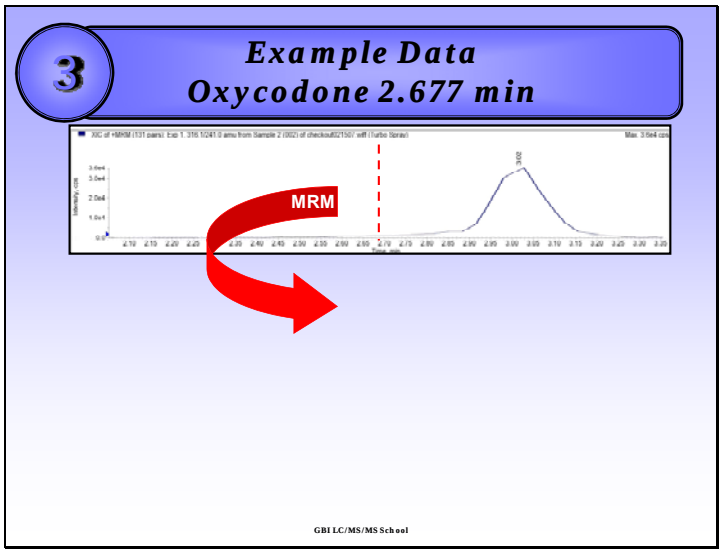

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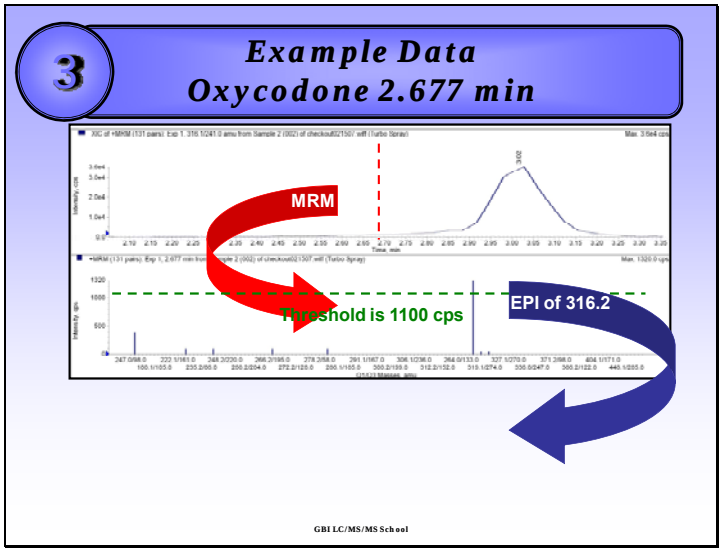

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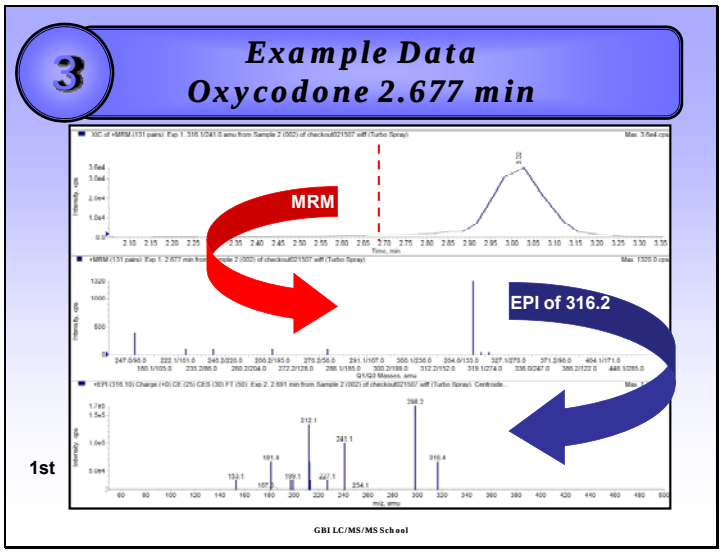

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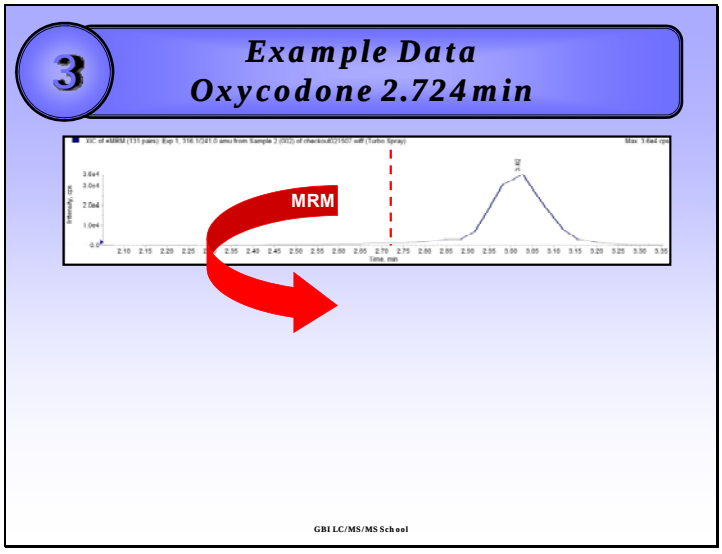

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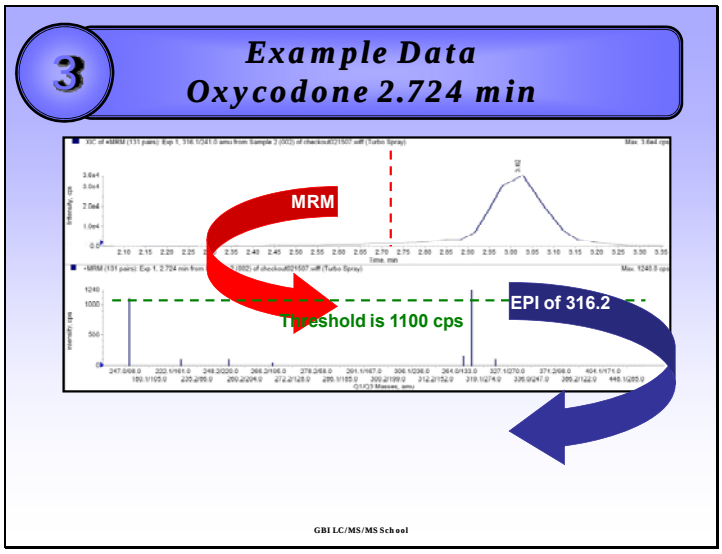

