Method Development and Validation for the Identification and Separation of Acetyl Fentanyl, Fentanyl, and Heroin

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Abstract

The identification of fentanyl and fentanyl analogs in heroin samples has become of growing importance in the United States. Acetyl fentanyl has now become a scheduled narcotic in many states, including Kentucky, and it is possible that other fentanyl analogs will become scheduled as well. The use of GC/MS and GC-FID for the identification of acetyl fentanyl has previously been proven difficult due to acetyl fentanyl, fentanyl and heroin having very similar retention times.

This paper includes the research for the development of a method for the separation and identification of fentanyl and acetyl fentanyl in heroin samples by GC/MS and GC-FID. The development of this method allows forensic laboratories to positively identify the presence of fentanyl and fentanyl analogs, in heroin samples that are submitted for testing. To determine the success of the parameter changes that were made throughout the research, the peak separation between the peaks of acetyl fentanyl, fentanyl, and heroin was calculated. Also, the peak resolution was determined for each method.

The results from this project demonstrate that the method developed during the research, through the modification of the temperature program, pressure program, and split ratio, can adequately separate acetyl fentanyl, fentanyl and heroin while still keeping the method runtime short for backlogged forensic labs. With this method acetyl fentanyl, fentanyl, and heroin can be positively identified in samples submitted to forensic laboratories for analysis. The method that

was developed throughout this research showed a 0.590 minute increase in the separation between acetyl fentanyl and heroin and a 1.518 minute increase in the separation between fentanyl and heroin. The method developed also showed an increase in peak resolution. The peak resolution between all of the substances increased at least two-fold. As well as the increased peak separation and resolution, the amount of carryover between heroin and acetyl fentanyl was decreased with the application of the new method.

This research was completed to develop and validate a method for use in the Kentucky State Police Eastern Laboratory. Although this method was developed for a particular laboratory that had specific compounds that they needed a method to separate, this method could easily be implemented in any laboratory and could be used for the separation of a variety of compounds. If this particular method did not work for a laboratory, the same process used to develop this method could easily be repeated in other laboratories to create a method that would meet their needs.

Introduction

Heroin is a semi-synthetic form of morphine, and has been considered one of the most addictive controlled substances (3). Heroin is a schedule I narcotic controlled substance that can be injected, smoked, or sniffed/snorted. It is highly addictive and causes both physical dependence and psychological dependence (6). When heroin enters the brain it binds to opioid receptors. These opioid receptors control many processes throughout the body, such as blood pressure, respiration, and arousal. Long-term use of heroin can also lead to brain damage (4).

Heroin has been a commonly analyzed substance in drug laboratories for years. Until recently, this analysis mainly consisted of positively identifying heroin in samples submitted to

forensic laboratories. The presence of fentanyl, a synthetic opioid, and its analogs, mainly acetyl fentanyl, in samples of heroin is becoming an increasing problem.

Fentanyl is a schedule II narcotic substance. It is a powerful μ-opioid receptor agonist that was first synthesized in 1960 by Paul Janssen. Fentanyl rapidly crosses the blood-brain barrier and has high lipid solubility with rapid onset and short duration of its effects (7). It was first utilized in the treatment of chronic pain and in anesthesia. (13) The increased presence of fentanyl mixed with other illicit substances is a major concern because fentanyl is much more potent than other opioids. Some studies estimate that fentanyl is 80 times more potent than morphine (10). Many fentanyl analogs including alfentanyl, acetyl fentanyl, and sufentanyl were also synthesized in addition to fentanyl itself. Much of the concern surrounding fentanyl analogs is due to their potential for dependence and misuse, their high potency and their associated risk of fatal overdose (10).

Compared to other fentanyl analogs, acetyl fentanyl is more commonly identified in forensic drug laboratories. The significant risk to public health that acetyl fentanyl presents has led to it being emergency scheduled into schedule I of the controlled substances act in May of 2015 (11). Acetyl fentanyl is estimated to be 80 to 100 times more potent than morphine (8). The lethal dose of acetyl fentanyl (9.3 mg/kg) is approximately seven times lower than that of fentanyl and 50 times lower than that of morphine (9). This is one of the reasons there has been an increase in the number of "heroin" related overdoses. Drug users are often unknowingly sold heroin that contains fentanyl or one of its analogs and this can lead to inadvertent overdose or death (8).

The large increase in the number of cases where acetyl fentanyl has been present in heroin samples has resulted in the need for gas chromatography mass spectrometry (GC/MS) and

gas chromatography with a flame ionization detector (GC-FID) methods for the identification and separation of acetyl fentanyl, fentanyl, and heroin. Previously, acetyl fentanyl and heroin have been difficult to separate and identify due to their similar retention times making it difficult for an analyst to positively identify both in a sample. With the very similar retention times a lot of carryover from heroin was seen in the mass spectra of acetyl fentanyl when samples containing both were analyzed, making identification of acetyl fentanyl difficult. Therefore the following research was performed in order to develop a method that could be applied in forensic laboratories. Once the new acetyl fentanyl/heroin method was developed through this project, it was compared to the method already in place for drug analysis at the Kentucky State Police (KSP) Eastern Laboratory. The retention times and peak resolutions of the two methods were compared to show the increase in separation of compounds for the two methods.

Materials

Reagents and Chemicals

Certified reference standards of acetyl fentanyl, fentanyl, and heroin were purchased from Cerilliant (Round Rock, TX) for use in analysis. Details concerning concentration and solvent can be found in Table 1. Methanol was purchased from Fischer Scientific (Pittsburgh, PA) and was used for the blanks throughout analysis.

Table 1: Drug Standard Information

Drug	Chemical Company	Concentration	Solvent
Acetyl Fentanyl	Cerilliant	1 mg/mL	Methanol
Fentanyl	Cerilliant	1µg/mL	Methanol
Heroin	Cerilliant	1 mg/mL	Acetonitrile

Sample Preparation for Identification/Separation

In order to determine the degree of separation of acetyl fentanyl and heroin in samples in which they are both present, solutions containing various amounts of acetyl fentanyl, fentanyl, and heroin were prepared by placing the three standards, at the concentration at which they were purchased, into GC vial inserts with final volumes of approximately 250μL. The sample solutions were referred to as "Test Mixes" and their composition can be found in Table 2. Also, samples of unknown concentration and combinations of drugs of interest were obtained, to simulate "street" samples of these drugs. These samples were referred to as "Unknowns" throughout the project and the composition of these solutions can be found in Table 2.

Table 2: Composition of Solutions

Sample Solution	Composition
Test Mix 1	Acetyl fentanyl, heroin
Test Mix 2	Acetyl fentanyl, fentanyl, heroin
Unknown 1	Acetyl fentanyl, fentanyl, heroin
Unknown 2	Acetyl fentanyl, fentanyl, heroin
Unknown 3	Fentanyl, heroin
Unknown 4	Fentanyl, heroin

Instrumentation

An Agilent Technologies 7890B gas chromatograph with a 5977A mass spectrometry detector (MSD) and flame ionization detector (FID) was used for analysis throughout this project. The GC/MS contained a DB-5MS Ultra Inert capillary column with a length of 15 meters, a diameter of 0.250 millimeters, and a film thickness of 0.25 micrometers.

Methods

The optimization of the GC/MS instrument parameters for the identification and separation of acetyl fentanyl, fentanyl, and heroin was the overall goal of this project. To begin,

data was collected using the standard method for drug analysis in use by the KSP Eastern Laboratory. The parameters for that method can be found in Table 3.

Table 3: Parameters for KSP GC/MS Method of Separation

Oven Temperature			Pressure			Injector	
		Hold Time			Hold Time	Mode	Split
Initial	100°C	0.50 min	Mode	Ramped		Split Ratio	50:1
Ramp	20°C /min	8.5 min	Initial	5 psi	0.5 min	Carrier Gas	Helium
Final	315°C	0 min	Ramp	150 psi/min to 40 psi		Injection Volume	1.00 µL

The oven temperature program, pressure program, and split ratio were adjusted throughout the project in order to achieve optimal separation of acetyl fentanyl, fentanyl and heroin in samples. Initially one parameter was adjusted at a time to determine how changing each parameter would affect the resulting spectra. Once that was determined multiple parameter changes at one time were tried until optimization occurred. The parameters for the final method that showed the greatest separation, and therefore the best identification of acetyl fentanyl and heroin can be found in Table 4.

Table 4: Parameters for Optimal GC/MS Method of Separation

(Acetyl Fentanyl/Heroin Method)

Oven Temperature			Pressure			Injector	
		Hold Time			Hold Time	Mode	Split
Initial	230°C	13.5 min	Mode	Ramped		Split Ratio	25:1
Isothermal Temperature Program			Initial	5 psi	0.5 min	Carrier Gas	Helium
			Ramp	150 psi/min to 10 psi		Injection Volume	1.00 µL

Results/Discussion

GC-FID

The GC-FID method currently in place in the KSP Eastern Laboratory was determined to adequately separate acetyl fentanyl and heroin, allowing the focus to be on developing a new GC/MS method for identification and separation of acetyl fentanyl and heroin. Figure 1 shows an example of one of the GC-FID chromatograms obtained for Test Mix 1. The retention times obtained through GC-FID analysis showed sufficient separation so that each of the peaks obtained could be individually associated with a controlled or non-controlled substance. In the case of this research, the peaks obtained could be associated with acetyl fentanyl, fentanyl, or heroin.

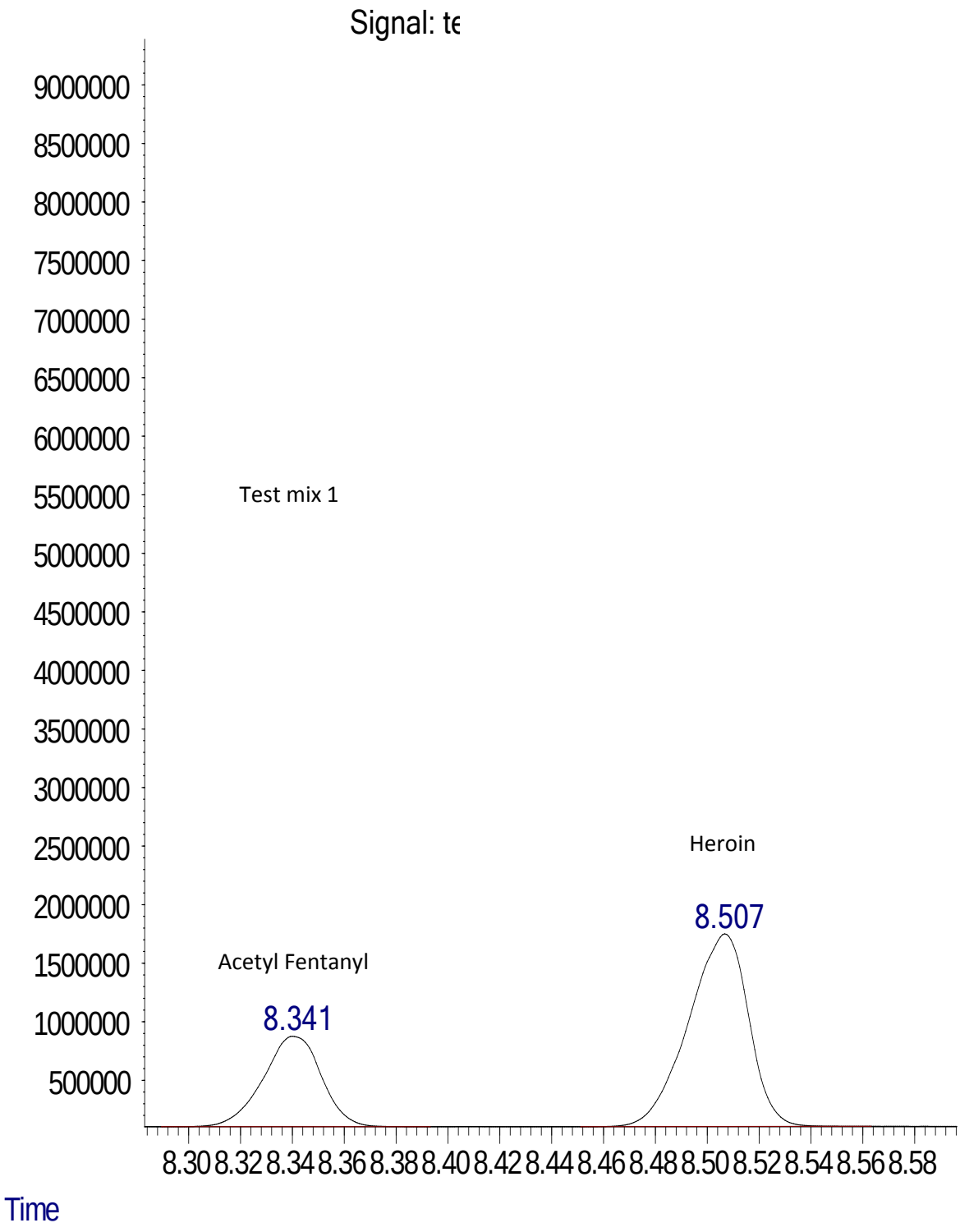

Figure 1: GC-FID Chromatogram for Test Mix 1 (zoomed)

GC/MS

The results of GC/MS portion of this research showed that an improved method for the identification and separation of acetyl fentanyl, fentanyl and heroin could be developed. The retention times and the peak resolutions obtained from the method currently in place by the KSP Eastern Laboratory were compared to those obtained from the new method (acetyl fentanyl/heroin method).

Each sample solution, ("Test Mix" or "Unknown") was run on the current KSP method in triplicate. The retention times from one of those runs are displayed in Table 5. This table contains only the retention times for a single run of the solutions. The total ion chromatogram (TIC) for Test Mix 1 and the mass spectra for the acetyl fentanyl and heroin present in the sample can be found in Figure 2A-2C. Similar mass spectra and TICs were obtained for all of the sample solutions, with the only differences due to different drugs contained in the samples. The TIC for Unknown 1 and the mass spectra for the acetyl fentanyl, fentanyl, and heroin present in the sample can be found in Figure 3A-3D.

