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Selecting and optimizing transitions for LC-MS/MS methods

When developing LC-MS/MS methods, laboratories choose which transitions to use. By making those choices carefully, laboratories can improve the sensitivity and robustness of their methods. This article presents best practices for selecting and optimizing precursor and product ions, along with the underlying theory behind these choices.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) has changed forensic toxicology testing over the last three decades and become a valuable alternative to gas chromatography mass spectrometry (GC-MS). Compared with GC-MS, LC-MS/MS offers shorter run times and simplified sample preparation.

Identification in both LC-MS/MS and GC-MS relies on the chromatographic retention time and the formation of product ions that provide structural information about the analyte. Where GC-MS relies on product ions formed in the source during ionization, LC-MS/MS instead uses multi-reaction monitoring (MRM) in a triple quadrupole system. In the first quadrupole, a charged precursor ion is selected and allowed to pass through to the second quadrupole, where it collides with gas to form product ions in a process called collision-induced dissociation (CID). Finally, a single product ion is selected in the third quadrupole and allowed to pass through to the detector. The instrument measures a combination of precursor and product ions (known as a transition, or MRM) for a few milliseconds and then moves on to the next transition in the list. Typically, a transition is measured a few times per second, allowing the construction of transition-specific chromatograms.

An analyte is quantified by comparing the peak area (or more rarely, peak height) with the area of the internal standard. This ratio is then compared with area ratios of one or more calibrators with known concentrations. An analyte is identified by calculating the area ratio between the peak area of the quantifying transition and the peak areas of one or more qualifying transitions, known as ion ratios. The sample ion ratios are compared with the ion ratio of a single calibrator, or an average of ion ratios from calibrators in a multi-point calibration set, and should be similar (typically, ±20%).

Requirements of the NLCP

Section L of the *NLCP Urine Laboratory Manual*[1] specifies requirements for confirmation methods. For LC-MS/MS methods using MRM, two transitions must be monitored for each analyte (one for quantification and one as a qualifier), and two transitions must be monitored for the internal standard (one for quantification and one as a qualifier). The transitions should be free of interferences and matrix effects and "should be relevant to the target analyte (e.g., should be a transition from the target analyte or minimally be a justifiable structure relative to the target analyte)."[1] Laboratories must also provide structural justification for the transitions and systematically evaluate instrument parameters.

Structural justification means identifying the structure of the precursor and product ions. This information is used to determine whether the transitions are fit for purpose and to better understand the risk of interference.

Importance of chromatographic separation

Although this article focuses on MS, it is important to remember the role of chromatography. Chromatographic separation is essential to separate analytes from interferences and reduce background noise. Optimizing the chromatographic separation is often the most effective tool in resolving interferences.

CID fragmentation mechanisms

In positive mode, the most common precursor ion is the protonated molecule [M+H]+, which breaks down during CID to form product ions. The mechanism typically includes breaking up the structure close to nitrogens or oxygens, resulting in a charged ion and a neutral fragment (which the mass spectrometer cannot detect). In negative mode, the precursor ion is typically the deprotonated molecule [M-H]−, with a similar fragmentation pattern. Because of charge location and transfer during fragmentations, the bonds broken during positive mode fragmentation might be the same (or not) as those broken during fragmentation in negative mode.

Fragmentation can also be much more complex, including multistep mechanisms and rearrangements, which often occur when a ring structure is broken during fragmentation. For a more in-depth discussion about LC-MS/MS fragmentation, the authors recommend a review by Demarque et al.[2] on fragmentation reactions using electrospray as well as an article by Bijlsma et al.[3] on fragmentation of drugs of abuse.

Selecting suitable transitions for LC-MS/MS

Vendor optimization software often helps laboratories choose a transition, in particular the product ions by identifying prevalent product ions, and optimizes analyte-specific parameters such as the collision energy. However, the final choice depends on multiple factors, including ion intensity and potential for interference. Common precursor and product ions for current NLCP analytes are found in **Exhibit 1**. The list is not exhaustive, and laboratories can certainly consider other product ions. In addition, some products ions, though included in the table, are less suitable as discussed below.

Ion intensity and signal to noise

It is intuitive that the transitions generating the most signal will also provide the best sensitivity. However, sensitivity also depends on the background noise of the transition, which can vary widely between transitions, thus making signal/noise ratios a better measure for product ion selection. A higher background is generally observed for product ions with lower masses and for certain common product ions related to many substances (e.g., *m/z* 91) or related to the mobile phases used.