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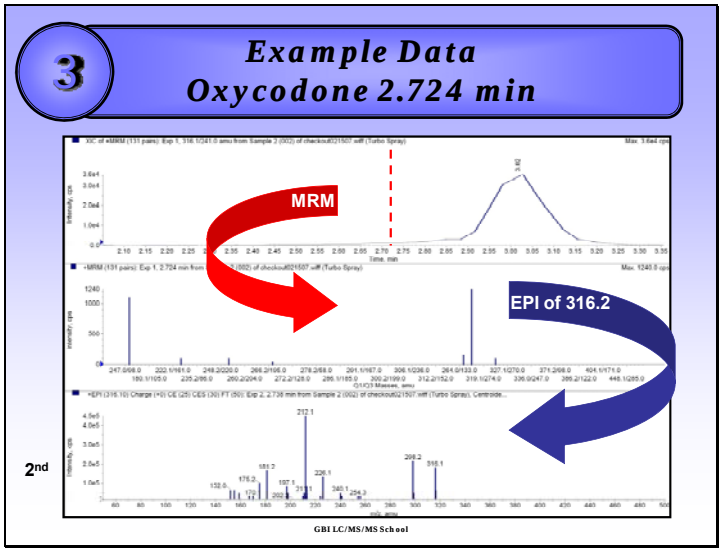

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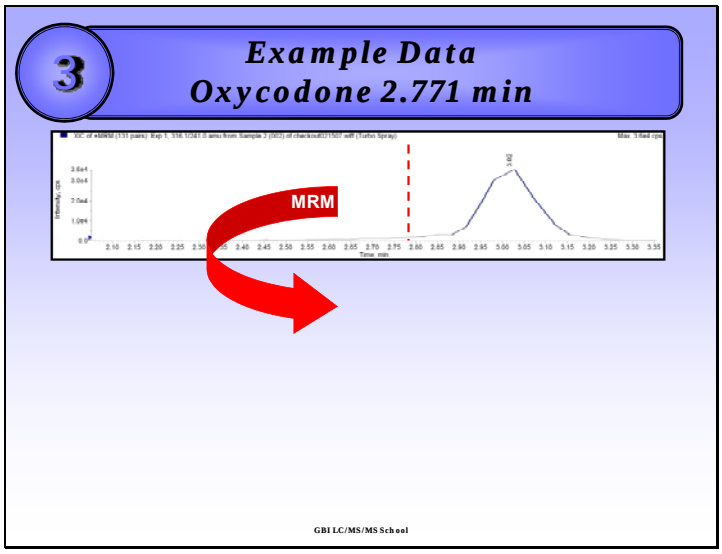

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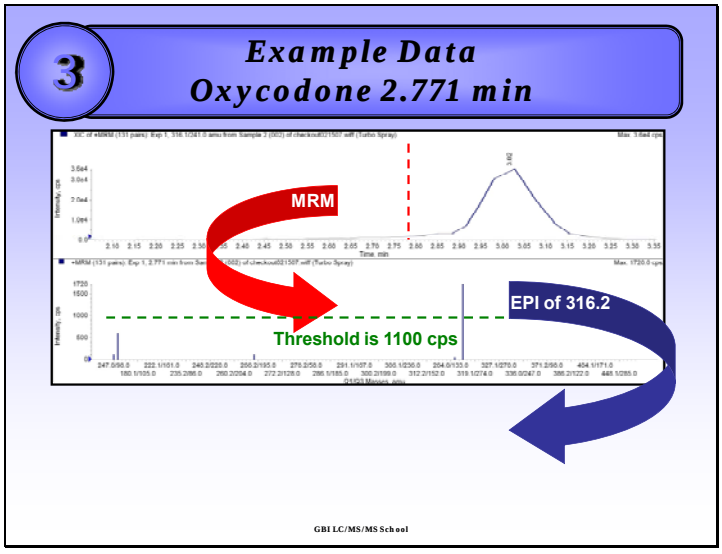

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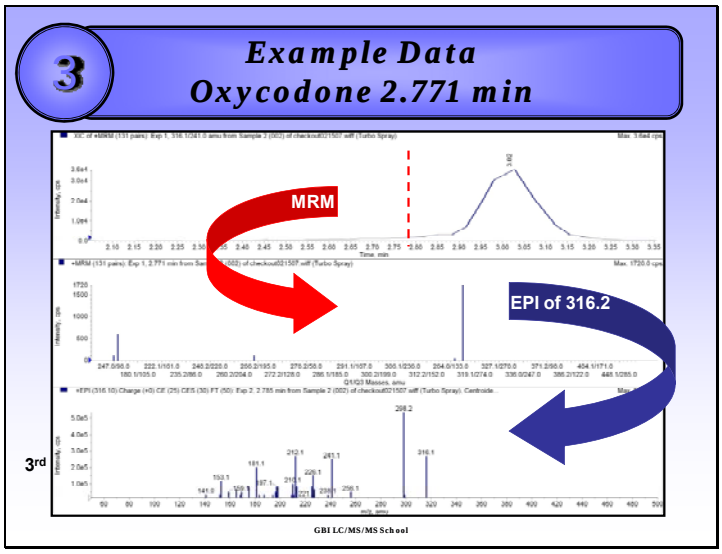

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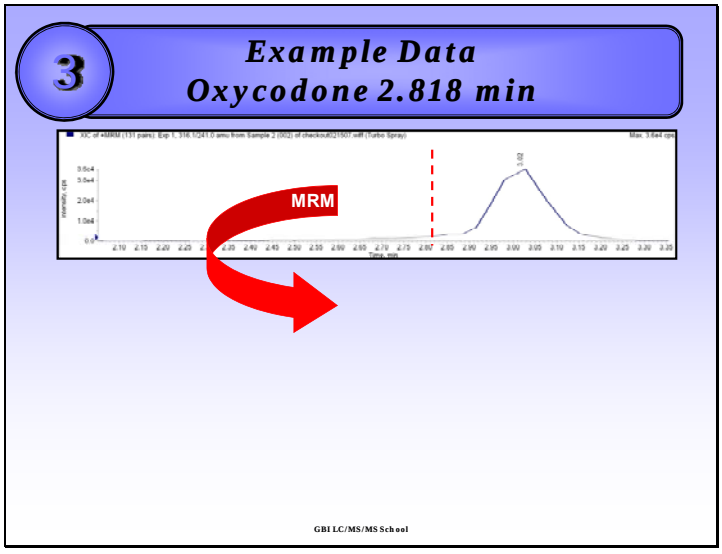