Table 5: KSP Method Retention Time (RT) Data for Run 1

	Heroin RT (min)	Acetyl Fentanyl RT (min)	Fentanyl RT (min)
Test Mix 1	8.146	8.303	-
Test Mix 2	8.140	8.303	8.525
Unknown 1	8.146	8.303	8.519
Unknown 2	8.152	8.292	8.519
Unknown 3	8.140	-	8.519
Unknown 4	8.140	-	8.519

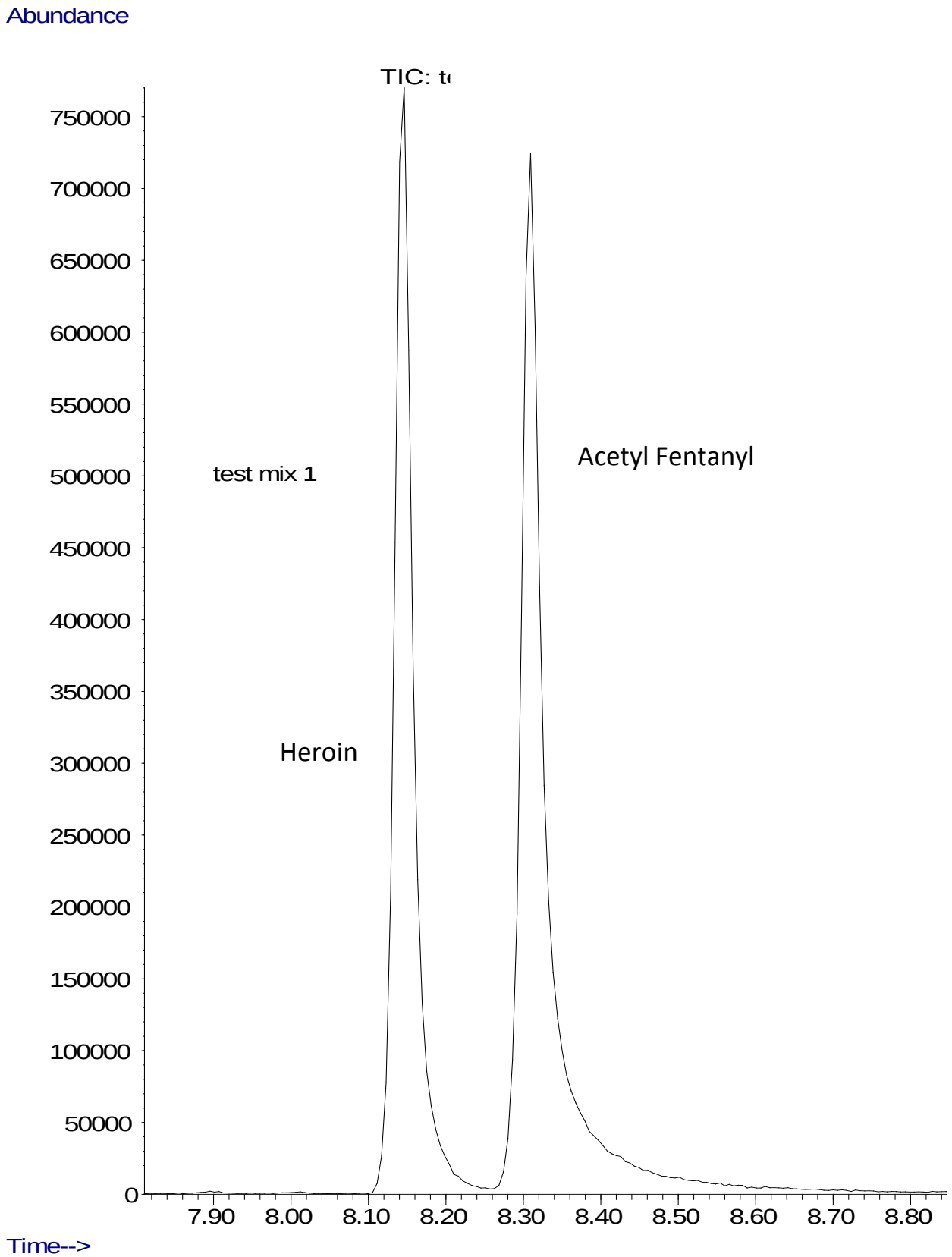

Figure 2A: Total Ion Chromatogram for Test Mix 1(zoomed)

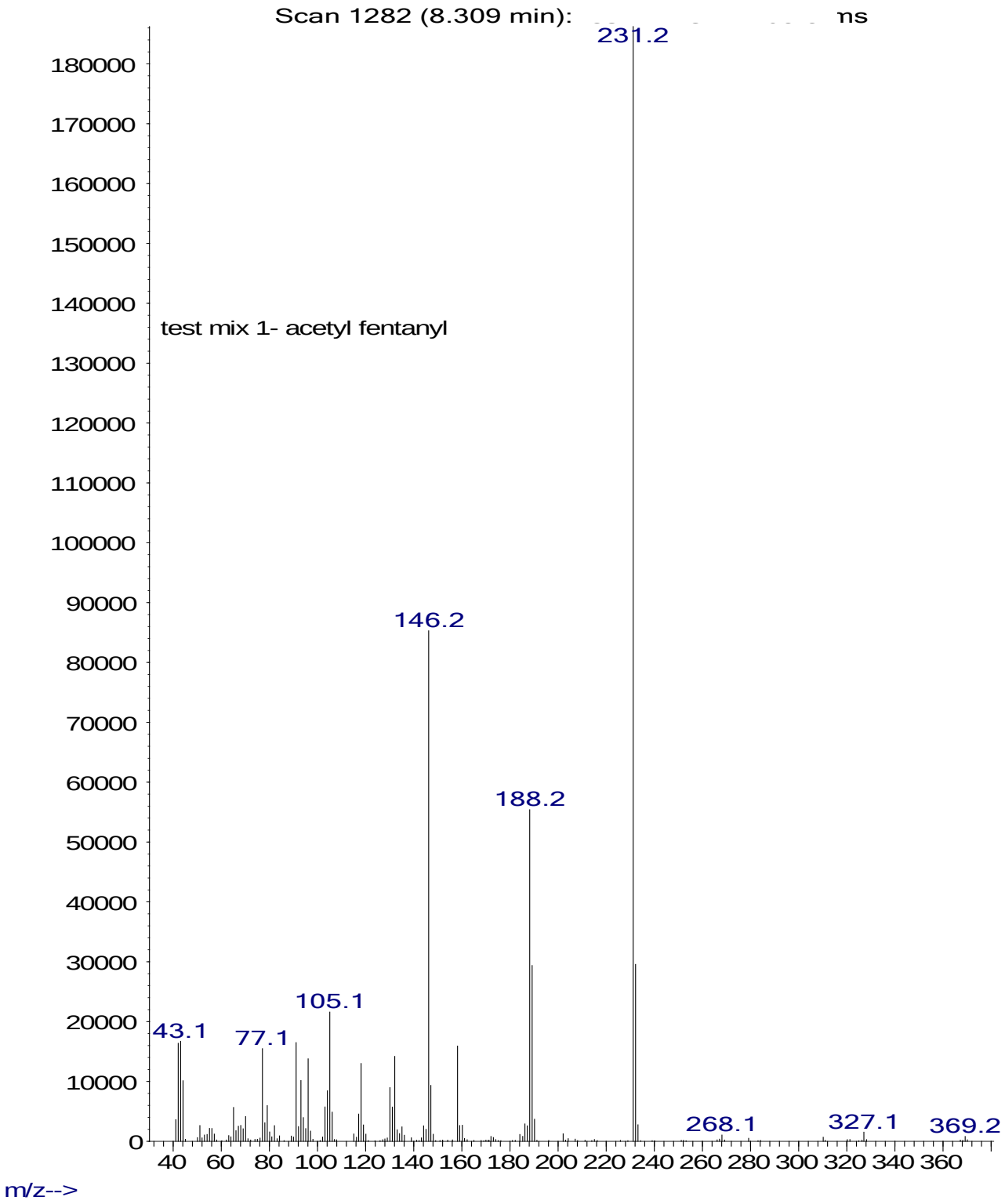

Figure 2B: Acetyl Fentanyl Mass Spectrum

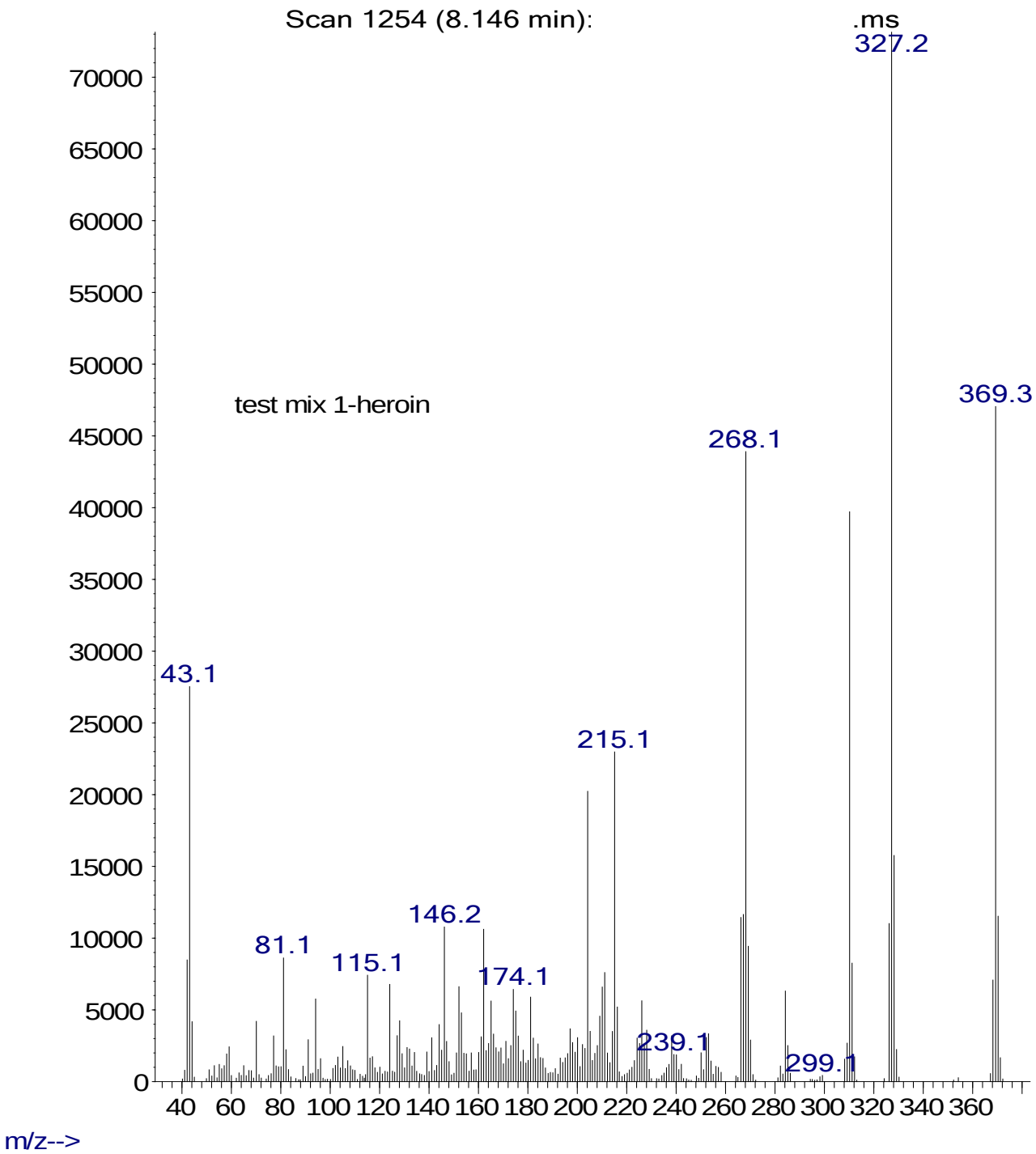

Figure 2C: Heroin Mass Spectrum

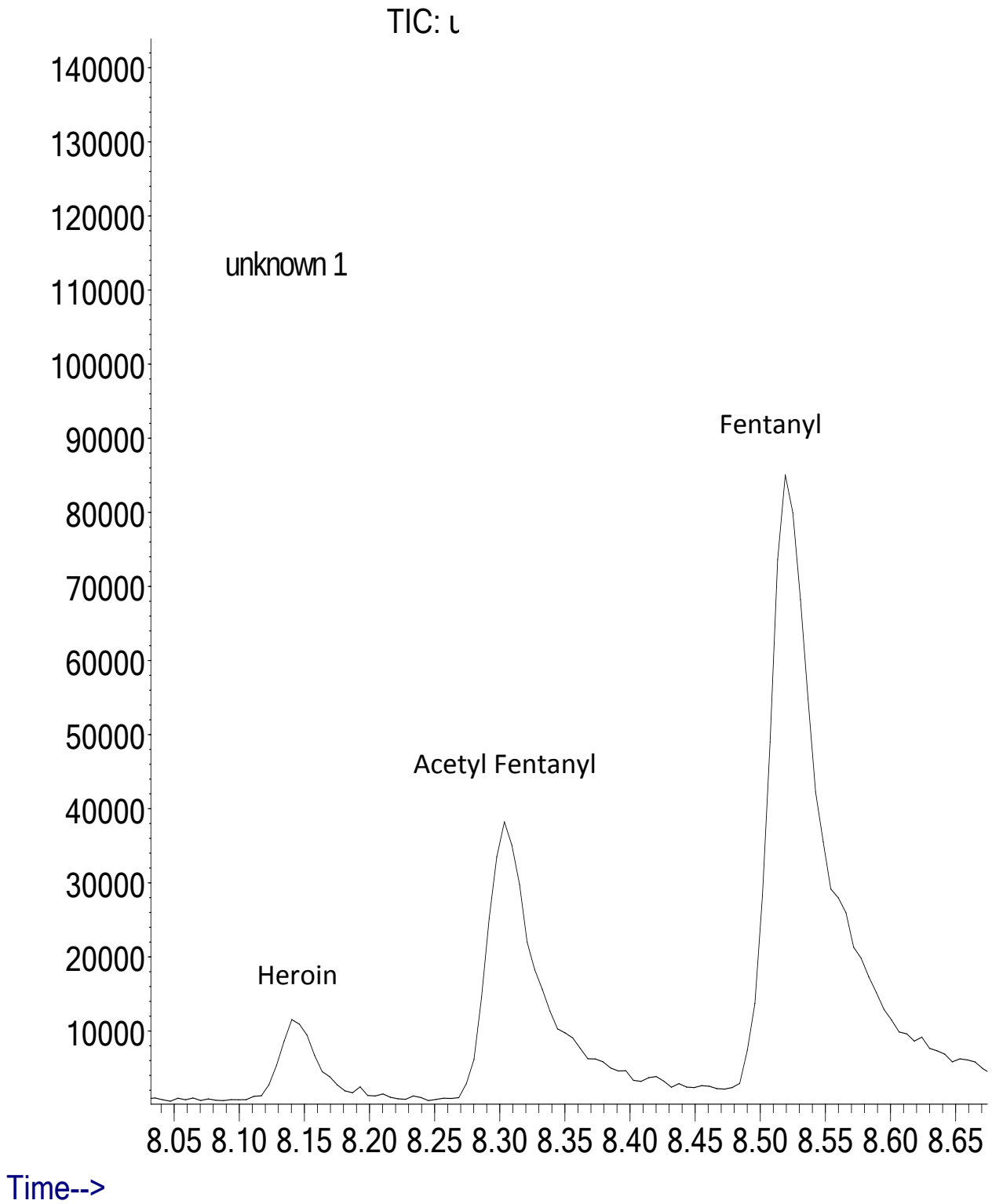

Figure 3A: Total Ion Chromatogram for Unknown 1 (zoomed)

