

1 Introduction

Melatonin is a hormone secreted by the brain to help regulate sleep. Since melatonin is found in certain foods, it can be sold as a dietary “over-the-counter” (OTC) supplement. Unlike pharmaceuticals, OTC drugs are not regulated by agencies such as the Food and Drug Administration (FDA). Hence, impurities present in OTCs are not required to be characterized, quantified, or reported. Impurities found in OTC drugs can be harmful and even cause death. For instance, in 1989 an epidemic referred to as eosinophilia-myalgia syndrome (EMS) resulted in the death of thirty people and affected as many as 1500 people. The epidemic was attributed to consumption of the OTC supplement tryptophan which was manufactured by a single chemical company. The synthesized tryptophan had at least six impurities which could have caused the onset of EMS. Since melatonin has structural similarities to tryptophan we have used the high resolution and accurate mass capability of time-of-flight mass spectrometry to identify and characterize impurities in OTC melatonin tablets.

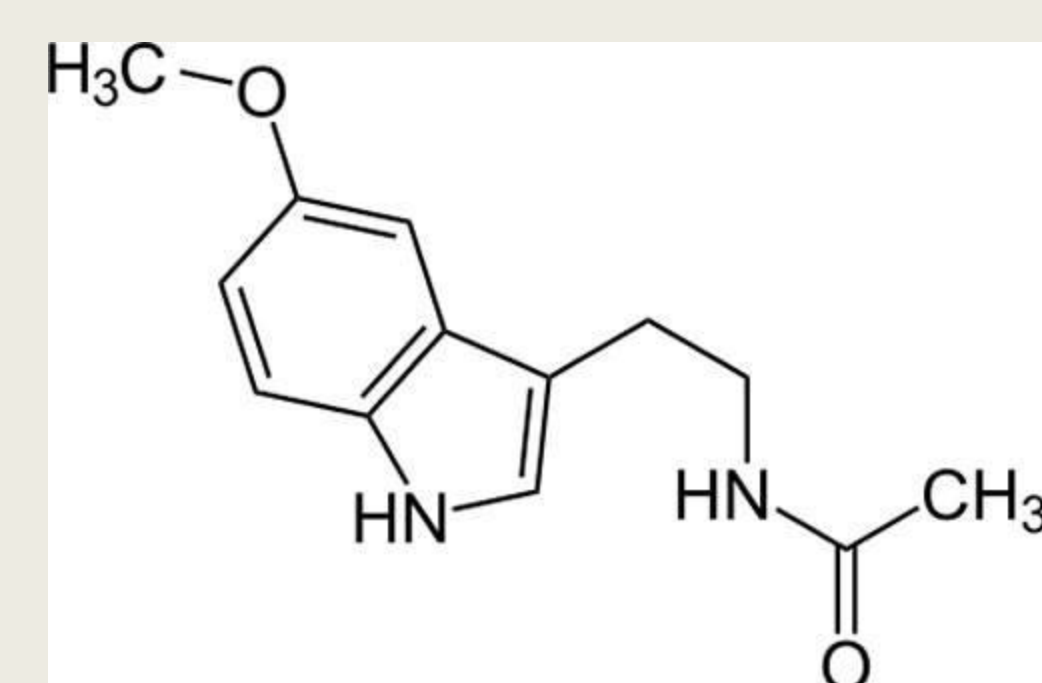


Figure 1. the structure of melatonin

2 Experimental conditions

Samples and standards:

OTC melatonin was obtained from a local grocery store. N1-acetyl-N2-formyl-5-methoxykynurenin (AFMK) was obtained from Cayman Chemicals (Michigan, USA)

Sample preparation conditions:

Melatonin tablet (containing 1 mg melatonin) was crushed in a mortar and pestle and dissolved in Milli Q water (10 ml). The mixture was shaken vigorously and then centrifuged at 6000 rpm for 10 min. The supernatant was injected on column.