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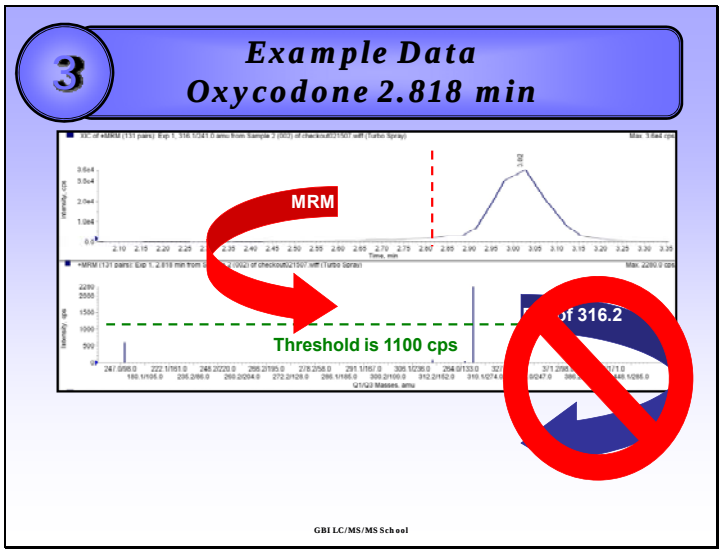

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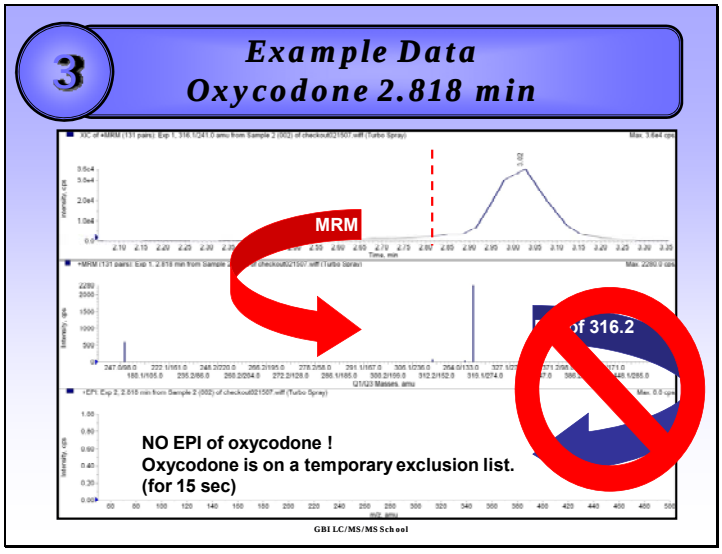

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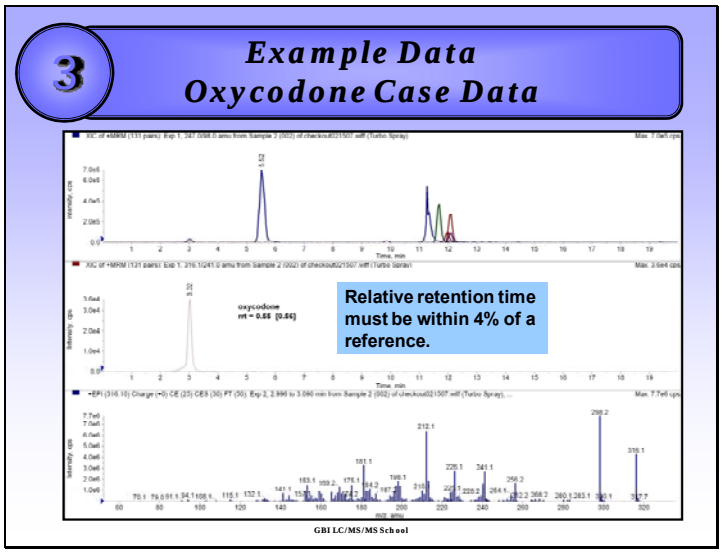

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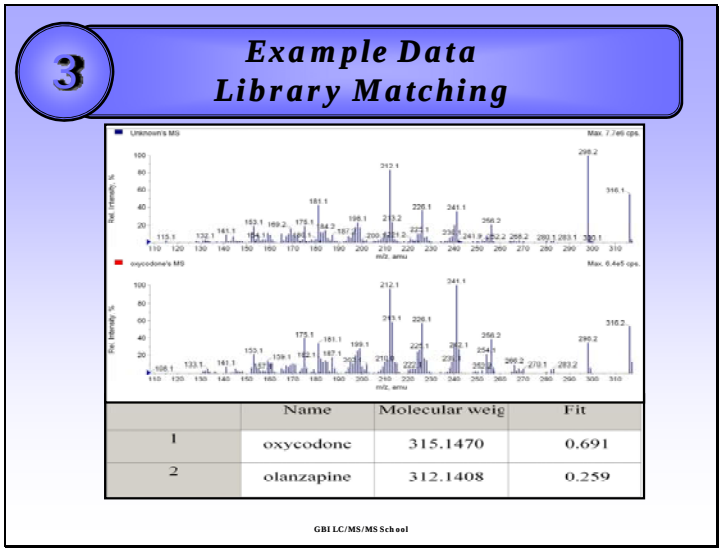

Slide 39

3 Library Matching Standard for a Match

- The following acceptance criteria may be used as guidelines, but not as absolutes, to identify a substance through its mass spectrum:
  1. **Mass spectra will be obtained using the appropriate ion transition.**
  2. **Usually the base peak will be the same and will contain the major ions and relative abundances.**
  3. **When compared to the reference spectrum, the spectra must contain strong similarities.**
  4. **Any differences between the reference spectrum and the unknown spectrum must be carefully evaluated for acceptability.**

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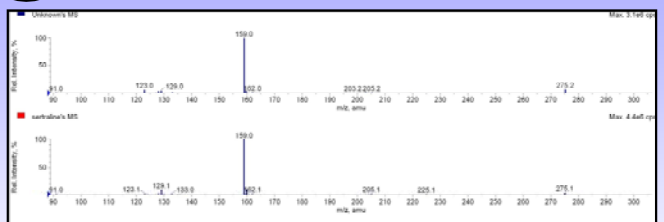
3 Library Matching Just enough?



Sertraline does not have a lot of ions, but even the small ions are always found.