Figure 3B: Acetyl Fentanyl Mass Spectrum

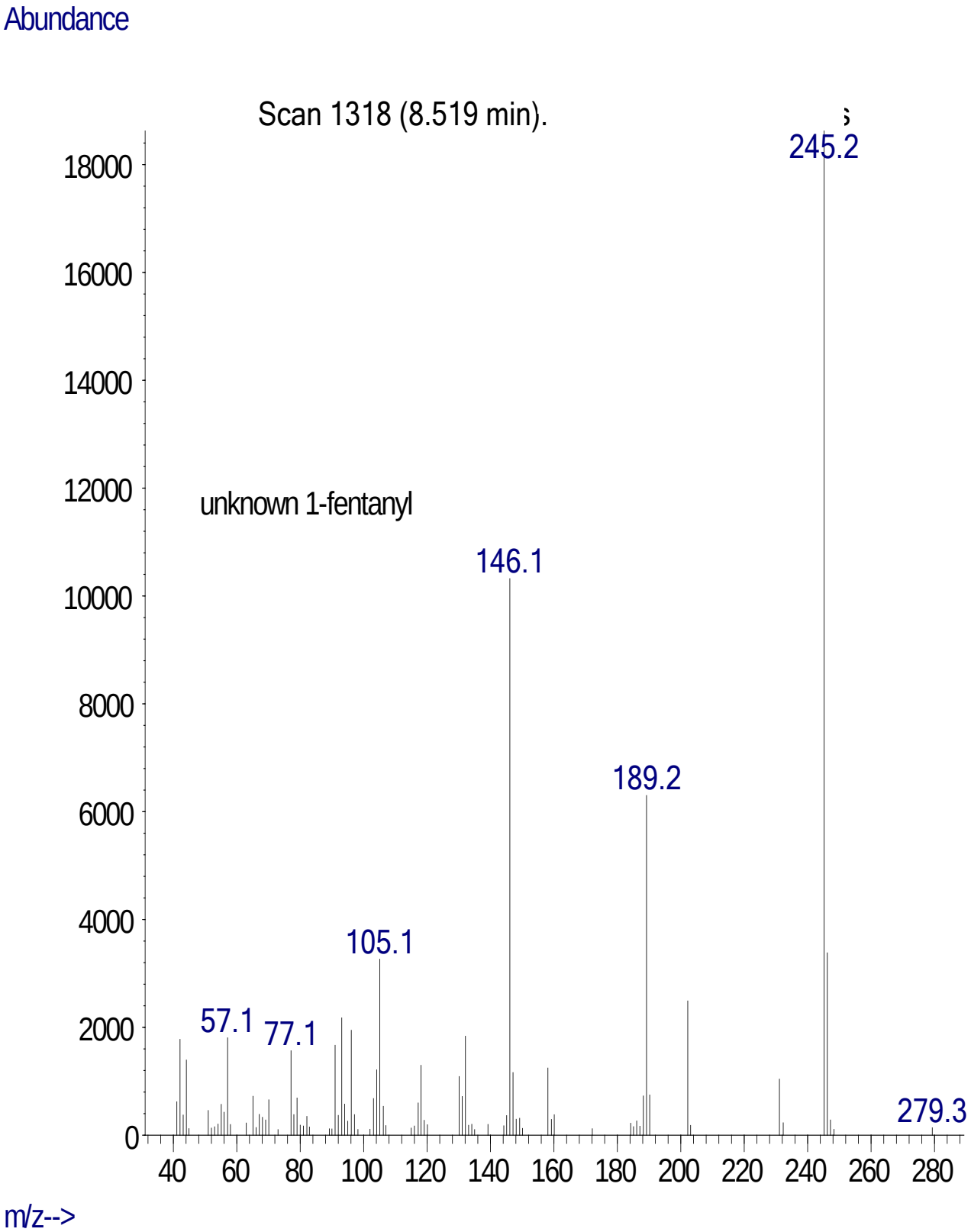

Figure 3C: Fentanyl Mass Spectrum

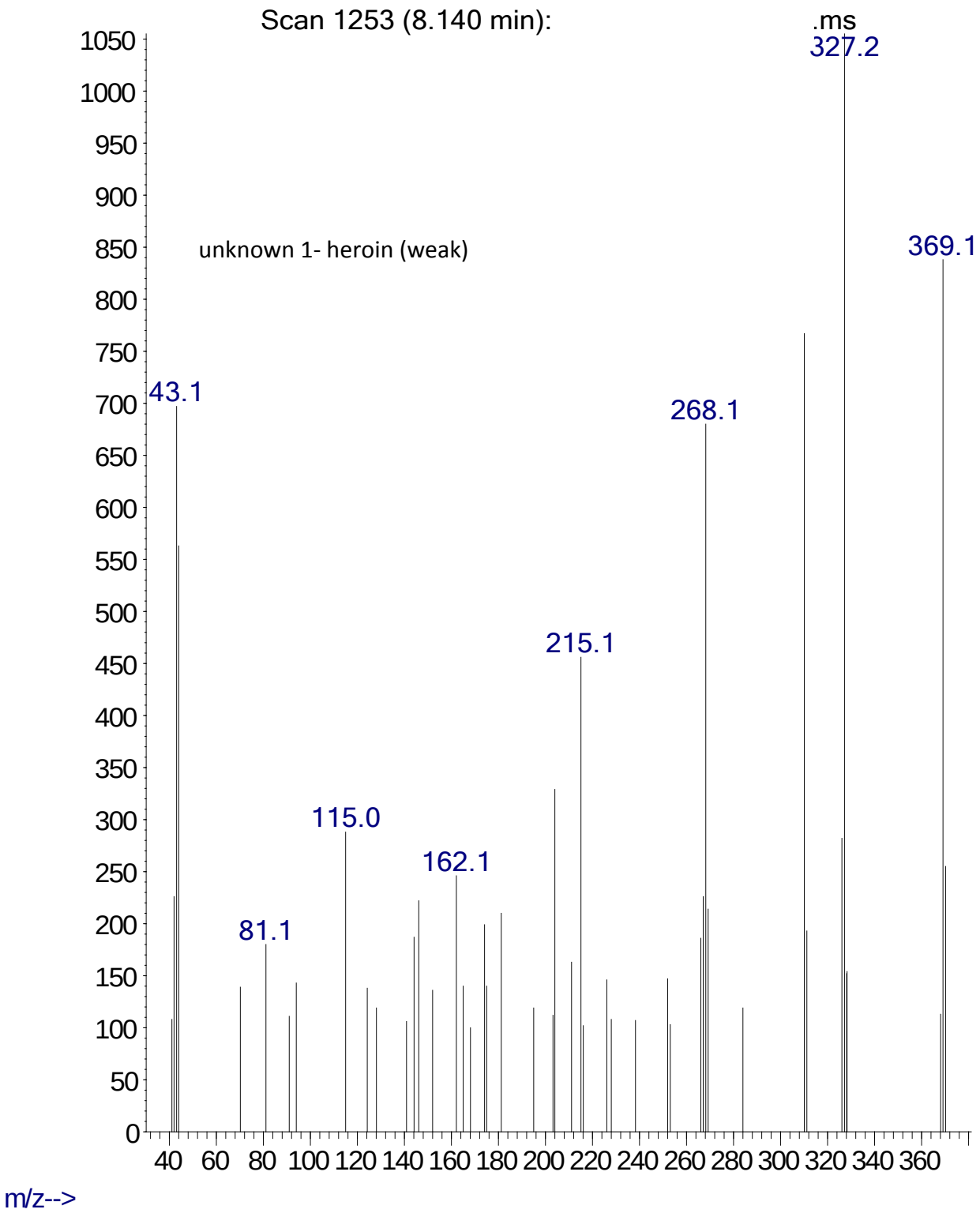

Figure 3D: Heroin Mass Spectrum

Each sample solution, “Test Mix” or “Unknown” was also run in triplicate on the new method developed (acetyl fentanyl/heroin method). The retention times for all of the sample solutions can be found in Table 6. The table contains the retention times for a single run. The TIC for Test Mix 1 along with the mass spectra for the acetyl fentanyl and heroin in the sample can be found in Figure 4A-4C. Similar TICs and mass spectra were obtained for all of the sample solutions as well, with the only differences coming from the drugs present in the samples. The TIC for Unknown 1 along with the mass spectra for the acetyl fentanyl, fentanyl, and heroin present in the sample can be found in Figure 5A-5D.

Table 6: Newly Developed Method Retention Time (RT) Data for Run 1

	Heroin RT (min)	Acetyl Fentanyl RT (min)	Fentanyl RT (min)
Test Mix 1	6.392	7.132	-
Test Mix 2	6.363	7.114	8.239
Unknown 1	6.362	7.097	8.257
Unknown 2	6.368	7.120	8.268
Unknown 3	6.345	-	8.239
Unknown 4	6.345	-	8.251

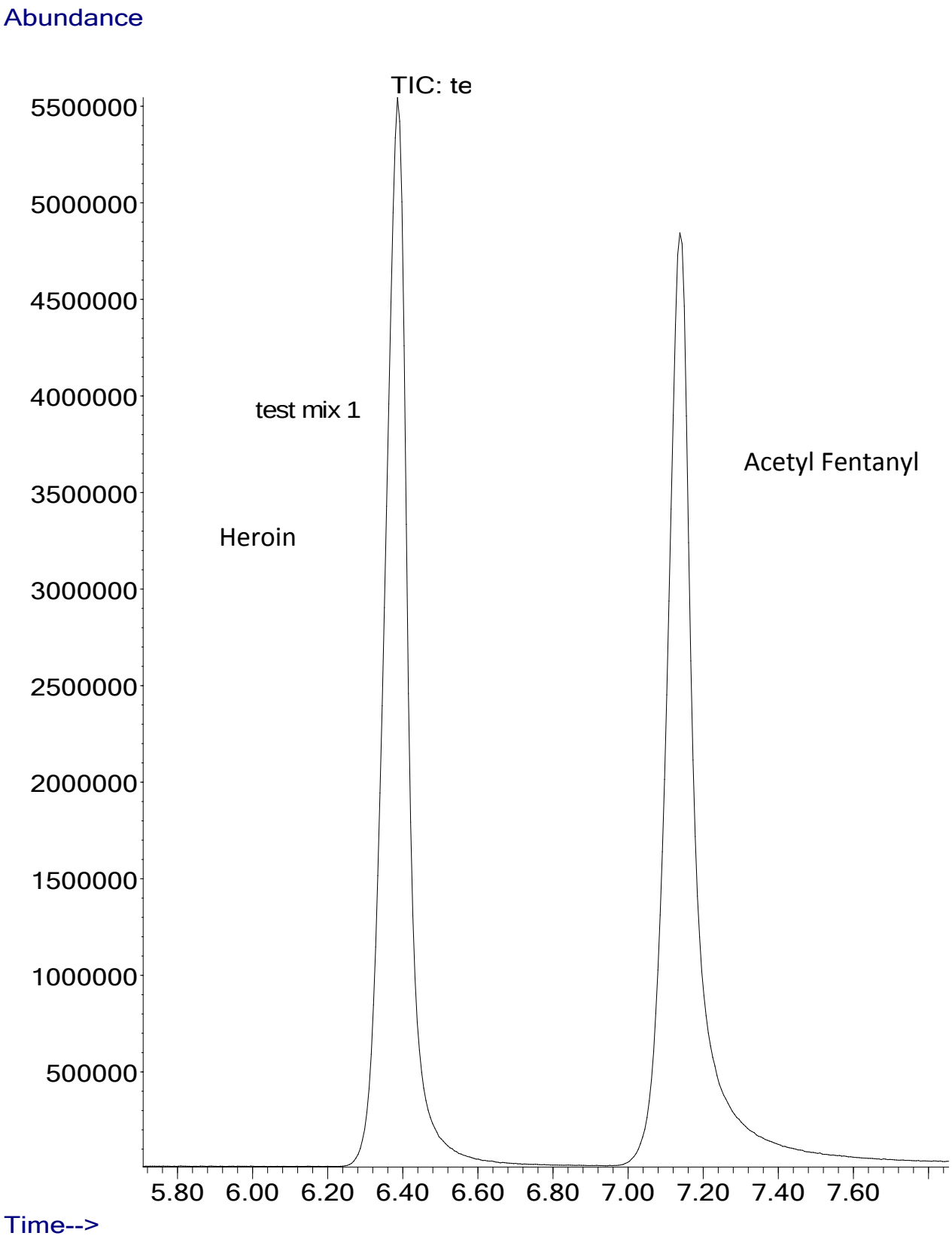

Figure 4A: Total Ion Chromatogram for Test Mix 1 (zoomed)