LC conditions:

LC Pump: PerkinElmer Flexar™ FX-10 LC pump
Column: PerkinElmer Brownlee Supra™ column C18 (1.9 μm, 2.1 x 50 mm)

Mobile phase A: water containing 0.1% formic acid

B: acetonitrile containing 0.1% formic acid

Gradient conditions: 0-5 min 20% B to 40%B in a linear gradient
5-7 min 40% B to 70% B

Flow rate: 0.4 mL/min

Mass Spectrometry conditions:

Mass spectrometer: PerkinElmer AxION™ 2 TOF

Ionization source: PerkinElmer Ultraspray™ 2 (Dual ESI source)

Pulse mode: 100-700 m/z
Trap mode: 100-700 m/z (D7:29, D8:43)
Calibrant: Internal calibrant (diluted Agilent tune mix)
Flow: 25 μL/min through the 2nd ESI sprayer.

3 Results

Pulse vs. Trap Acquisition Mode:

The AxION™ 2 TOF is fitted with a hexapole ion guide that traverses the different vacuum regions of the mass spectrometer for maximum ion transmission. In pulse mode operation, the ion guide solely transmits the ions efficiently into the pulser region. However, in trap mode operation, the hexapole ion guide can be used to trap ions. The ions are trapped by raising the potential of the ion guide exit lens above the ion guide DC offset potential. The trapped ions are then released into the pulser region after a certain delay. As the ions move from ion guide to pulser region, there is some separation of ions based on m/z . By timing the gating and pulsing of the ions, one can selectively accumulate and transmit a defined m/z range of ions into the TOF region drift tube selectively enhancing the intensity of the selected m/z range. Using the AxION™ 2 TOF in trap mode can significantly increase the S/N of ions 3 to 5 fold in comparison to pulse mode.

Figure 2 shows the analysis of melatonin tablet in pulse versus trap mode. In trap mode, impurities are detected at higher S/N (3-5 fold higher) than in pulse mode and additional impurity peaks that were not visible in pulse mode were detected.