	Name	Molecular weight (a	Fit
1	sertraline	305.0738	0.985

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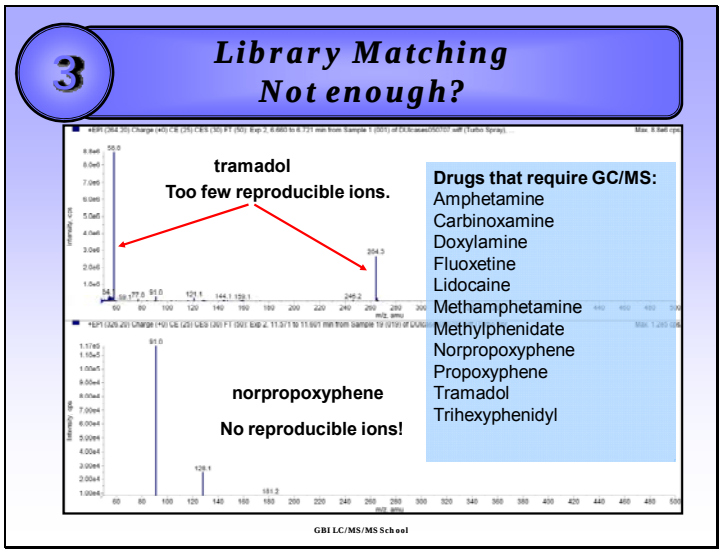
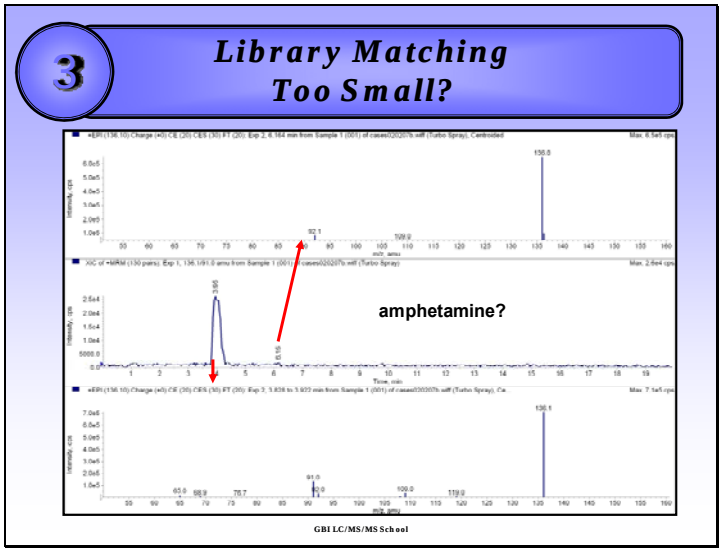


Slide 42



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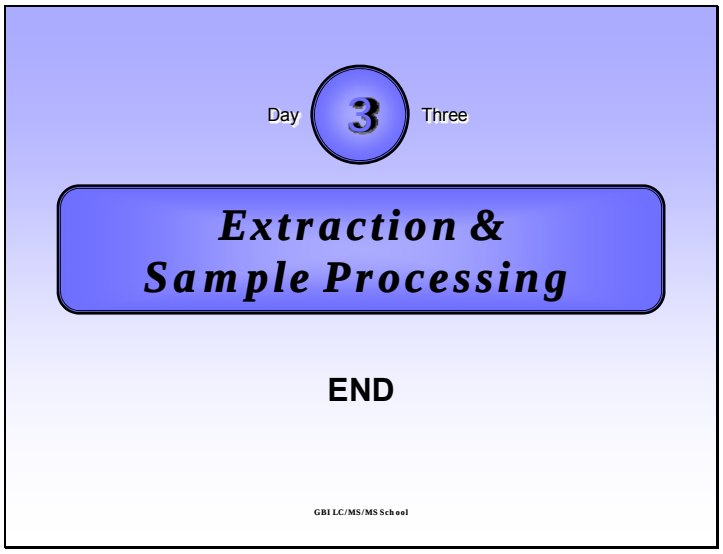
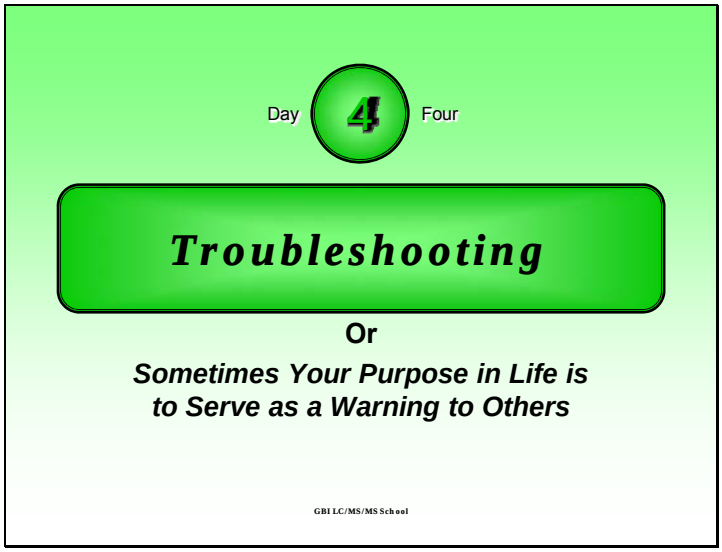
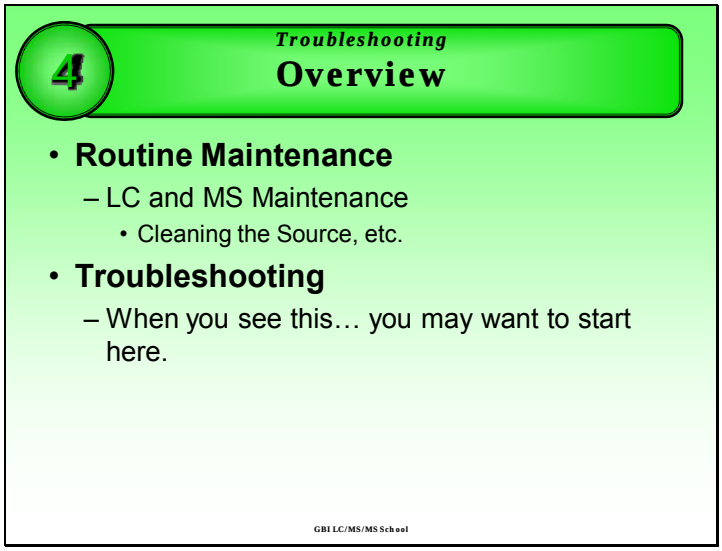