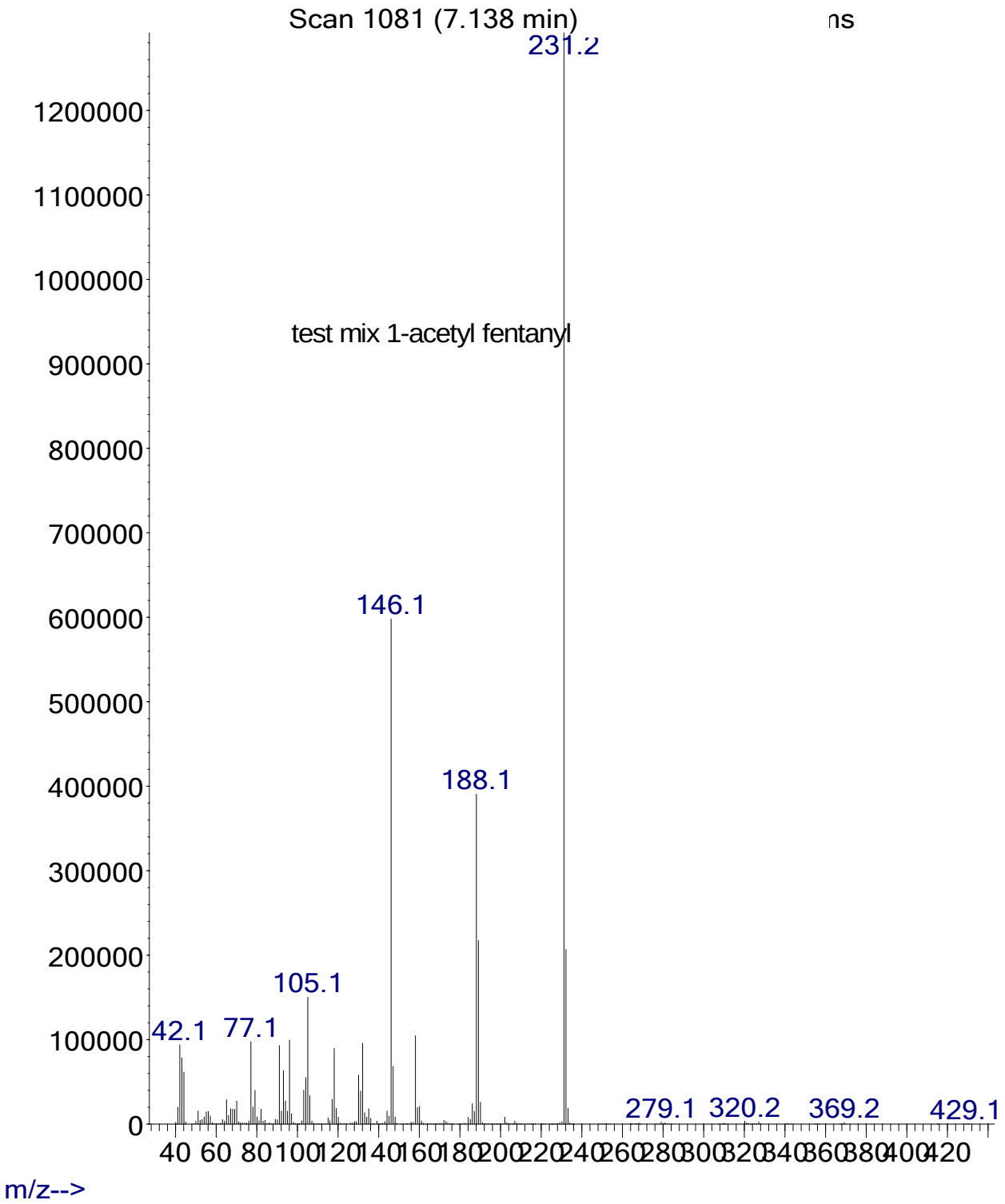

Figure 4B: Acetyl Fentanyl Mass Spectrum

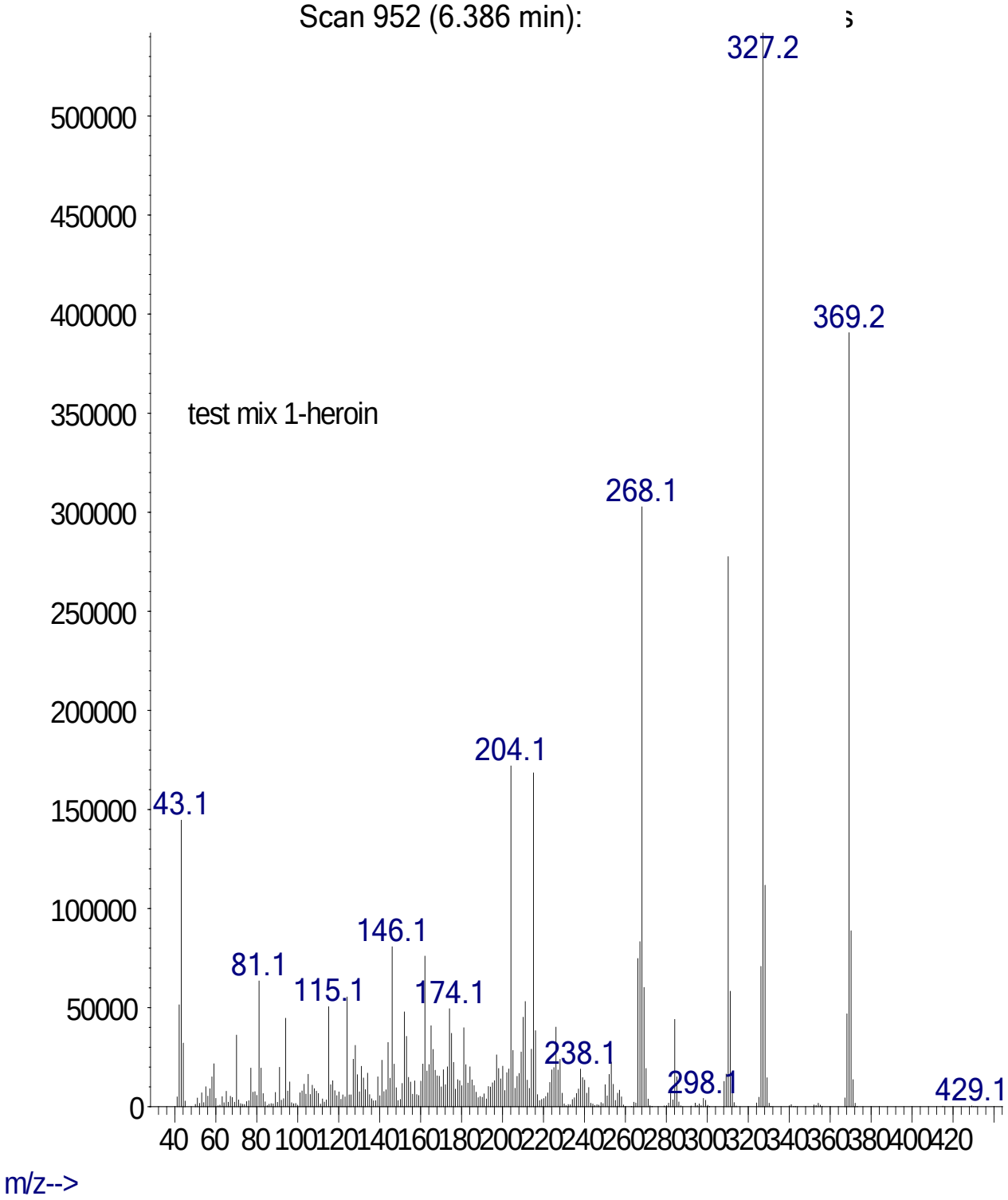

Figure 4C: Heroin Mass Spectrum

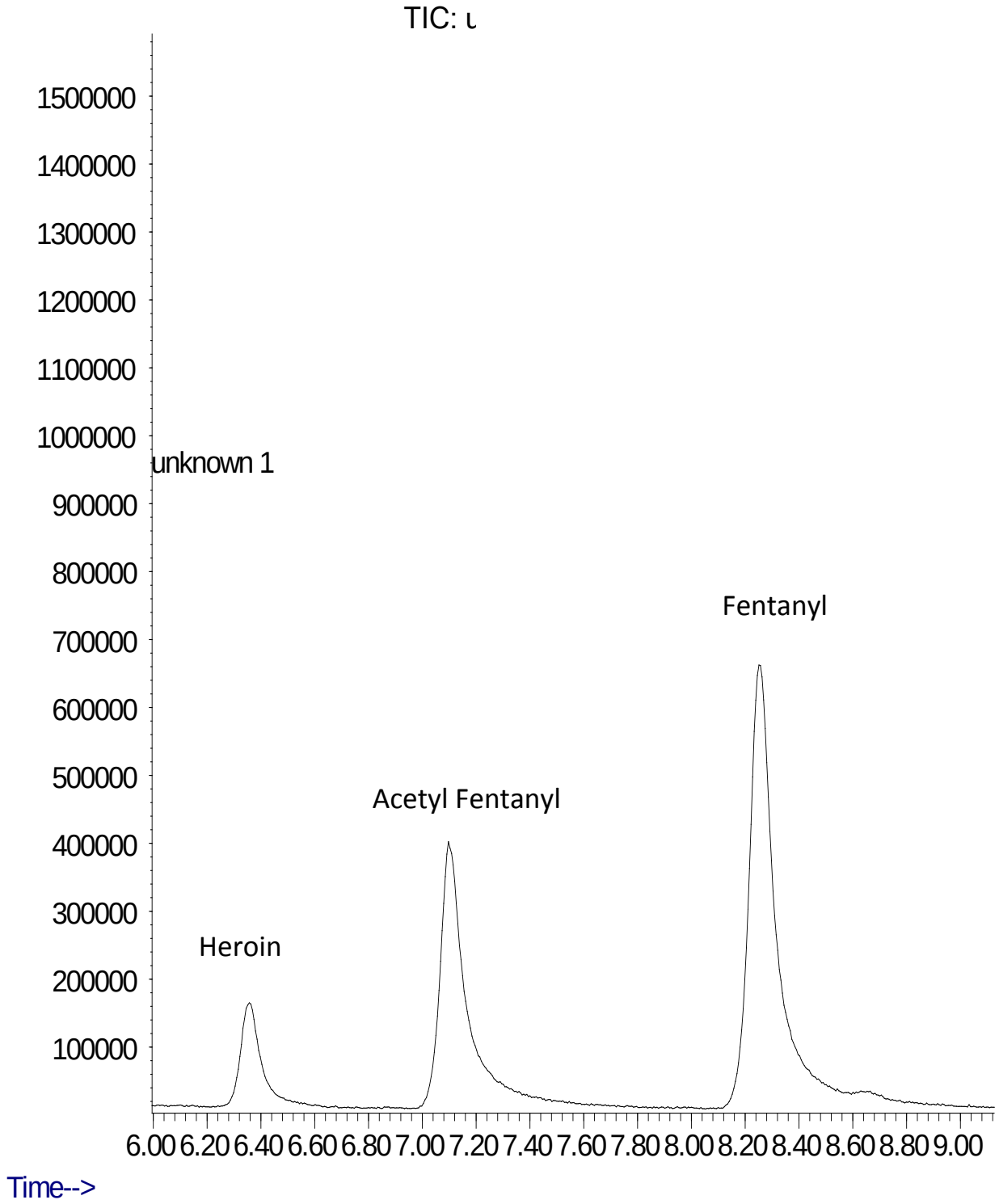

Figure 5A: Total Ion Chromatogram for Unknown 1(zoomed)