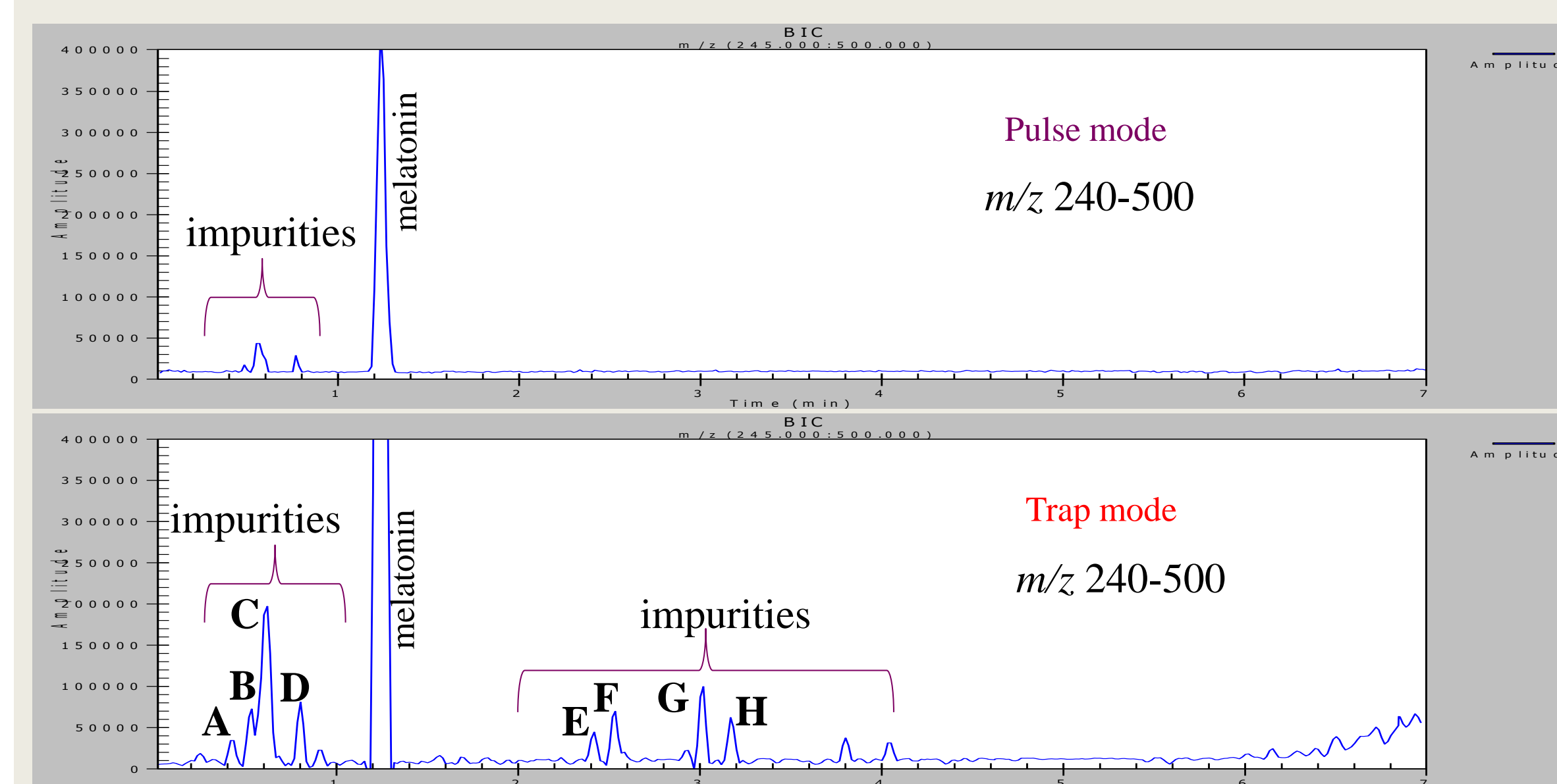


Figure 2. BICs of melatonin in pulse versus trap mode of operation

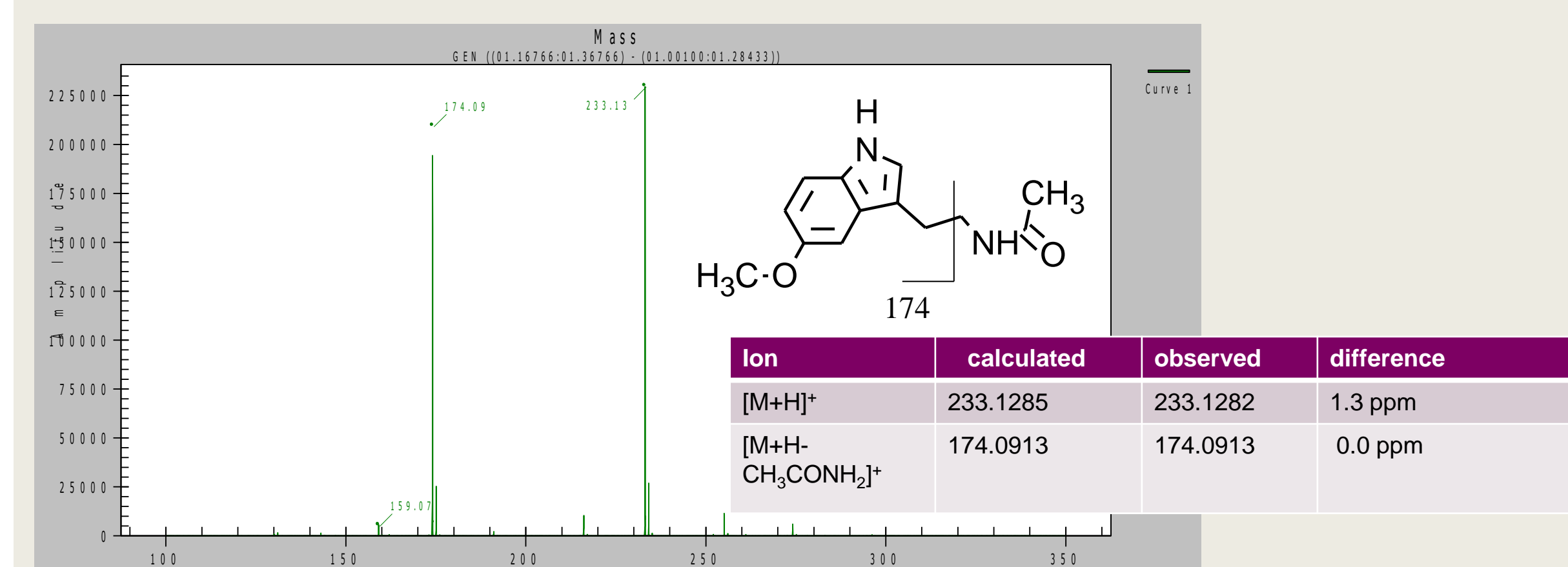


Figure 3. melatonin spectrum and mass accuracy

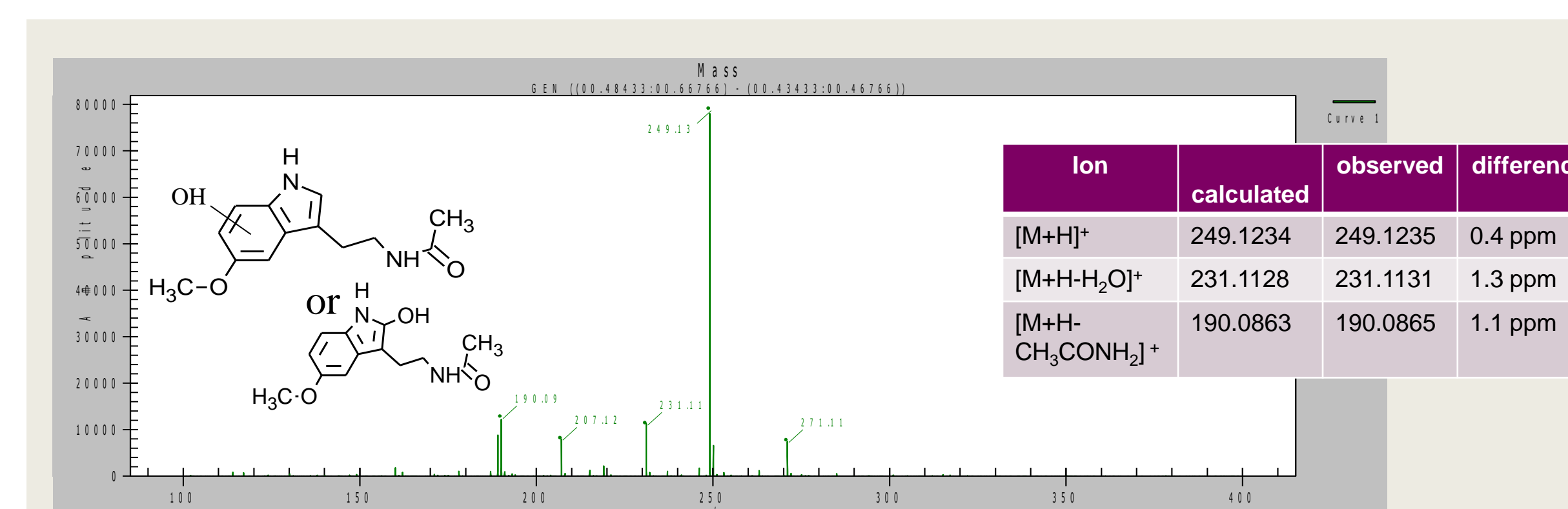


Figure 4. MS spectrum and mass accuracy of peaks B and C (isomers) from Figure 2

Peaks B and C were identified as oxidation products of melatonin based on accurate mass of the [M+H]⁺ ions and fragment ions (Figure 4). The difference in mass of the fragment ion [M+H-CH₃CONH₂]⁺ (m/z 190) in peaks B/C to the fragment ion m/z 172 