Day 4

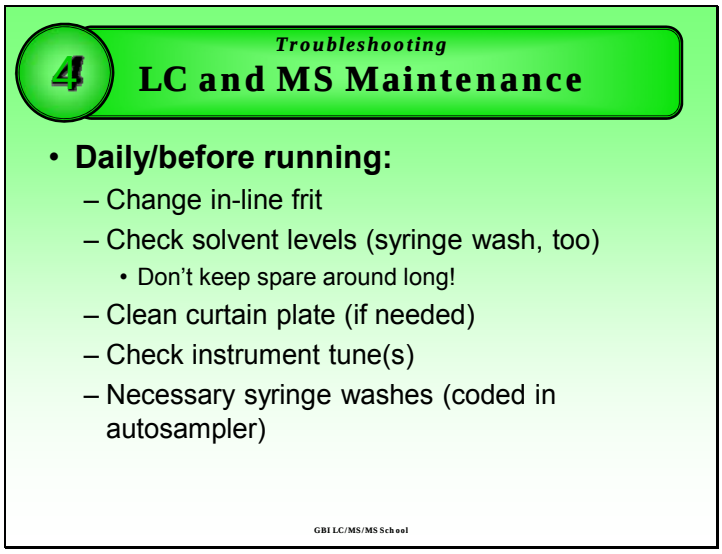
Slide 1



Slide 2

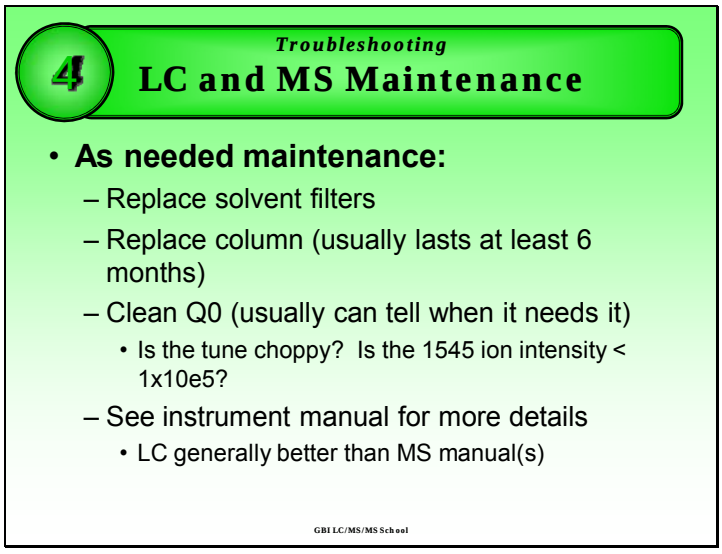


Slide 3

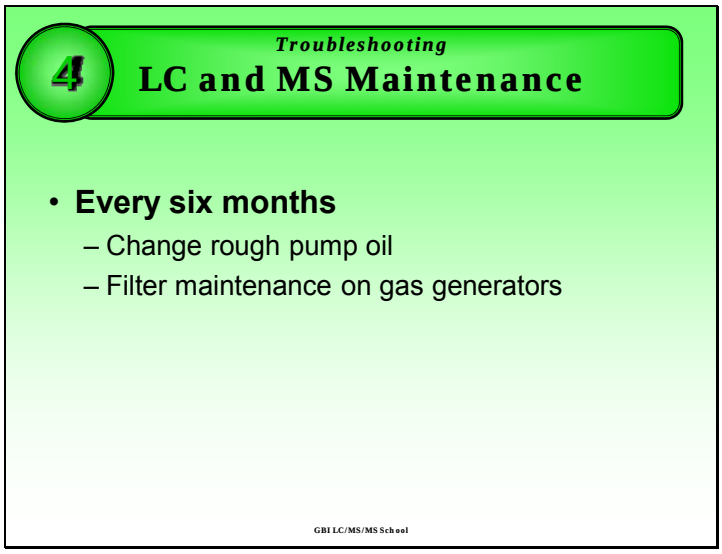


Our syringe washes are at least 2 per injection of 0.5 mL each. Normally, we use around 4 or more.

Slide 4



Slide 5



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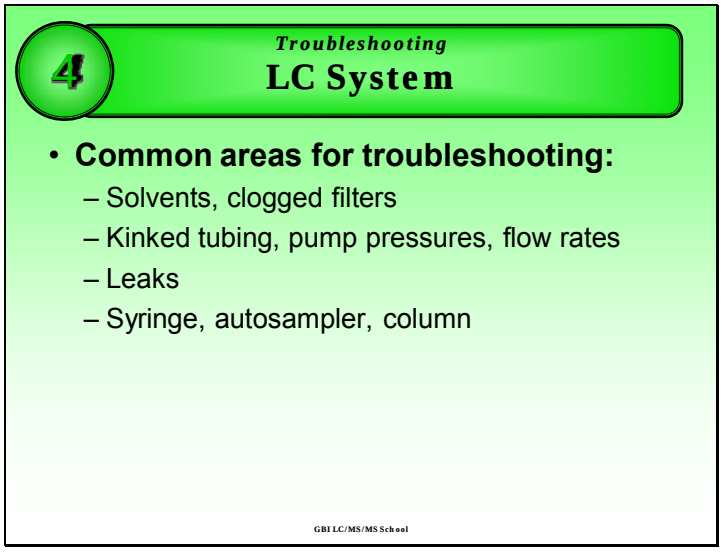

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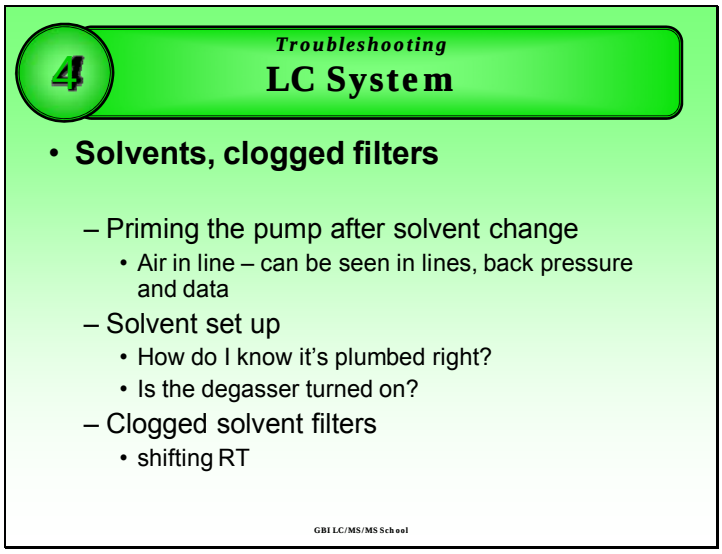
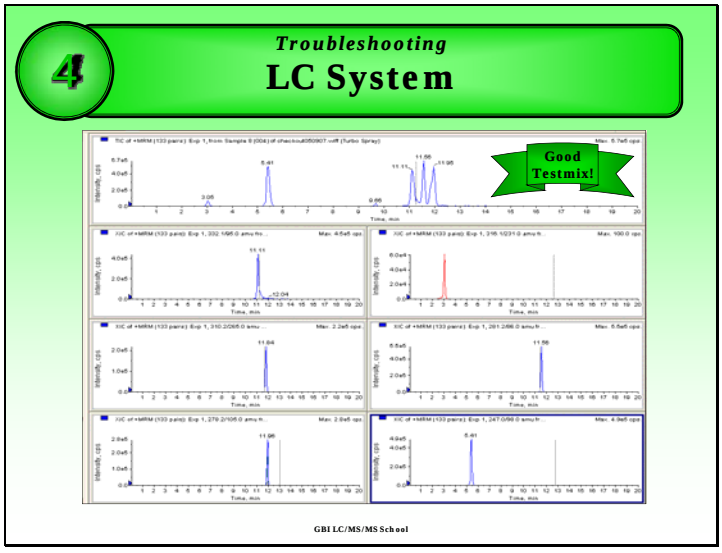
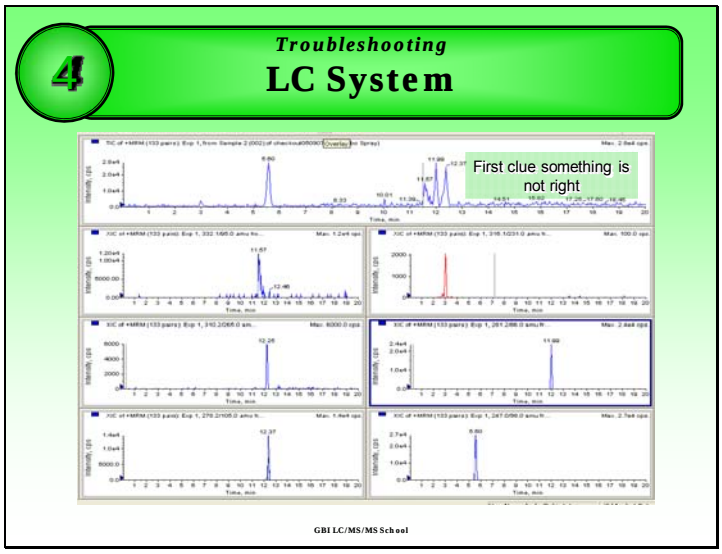