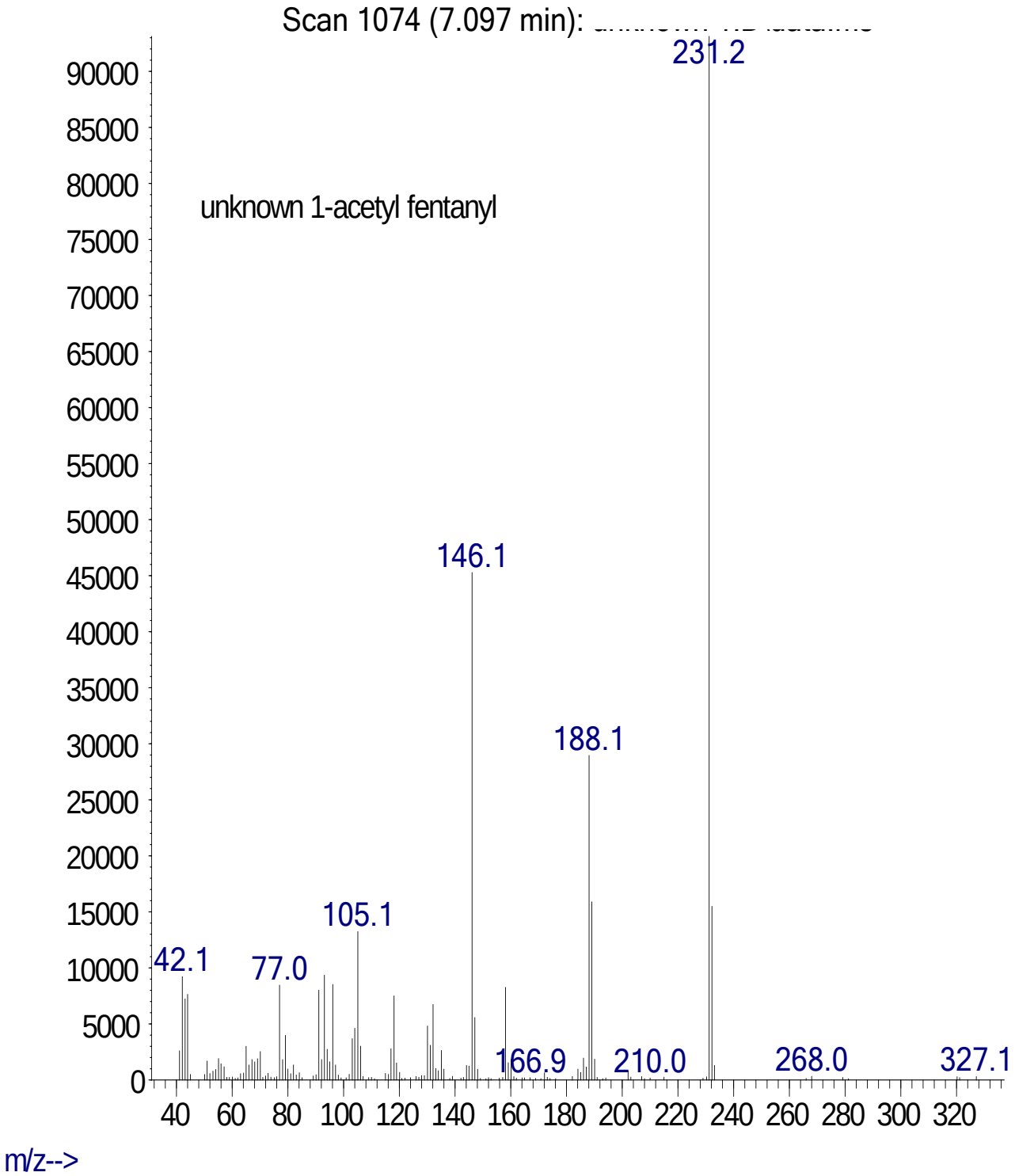

Figure 5B: Acetyl Fentanyl Mass Spectrum

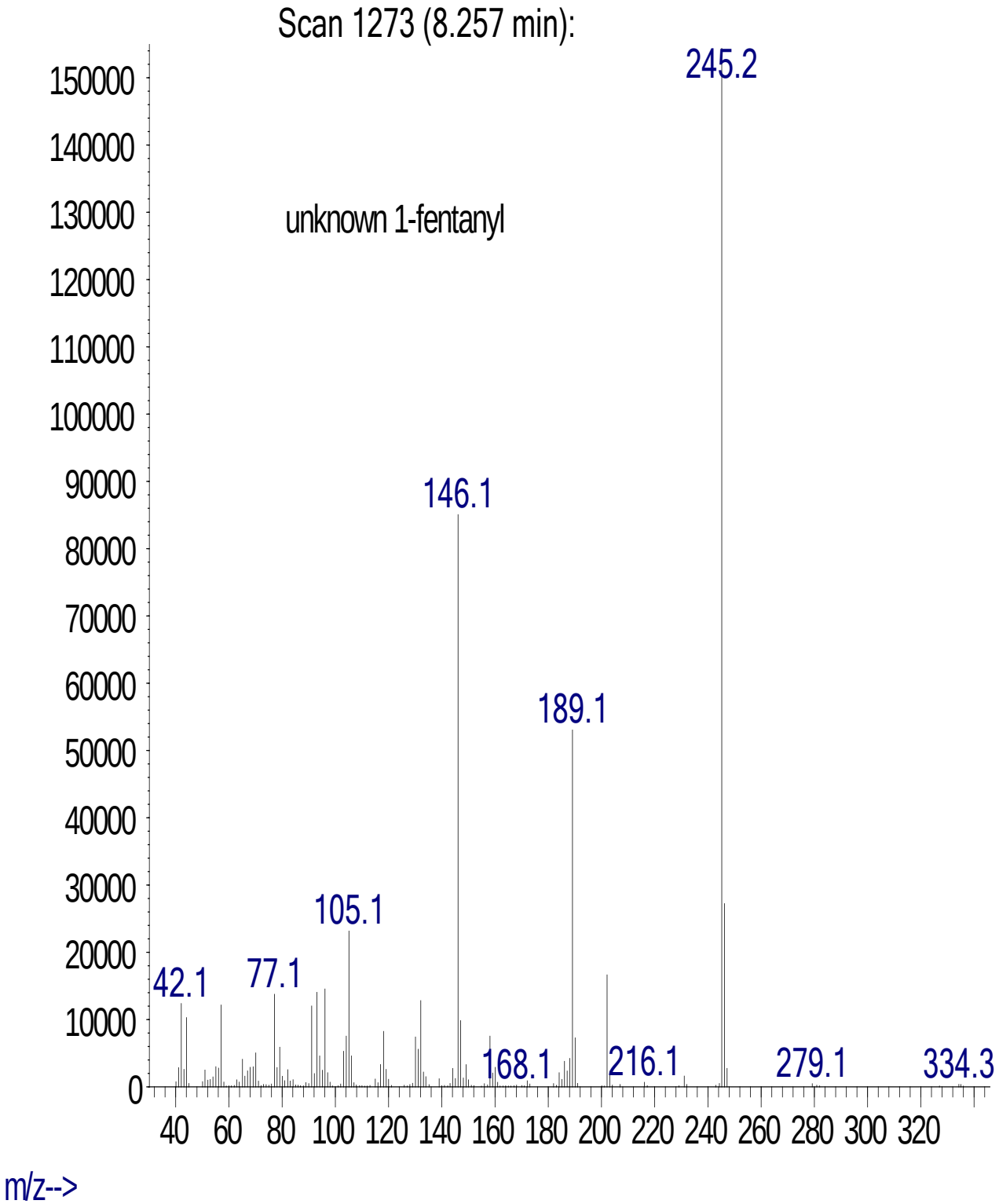

Figure 5C: Fentanyl Mass Spectrum

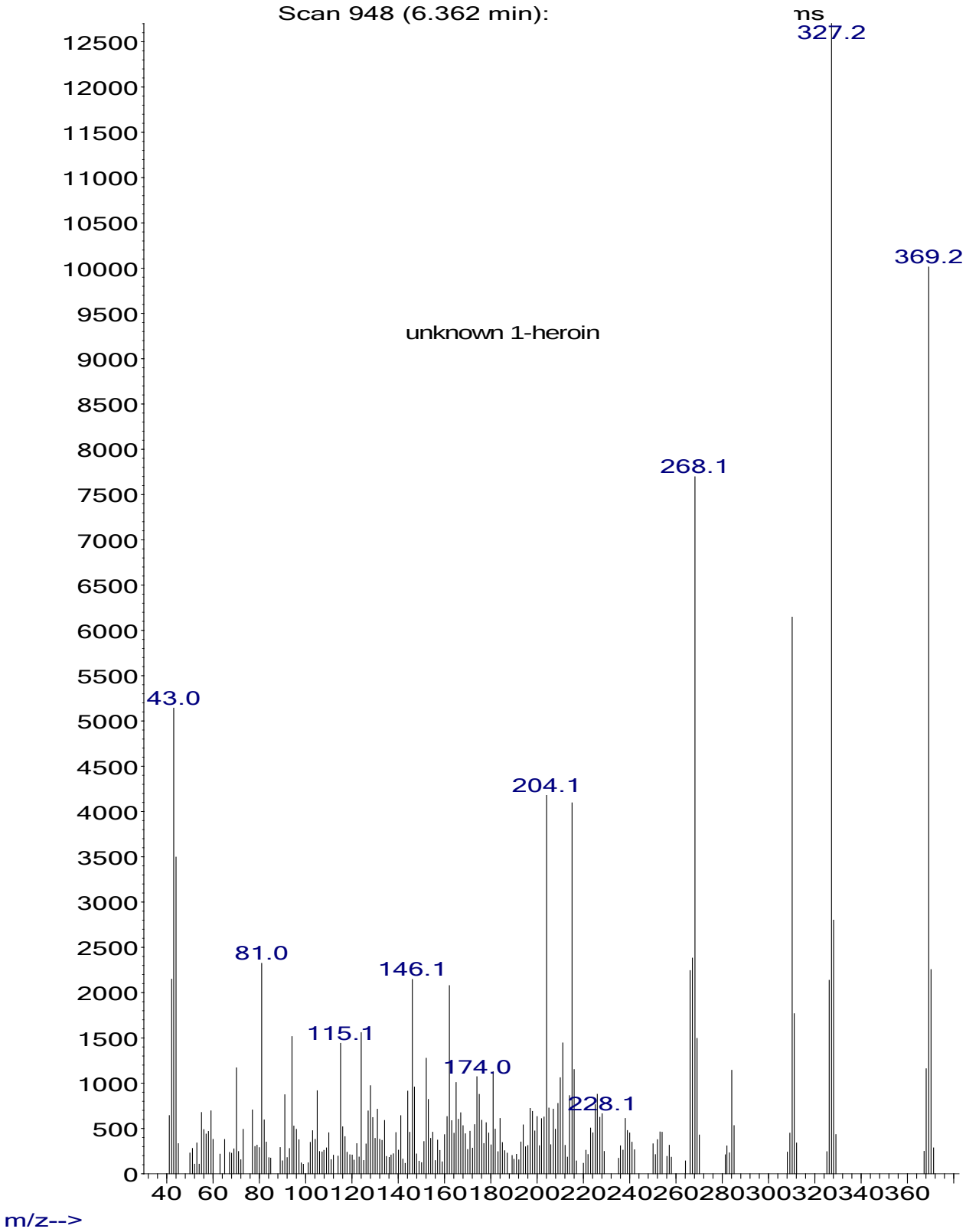

Figure 5D: Heroin Mass Spectrum

As can be seen by visually comparing the TIC of Test Mix 1, from the initial method (Figure 2A) to the one obtained from the new method (Figure 4A), the two peaks present in the chromatograms are better resolved. To ensure the ability to identify each component of the samples was not lost with the changes made to the method, each mass spectrum obtained was library matched to an internally lab-generated computer library. An example of one such library match can be found in Figure 6.

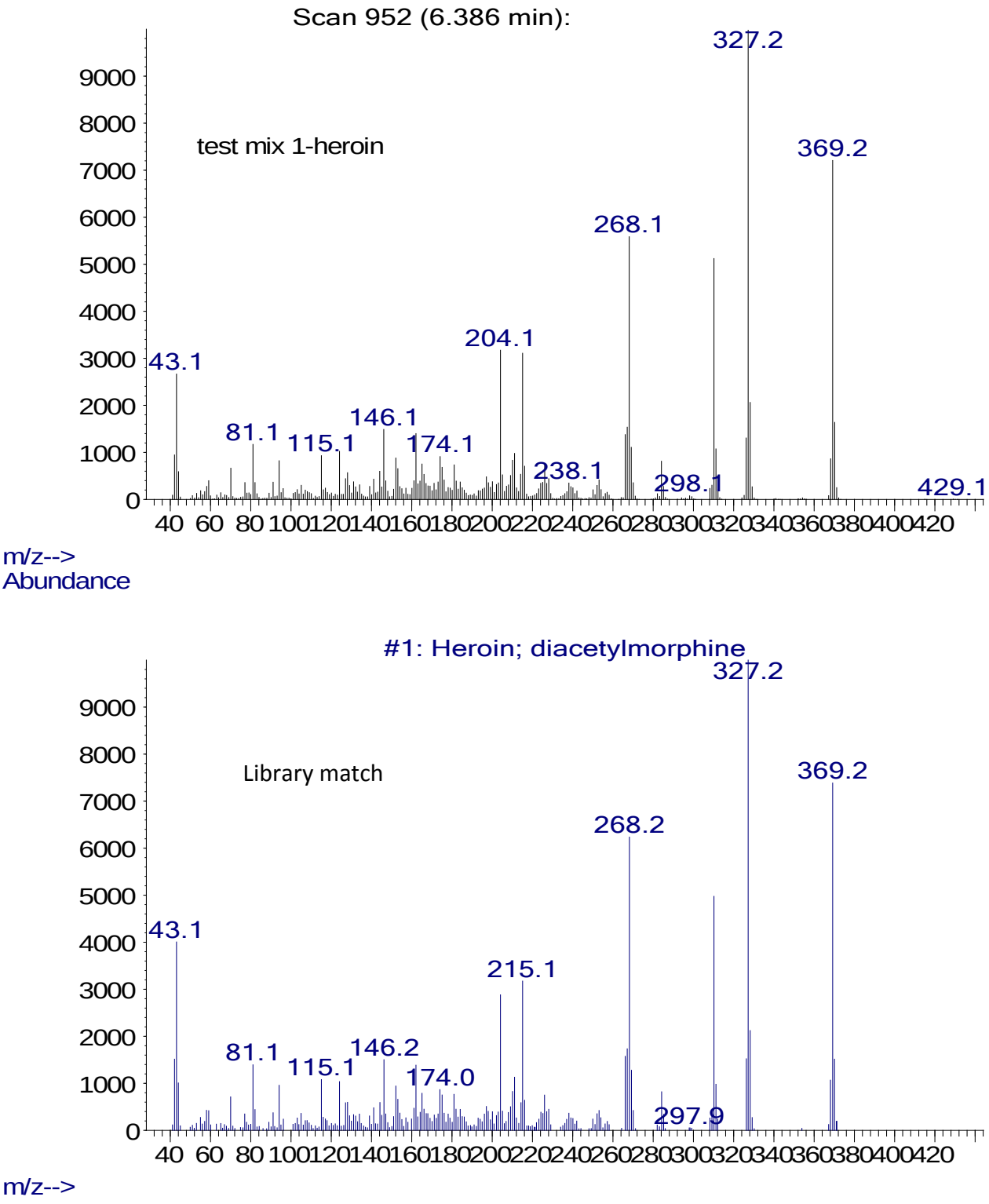

Figure 6: Library Match for Heroin in Test Mix 1 for New Method

The retention times obtained for each method were used to determine the amount of separation between each compound in the sample solutions. The average retention time for each drug was calculated by averaging all the retention times obtained for each drug throughout the research. This was completed for each method and the retention times can be found in Table 7. The obvious difference in retention times between the two methods was the first indication that the new method would be successful for separation of acetyl fentanyl, fentanyl and heroin.