in melatonin spectrum (Figure 3) suggests the peaks B/C are hydroxy products of melatonin and oxidation has occurred at the indole ring or aromatic ring of melatonin. Since the hydroxy products are hydrophilic compared to melatonin, they will elute earlier than melatonin. The results are consistent with previously published work by Williamson, *et al* (1).

Peaks A and D have identical accurate masses, but have different spectra and elution times. We speculate the structure of peak A to be a di-oxidation product of melatonin based on accurate mass of [M+H]⁺ and its fragments. (Figure 5)

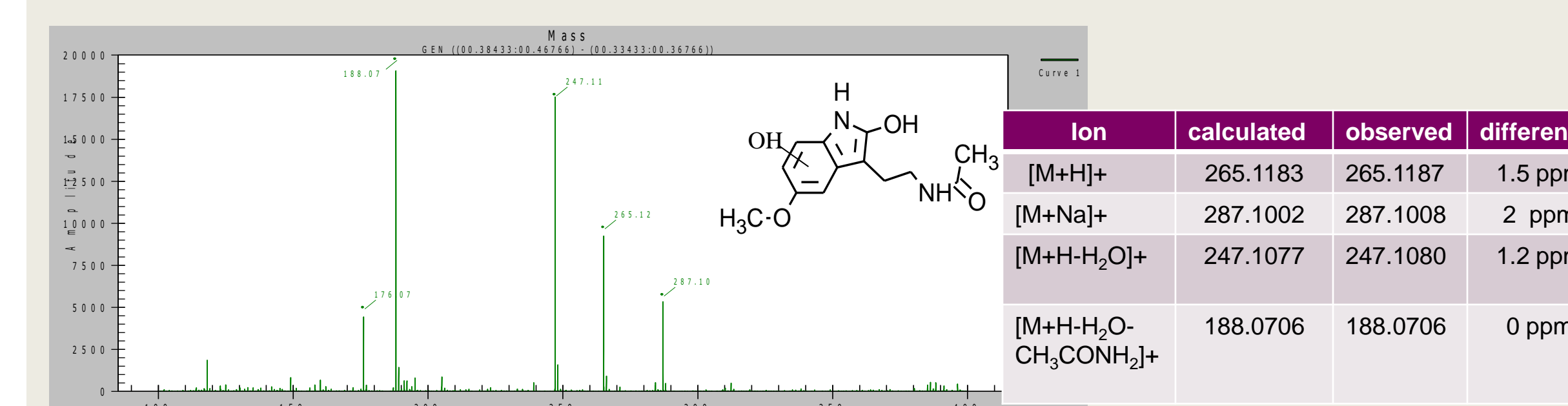


Figure 5. MS spectrum and accurate mass of peak A from Figure 2

The structure of peak D was speculated to be a substituted indoline compound by Williamson, *et al* (1) (Figure 6) who used a triple quadrupole for analysis. The elemental composition (C₁₄H₂₀N₂O₃) of the indoline structure proposed by Williamson, *et al* (1) would have an accurate mass of 265.15467 which is ~140 ppm higher than the mass obtained for peak D by the AxION™ 2 TOF (Figure 7), thus suggesting the proposed structure may be incorrect.