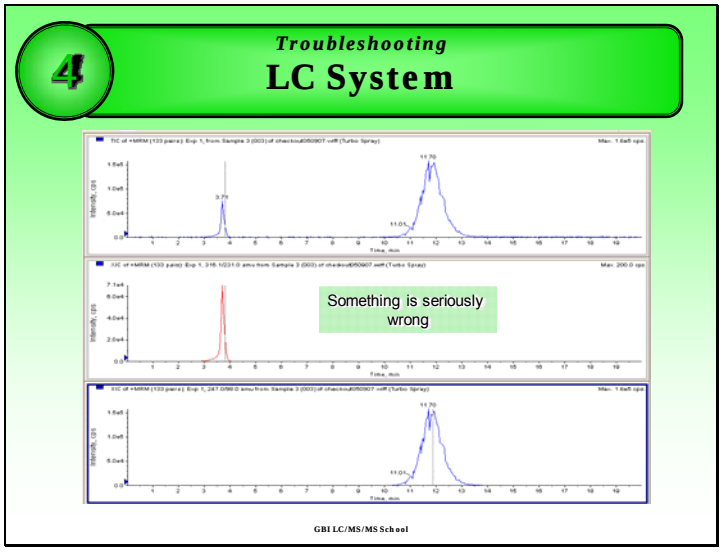
Slide 8



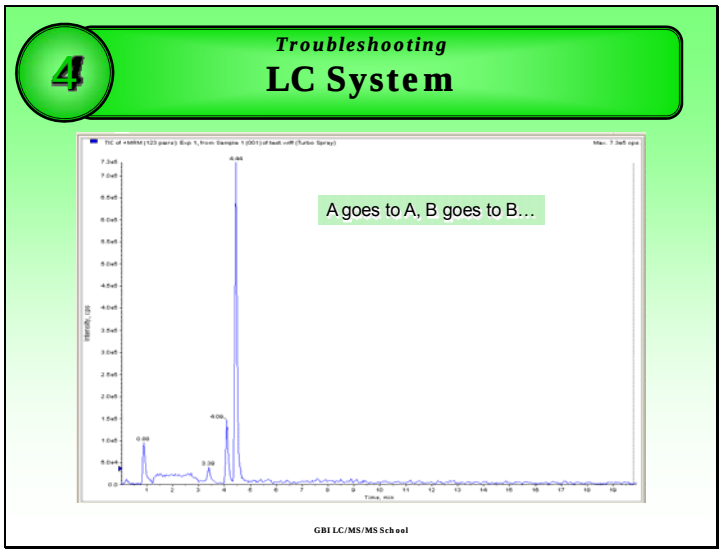
Slide 9



Slide 10



Slide 11



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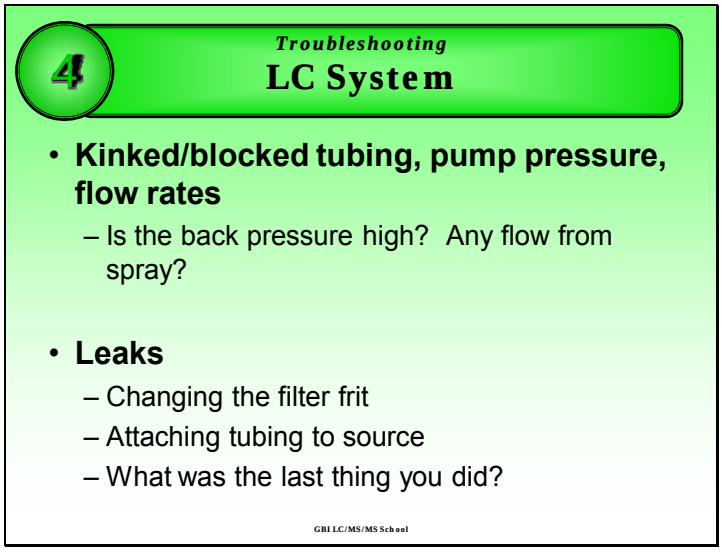