Table 7: Overall Average Retention Times (in minutes)

	Heroin	Acetyl Fentanyl	Fentanyl
Current Method	8.145	8.302	8.520
New Method	6.370	7.125	8.257

After the retention time's for each sample were determined the separation between the different components of each sample solution was calculated. This was determined by calculating the difference in retention times for each component of each sample. After this was completed for each individual run, the separation values were averaged. The averages for the current method can be found in Table 8 and the averages for the new method can be found in Table 9.

Table 8: Average Separation of Drugs Using Current Method (in minutes)

	Acetyl Fentanyl/Heroin	Fentanyl/Acetyl Fentanyl	Fentanyl/Heroin
Test Mix 1	0.161	-	-
Test Mix 2	0.159	0.214	0.373
Unknown 1	0.149	0.226	0.375
Unknown 2	0.153	0.224	0.377
Unknown 3	-	-	0.375
Unknown 4	-	-	0.375
Overall Average	**0.156**	**0.221**	**0.375**

Table 9: Average Separation of Drugs Using New Method (in minutes)

	Acetyl Fentanyl/Heroin	Fentanyl/Acetyl Fentanyl	Fentanyl/Heroin
Test Mix 1	0.742	-	-
Test Mix 2	0.746	1.131	1.877
Unknown 1	0.753	1.148	1.901
Unknown 2	0.744	1.149	1.893
Unknown 3	-	-	1.895
Unknown 4	-	-	1.900
Overall Average	**0.746**	**1.143**	**1.893**

As can be seen from Table 8, the overall average of separation between acetyl fentanyl and heroin in samples was 0.156 minutes using the current method. This was increased to a separation of 0.746 minutes using the newly developed method. The average of 0.156 minutes of separation between acetyl fentanyl and heroin and 0.375 minutes between heroin and fentanyl using the KSP method did not allow for the individual identification of both acetyl fentanyl and heroin in samples that contained both. Identification of acetyl fentanyl could not be made because many ions of heroin would carry over into the acetyl fentanyl mass spectrum. With the current method several of the smaller ions present in heroin carried over into acetyl fentanyl as well as larger ones such as 268, 327, and 369. However, with the increased separation obtained using the new method, both acetyl fentanyl and heroin could be identified when present in samples together.

The newly developed method resulted in a 0.590 minute increase in the separation between acetyl fentanyl and heroin and a 1.518 minute separation between fentanyl and heroin. With this increased separation between elution of acetyl fentanyl, fentanyl, and heroin each substance could be positively identified in samples together. The amount of carryover from heroin in the mass spectra of acetyl fentanyl and fentanyl greatly decreased. Only a few of the smaller heroin ions and the 369 ion carried over into the spectra for the other substances.

The peak resolution was also calculated for the two methods using Equation 1, where t_1 is the retention time for substance one, t_2 is the retention time for substance two, w_1 is the peak base width for substance one and w_2 is the peak base width for substance two. The average peak resolution for each solution for the current KSP method can be found in Table 10 and the average peak resolution for new method can be found in Table 11.

$$Peak\ Resolution = \frac{2(t_2 - t_1)}{w_1 + w_2} \qquad \text{Equation 1}$$

Table 10: Average Peak Resolution from Current KSP Method

	Acetyl Fentanyl/Heroin Resolution	Acetyl Fentanyl/Fentanyl Resolution	Fentanyl/Heroin Resolution
Test Mix 1	5.106	-	-
Test Mix 2	4.647	4.741	9.275
Unknown 1	3.836	4.968	9.264
Unknown 2	3.999	5.300	9.925
Unknown 3	-	-	11.047
Unknown 4	-	-	12.009
Overall Average	**4.397**	**5.003**	**10.304**

Table 11: Average Peak Resolution from New Method (Acetyl Fentanyl/Heroin Method)

	Acetyl Fentanyl/Heroin Resolution	Acetyl Fentanyl/Fentanyl Resolution	Fentanyl/Heroin Resolution
Test Mix 8	10.461	-	-
Test Mix 9	10.865	12.876	22.839
Unknown 1	9.586	12.783	22.801
Unknown 2	9.635	13.031	23.244
Unknown 3	-	-	23.366
Unknown 4	-	-	23.567
Overall Average	**10.136**	**12.897**	**23.163**

The peak resolution for each of the sample solutions increased when they were analyzed using the new method. The peak resolution for all of the samples showed at least a two-fold increase in peak resolution. The increase in peak resolution is indicative of an increase in the amount of separation between peaks. Although the peak resolutions calculated were much larger than what would normally be considered acceptable, this is a result of the method being developed specifically for the separation of certain substances. So for this research, the peak resolution was looked at simply to reinforce the idea that more separation occurred with the new method.

Method Validation

The validation portion of this experiment involved validating the method according to the KSP Controlled Substances Section policy for the validation of a new method. This involved running a few standards of interest on the method and successfully library matching each standard to two libraries, with only one of them being computerized. In order for the library match to be considered valid all of the significant ions had to be present in the mass spectra of each standard. The TIC, mass spectrum, and library match for the acetyl fentanyl and heroin standards on the new method can be found in Figure 7A-7I. The retention time for each standard can be found in Table 12. The library that was used to match each standard was an in house library created by the KSP Eastern Laboratory created using standards on that specific instrument.

Table 12: Retention Times for Method Validation

Standard	Retention Time (minutes)
Acetyl Fentanyl	7.149
Fentanyl	8.333
Heroin	6.405