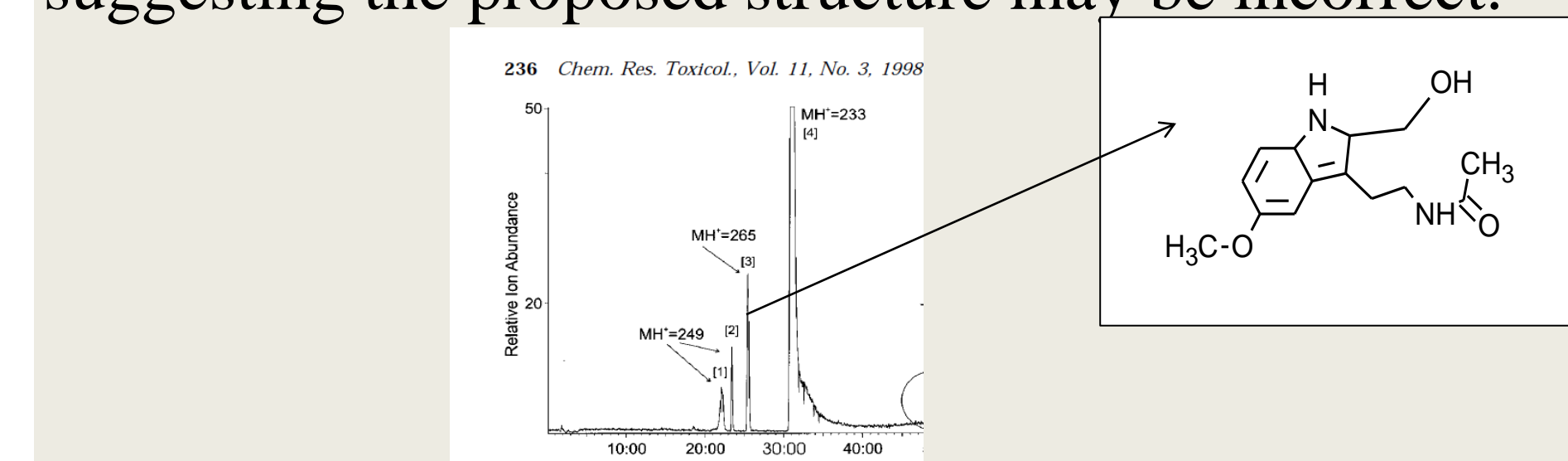


Figure 6. Structure of peak D speculated by Williamson, *et al* (1).