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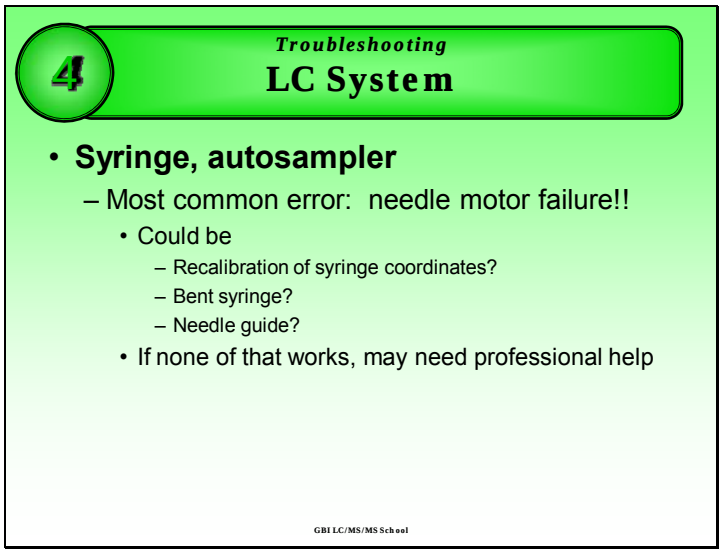
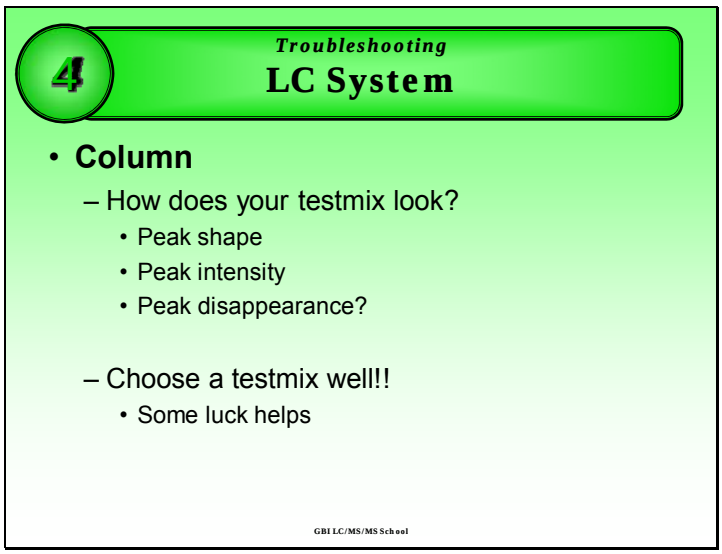
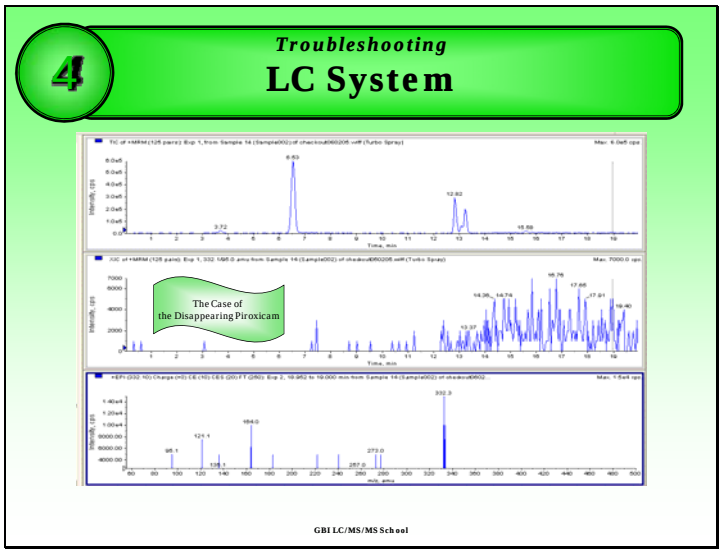


Professional help: #4, multiple needle motor failures. Service rep tried recalibration of the syringe arm; still didn't work. Ultimately sent back to the manufacturer and got a replacement.