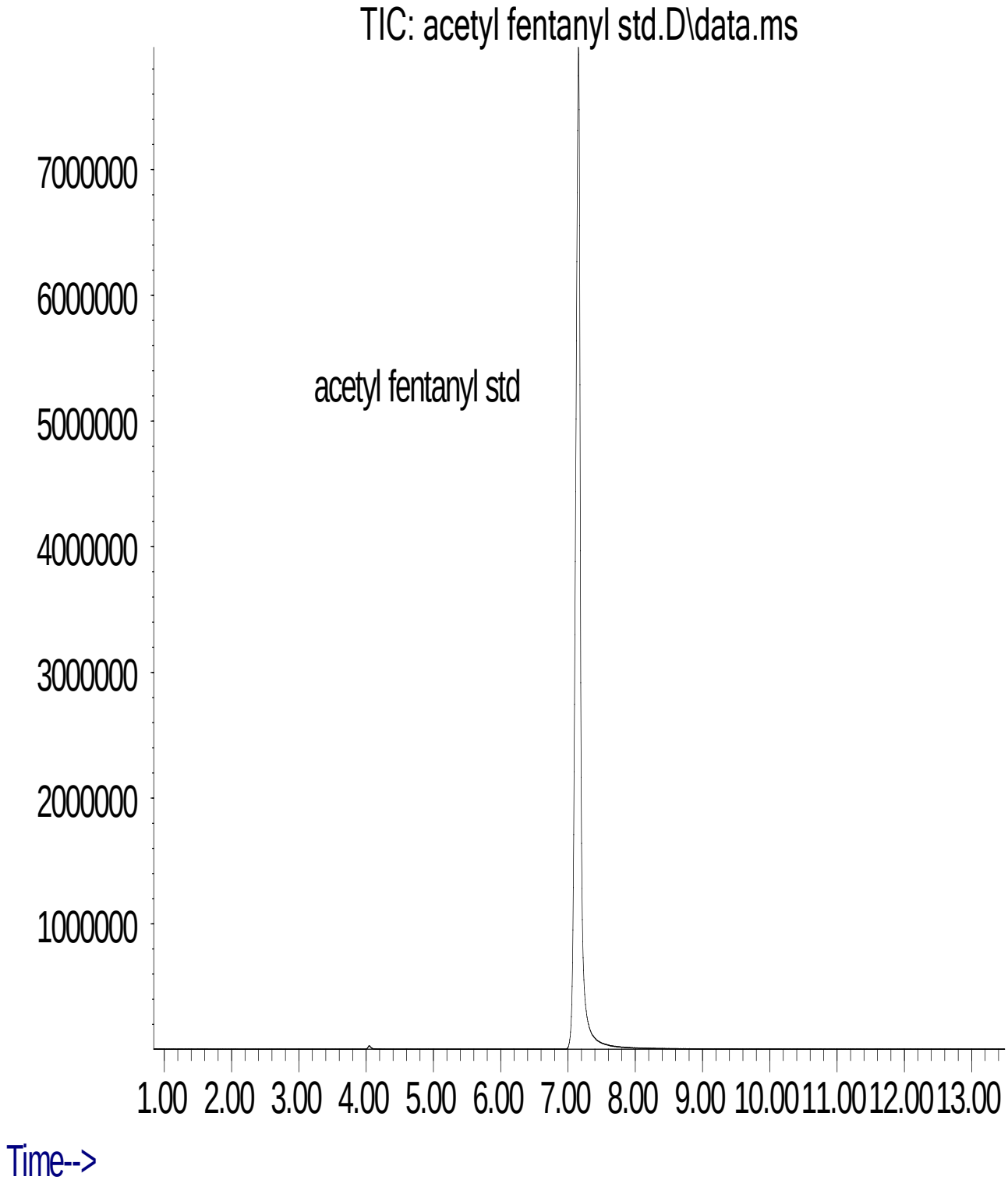

Figure 7A: Total Ion Chromatogram for Acetyl Fentanyl Standard

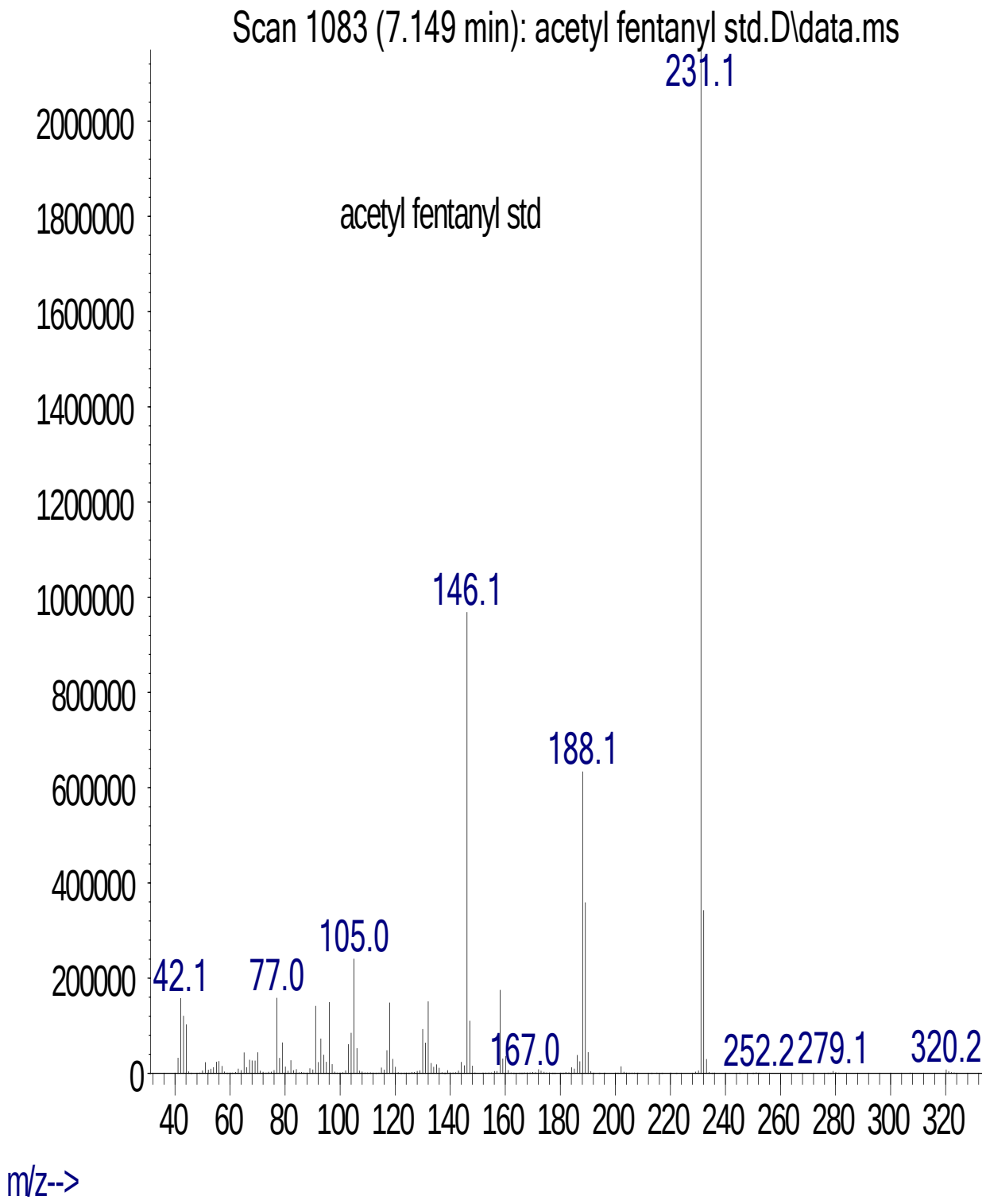

Figure 7B: Mass Spectrum for Acetyl Fentanyl Standard

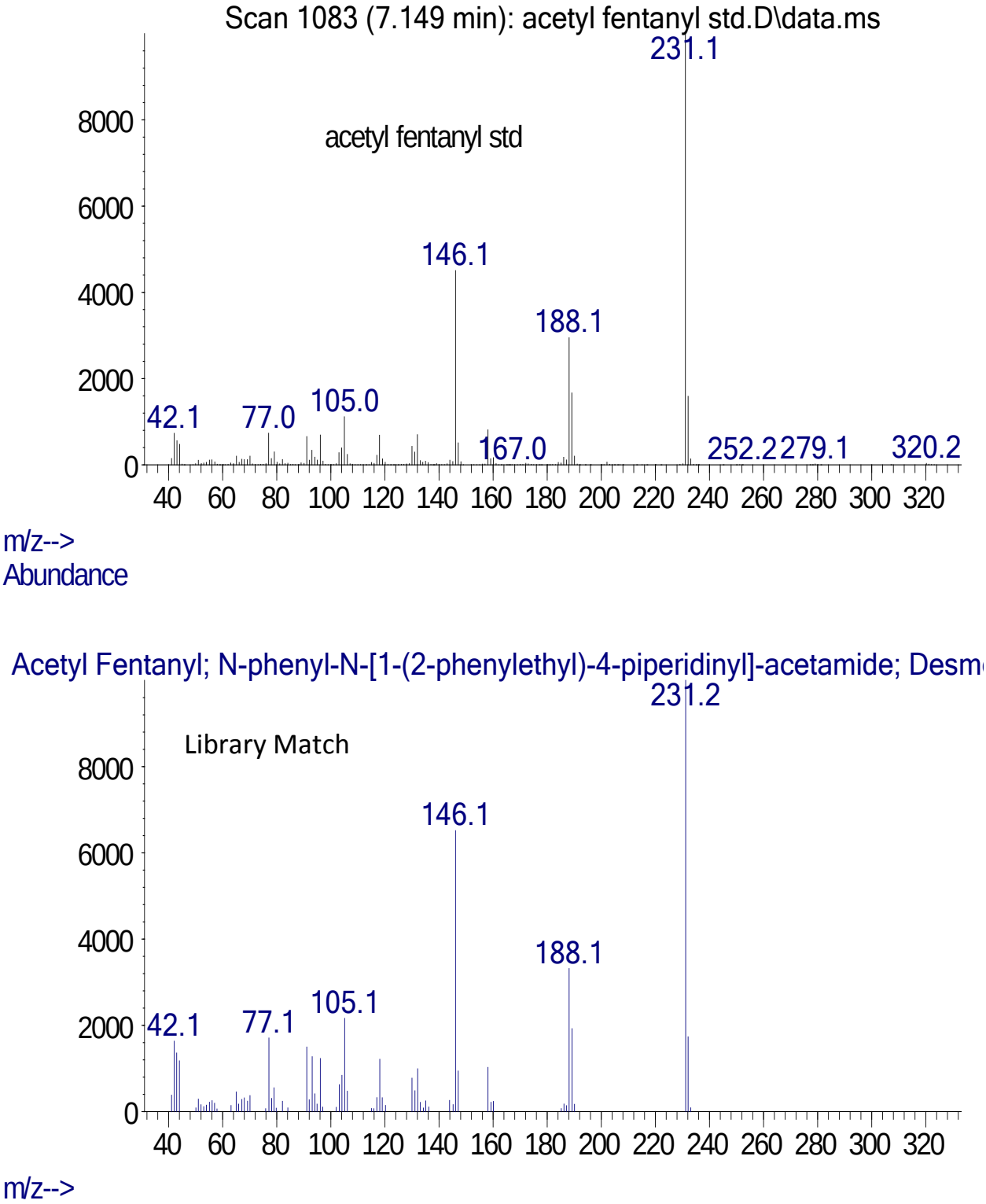

Figure 7C: Mass Spectrum and Library Match for Acetyl Fentanyl Standard

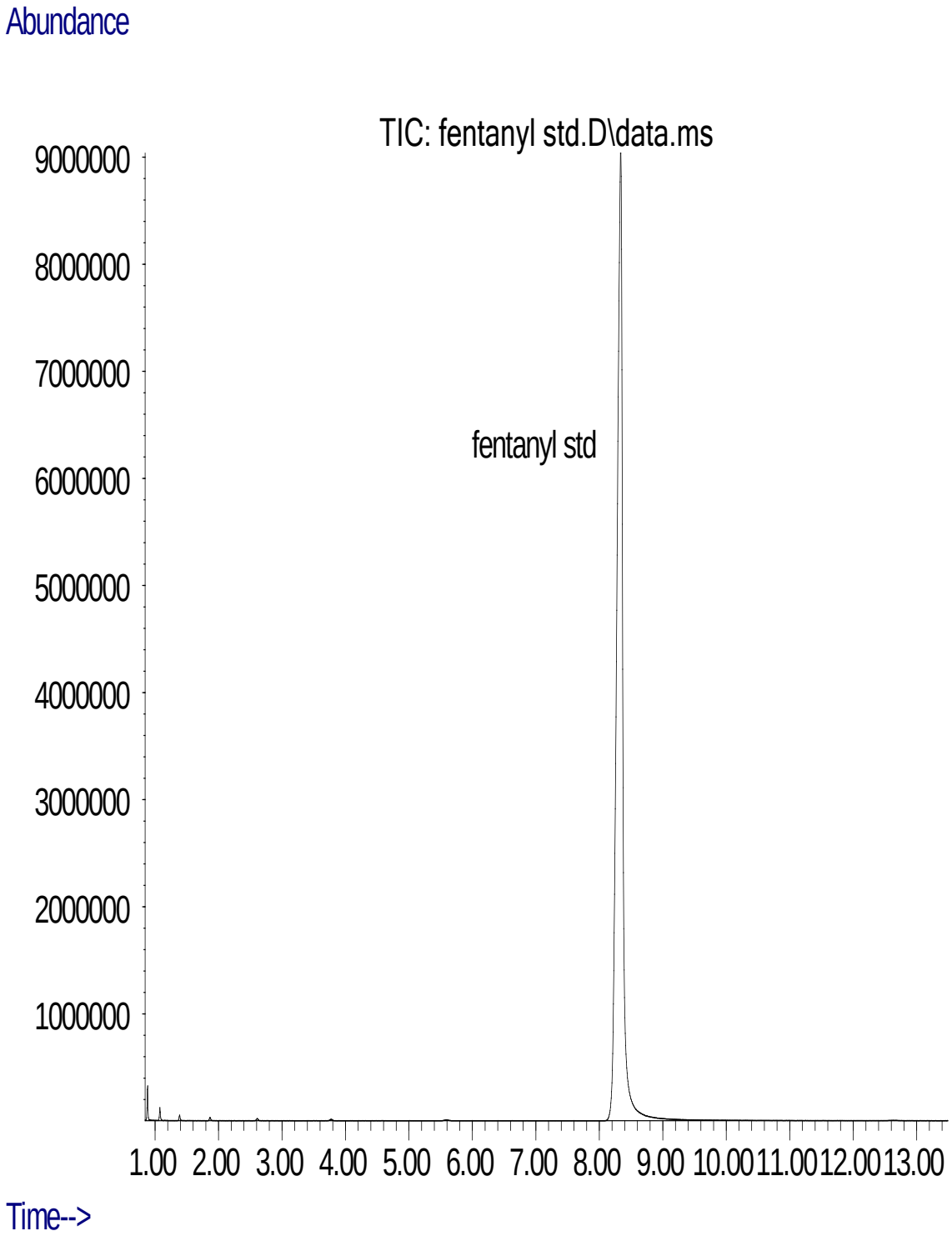

Figure 7D: Total Ion Chromatogram for Fentanyl Standard

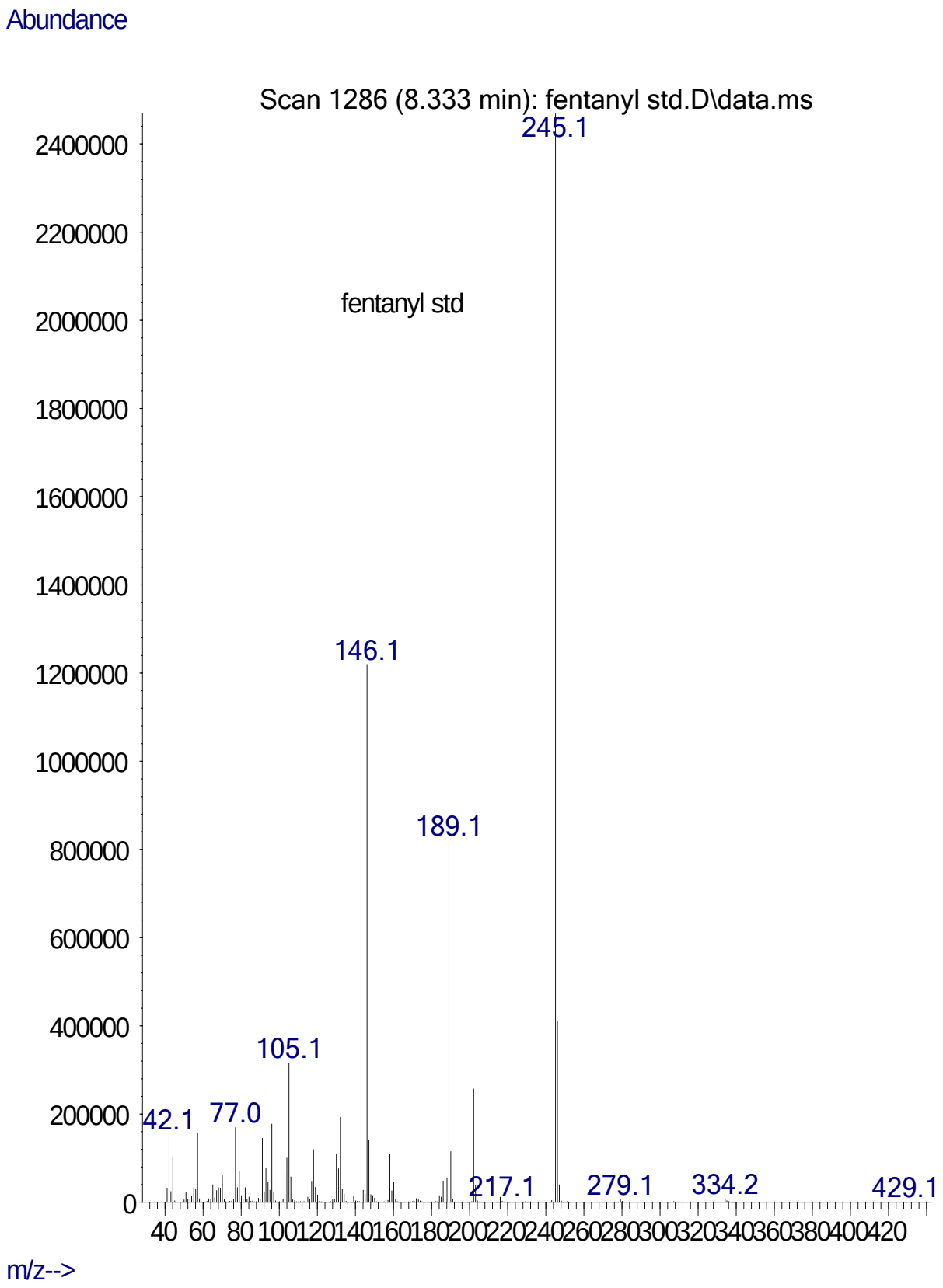

Figure 7E: Mass Spectrum for Fentanyl Standard

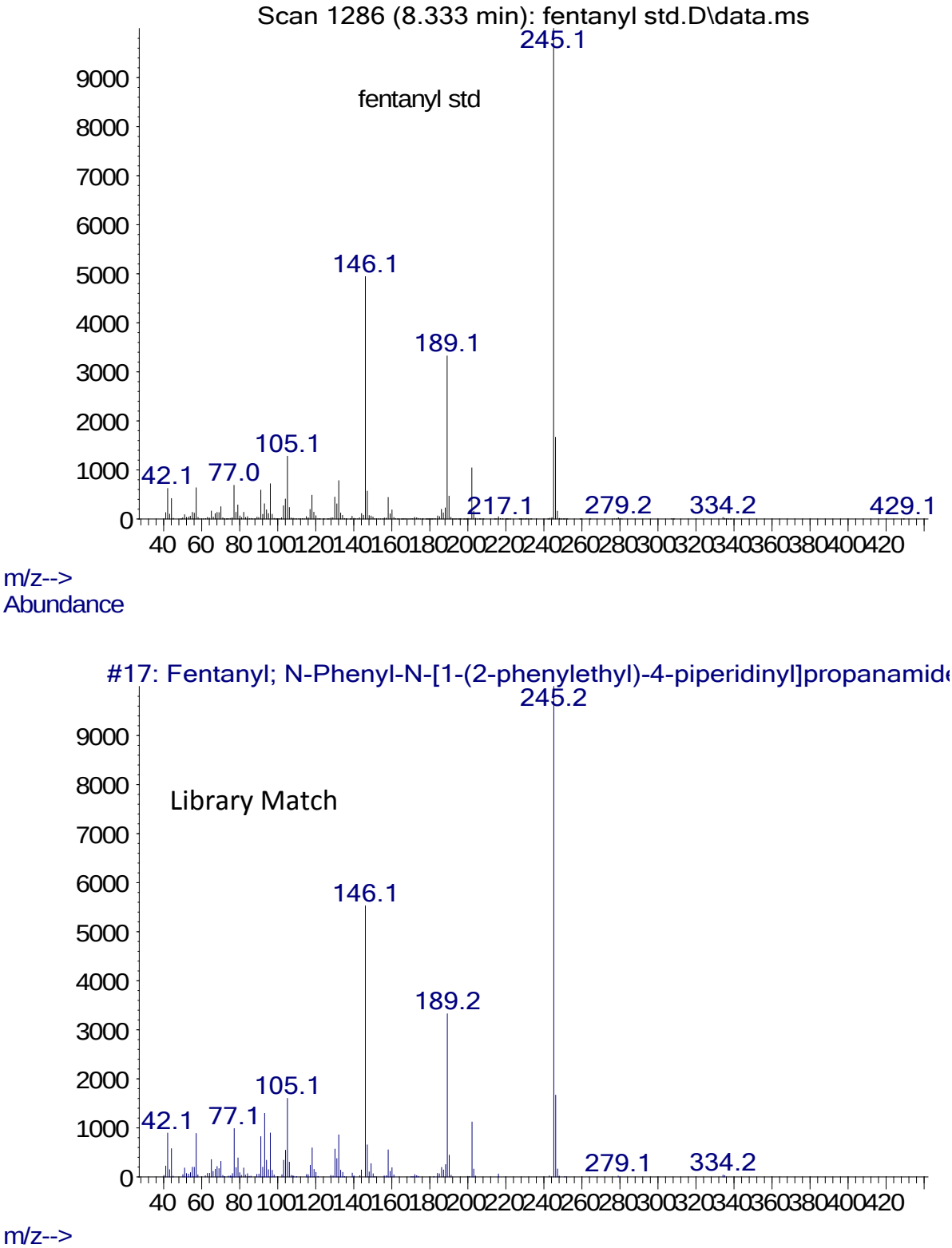

Figure 7F: Mass Spectrum and Library Match for Fentanyl Standard

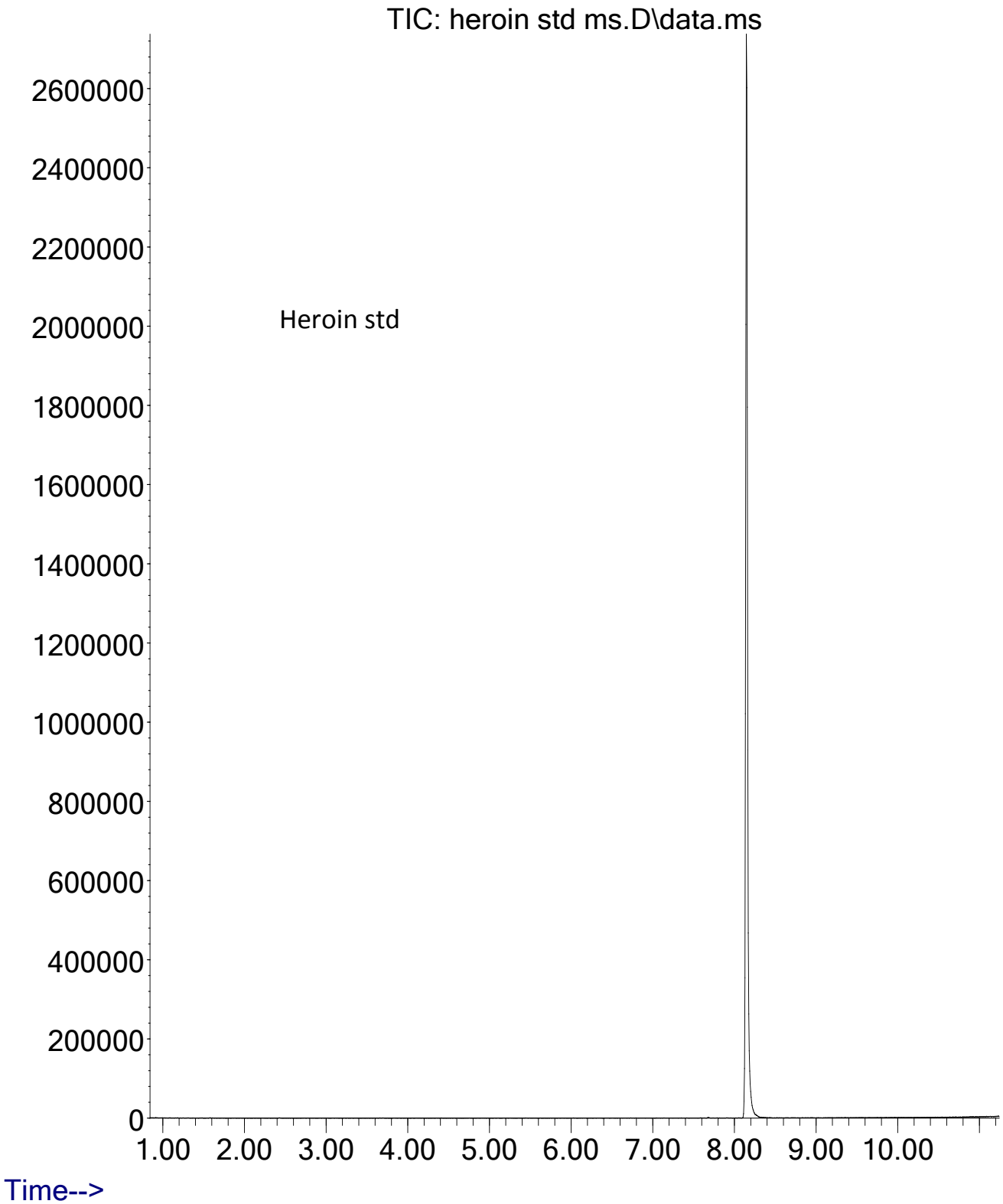

Figure 7G: Total Ion Chromatogram for Heroin Standard

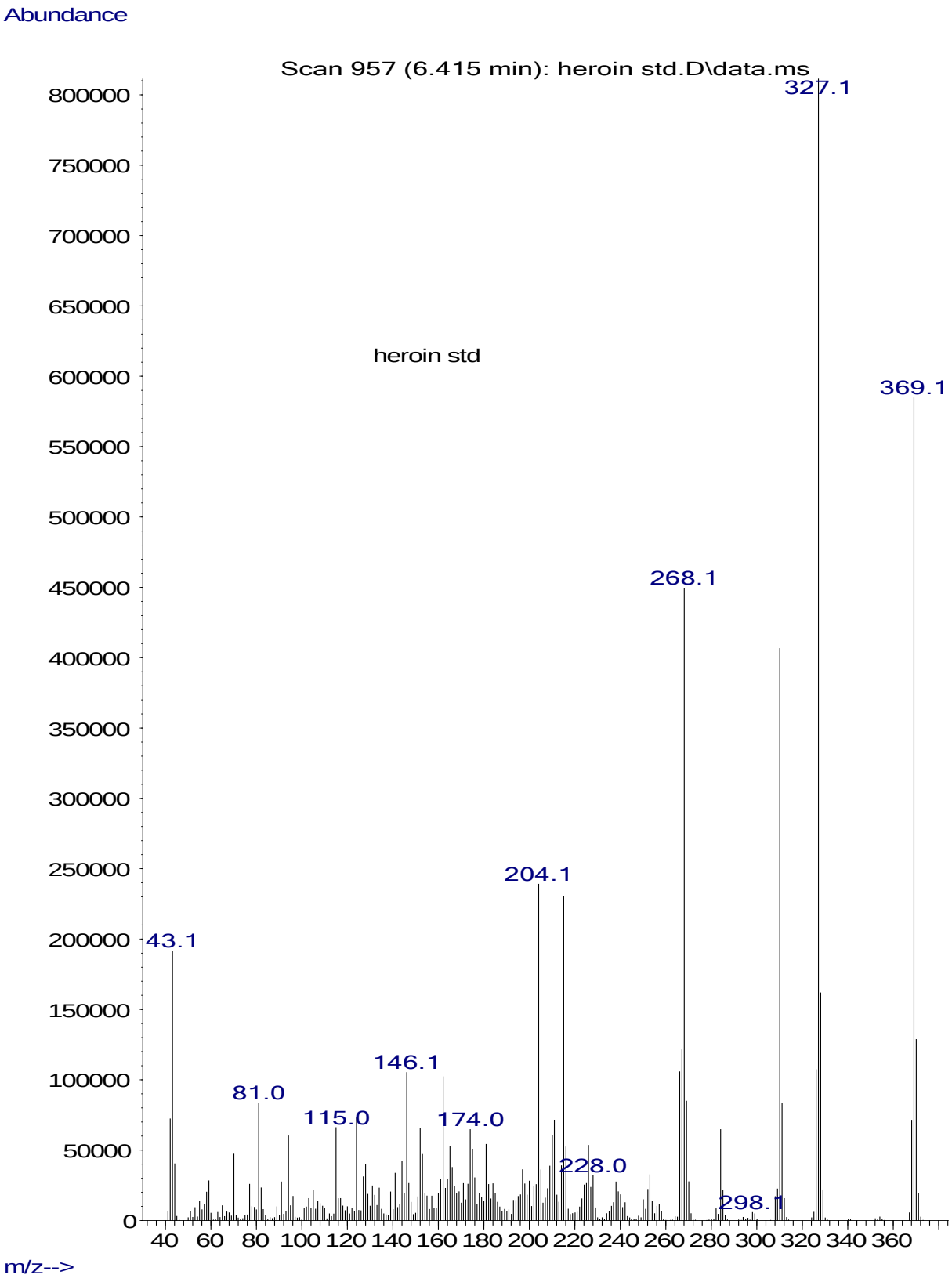

Figure 7H: Mass Spectrum for Heroin Standard

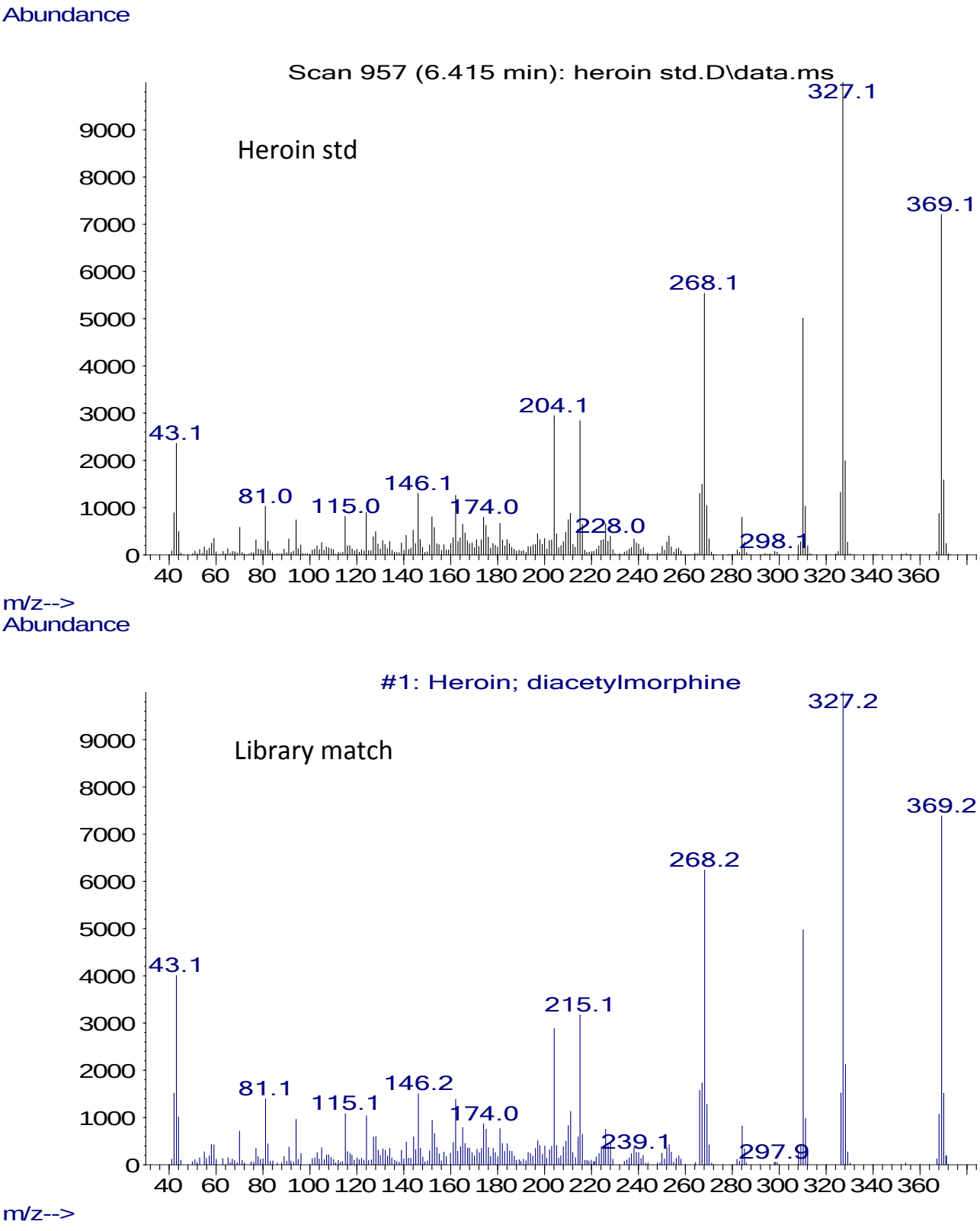

Figure 7I: Mass Spectrum and Library Match for Heroin Standard

Conclusion

Developing a method for the identification and separation of acetyl fentanyl and heroin is of growing importance due to the increasing number of reports where acetyl fentanyl is being found in heroin. The development of this method increased in importance for drug analysts in Kentucky when acetyl fentanyl became a schedule I narcotic earlier this year (1). Even more recently, acetyl fentanyl was given temporary placement as a schedule I controlled substance by the Drug Enforcement Administration. With the individual scheduling of acetyl fentanyl, it would be a good assumption that other fentanyl analogs will also be individually scheduled. The objective of this research project was to develop a method that could successfully separate acetyl fentanyl and heroin so that both could be positively identified in samples together.

Although this research project was successful in developing and validating a new method for the separation of acetyl fentanyl in heroin, more research could be done to further improve and expand the method. In future studies, if the few remaining ions, 369 and some smaller ions, from heroin that carry over into the acetyl fentanyl and fentanyl spectra could be eliminated, it would be very beneficial. Also, if the source of the other extra ions present in the mass spectra, 279, 320, 429, and 503 could be determined that would be helpful. Another topic for further study would be if the method developed in this research project could be applied to other drugs that have similar retention times and determining if the method developed would work to separate those as well.

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