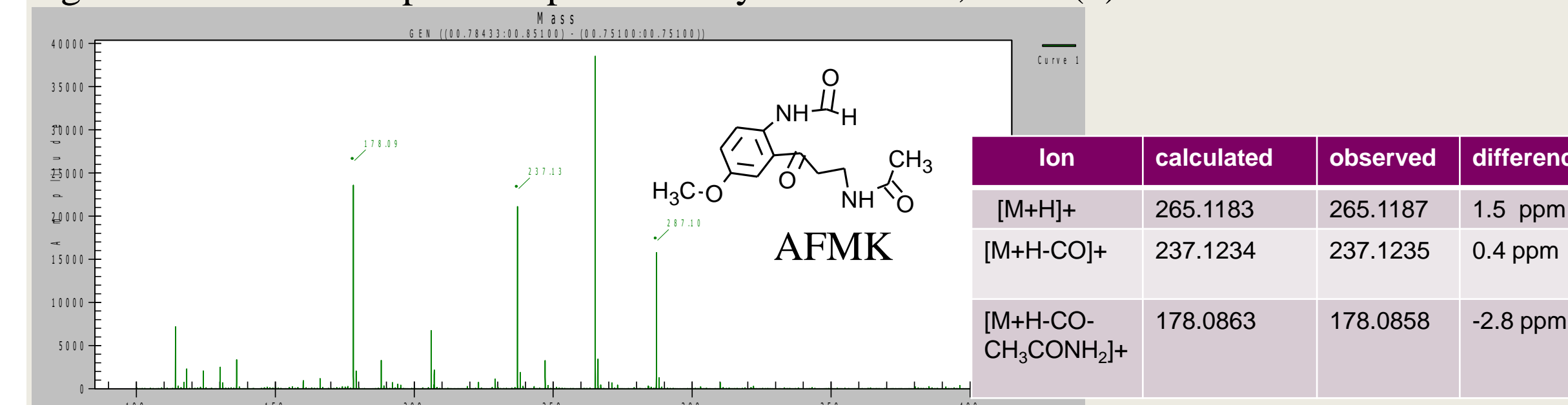


Figure 7. MS spectrum of peak D

For these reasons, we speculated the structure of peak D to be N1-acetyl-N2-formyl-5-methoxykynurenin (AFMK). We confirmed the structure of peak D by analyzing a synthesized standard of AFMK which matched both the retention time and accurate mass spectrum of peak D (data not shown).

Peaks E and F are most likely dimers of melatonin based on accurate mass data (Figure 8)

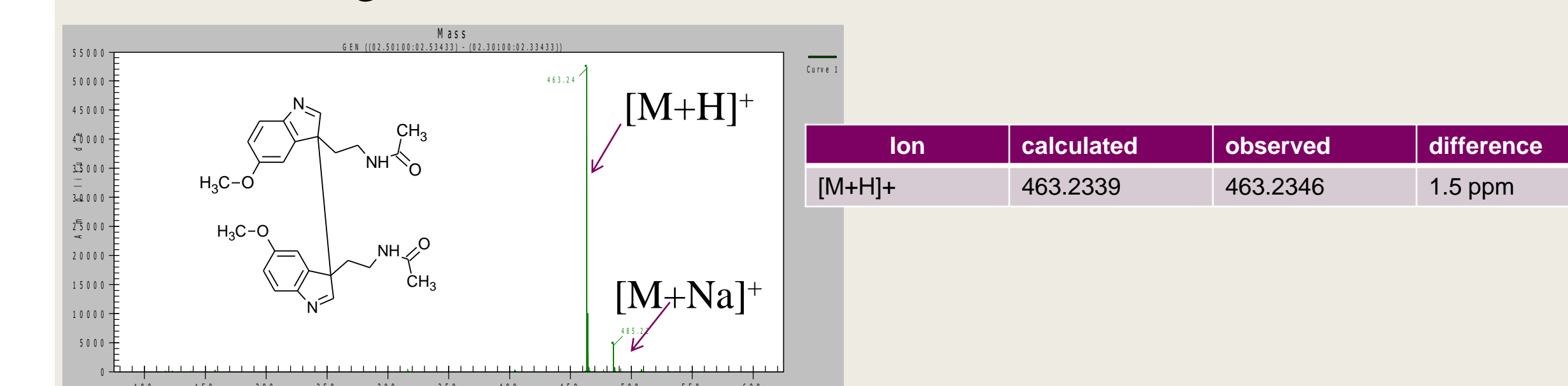


Figure 8. Spectrum of peaks E and F

Peaks G and H are likely formaldehyde dimers of melatonin (data not shown). These dimers were also observed by Williamson, *et al* (1) who further confirmed their presence by synthesis and NMR spectroscopy.

1. Williamson, B. L., *et al*. Chemical Research Toxicology, 1998, 11, 234-240

4 Summary and Conclusions

- Operating the TOF MS in trap (vs. pulse) mode provided a 3 to 5 fold improvement in sensitivity. The increased sensitivity allowed additional impurities to be detected and measured in an OTC melatonin sample that were not observed in pulse mode.
- The accurate mass measurement capability of the AxION 2 TOF could easily distinguish between elemental compositions C₁₄H₂₀N₂O₃ (m/z = 265.1547) and C₁₃H₁₆N₂O₄ (m/z = 265.1183). This provided the confirmation that a previously published structure proposed from data provided by a triple quadrupole MS was incorrect for a specific melatonin impurity.
- The significant improvement in sensitivity gained from operating the AxION™ 2 TOF in trap mode along with the mass measurement accuracy allowed the identification of additional melatonin impurities. These included N1-acetyl-N2-formyl-5-methoxykynurenin (AFMK) as well as melatonin dimers not observed in previously published work.