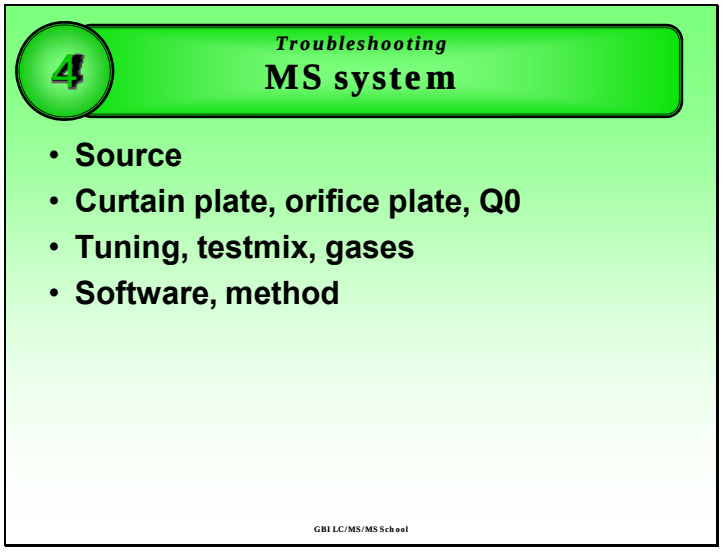
Slide 14



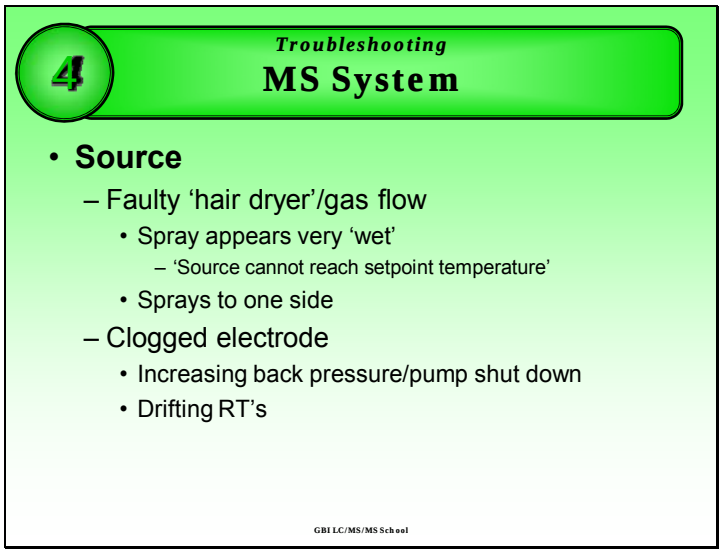
Slide 15



Slide 16

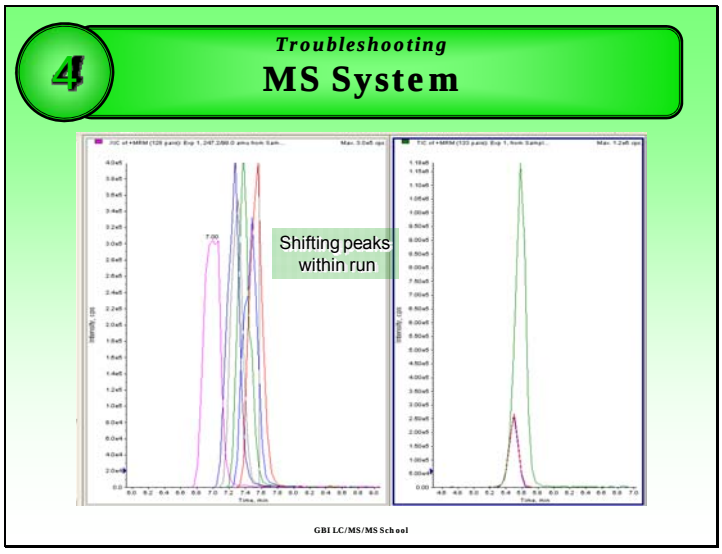


Slide 17



Error message on #1 =
Spraying to one side = o-ring missing on source interface

Slide 18



Slide 19

Troubleshooting
MS System

- **Curtain plate, orifice plate, Q0**
  - Curtain plate (3200) in correct position
    - Not flush with mount – will lose vacuum
  - Orifice plate
    - Be careful when clean curtain plate (very wet Kimwipes – can be all bad)
  - Q0
    - Choppy tune peak shapes?
    - Dirty background noise?
    - Trouble getting reproducible areas?

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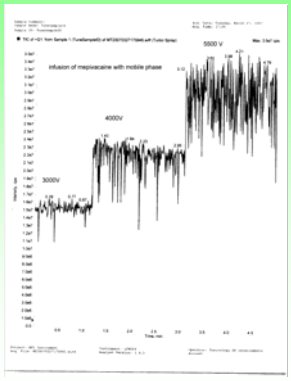
Troubleshooting
MS System



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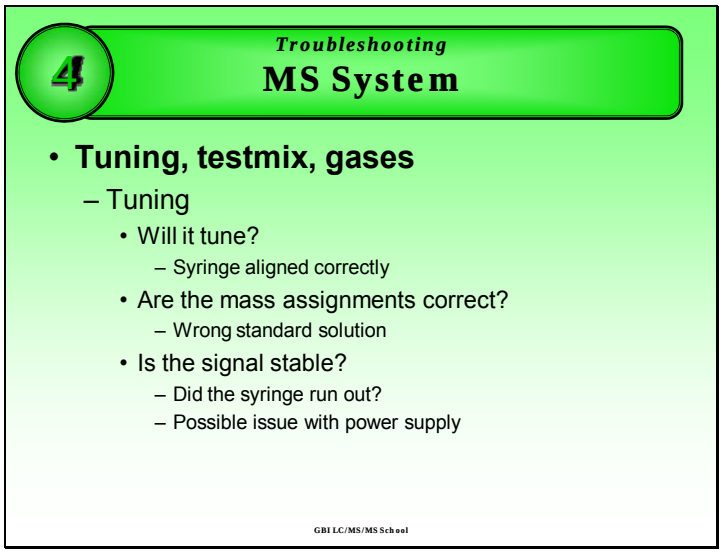
Slide 21



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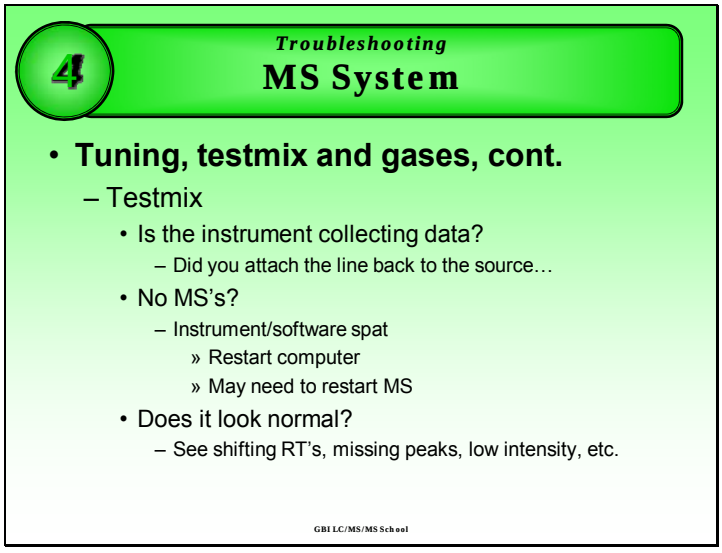

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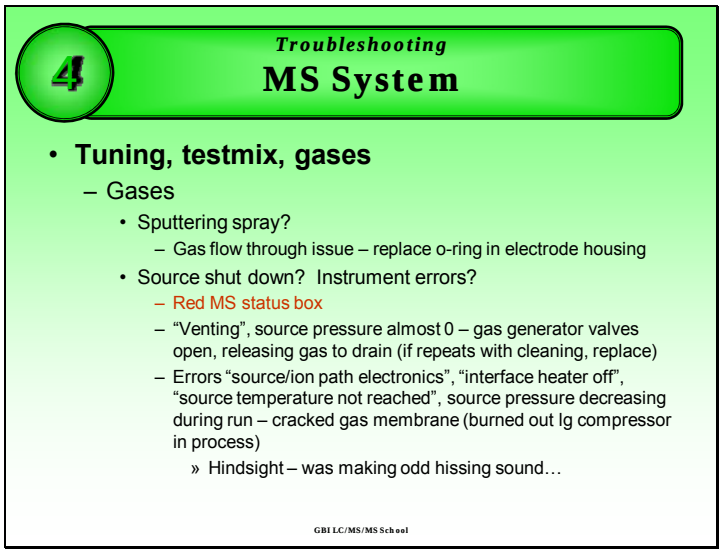

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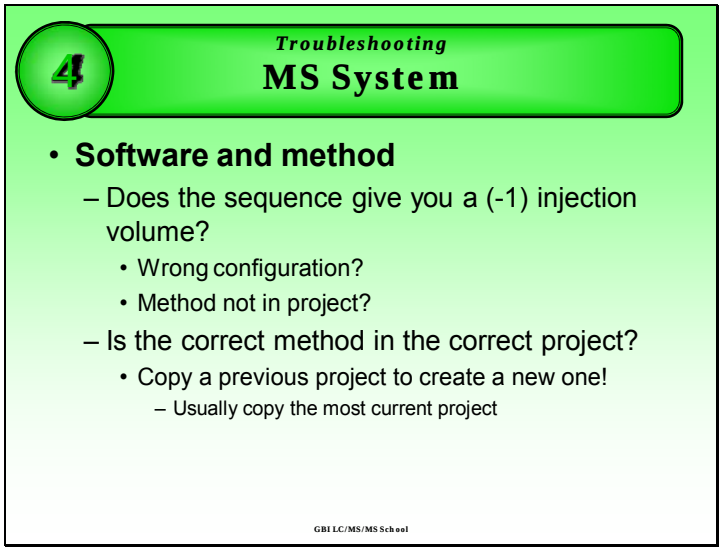

Slide 25