Less suitable product ions

The NLCP laboratory manual states "It is not appropriate to use trivial loss fragments, such as derivatizing agent derived fragments or adduct ions (e.g., dimers)." Trivial losses are small losses of little value when differentiating an analyte from different interferences, such as water loss (−18 Da, typically from aliphatic hydroxyl groups), ammonia loss ($-NH_3$, −17 Da), and carbon dioxide loss ($-CO_2$, −44 Da, typically from terminal carboxylic acids).The *m/z* 299 product ion from tetrahydrocannabinol-9-carboxylic acid (THC-COOH) is one example of a trivial loss. Trivial loss product ions are not suitable because interfering substances with the same precursor mass also are likely to produce the same trivial loss.

In addition, product ions with a very low mass (<50 Da) typically represent very small parts of the molecule and should be avoided because they are also expected to be produced by many interfering substances and are more likely to be present in the chromatography system background.

In some instances, higher sensitivity can be achieved by using the sodium $[M+Na]^+$ or ammonium $[M+NH_4]^+$ adducts as the precursor instead of $[M+H]^+$. However, this practice is generally avoided in LC-MS/MS because the adducts change the fragmentation and often make interpretation of product ions very complicated.

Derivatization in LC-MS/MS

Derivatization in LC-MS/MS is very rare. Oxime derivatives have been used for semi-synthetic opioids,[4] and sensitivity in oral fluid is increased by derivatizing THC-COOH.[5] If derivatization is used, product ions corresponding only to the derivatizing reagent or corresponding to a loss of only the derivatizing reagent are not acceptable. It is expected that a derivatizing reagent would also derivatize other interfering substances in the sample, which would then show the same derivatizing reagent losses.

^{13}C isotope precursor ions

The mass spectrometer is saturated at high concentrations, resulting in a loss of linearity. In those cases, ^{13}C isotope $[M+2]^+$ can be used as the precursor ion. This reduces the signal in the detector and allows for an extended linear range. In fact, calibration with both the protonated molecule $[M+1]^+$ and the ^{13}C isotope $[M+2]^+$ can be used in the same method. This approach was used to measure methamphetamine from 50–200,000 ng/mL (4,000-fold).[6] However, this approach is not permitted by NLCP because there is a requirement that each calibration curve must have a calibrator at the cutoff concentration.[1]

Exhibit 1. Product ions identified by literature searches.

Analyte	Precursor (*m/z*)	Formula	Product (*m/z*)	Formula	Sources	Notes
Δ^9 - & Δ^8-tetrahydro-cannabinol (THC)	315.2	$C_{21}H_{31}O_2^+$	123.0	$C_7H_7O_2^+$	7-9	Used for Δ^9-THC
			135.1	$C_{10}H_{15}^+$	8	Used for Δ^8-THC
			193.1	$C_{12}H_{17}O_2^+$	7-11	
			259.2	$C_{18}H_{24}O_2^+$	10, 11	
THC carboxylic acid (THC-COOH) Positive mode	345.2	$C_{21}H_{29}O_4^+$	193.1	$C_{12}H_{17}O_2^+$	3, 7, 12	
			229.1	$C_{15}H_{17}O_2^+$	3	
			257.2	$C_{18}H_{24}O_2^+$	3	
			299.2	$C_{20}H_{27}O_2^+$	3, 7, 12	Carbon dioxide loss
THC-COOH Negative mode	343.2	$C_{21}H_{27}O_4^-$	191.1	$C_{12}H_{15}O_2^-$	8, 13, 14	
			245.2	$C_{16}H_{21}O_2^-$	8, 13-16	
			299.2	$C_{20}H_{27}O_2^-$	13-16	Carbon dioxide loss
Cocaine	304.2	$C_{17}H_{22}NO_4^+$	82.1	$C_5H_8N^+$	7, 9, 10, 12, 17, 18	
			105.0	$C_7H_5O^+$	7, 17-20	
			150.1	$C_9H_{12}NO^+$	11, 20	
			182.1	$C_{10}H_{16}NO_2^+$	9-12, 17-19	
Benzoylecgonine	290.1	$C_{16}H_{20}NO_4^+$	105.0	$C_7H_5O^+$	7, 9, 11, 12, 18-21	
			119.0	$C_8H_7O^+$	20	
			168.1	$C_9H_{14}NO_2^+$	7, 9, 11, 12, 18, 19, 21	

Analyte	Precursor (*m/z*)	Formula	Product (*m/z*)	Formula	Sources	Notes
Hydroxy cocaine	320.1	$C_{17}H_{22}NO_5^+$	82.1	$C_5H_8N^+$	7, 18	
			121.0	$C_7H_5O_2^+$	19, 20	
			182.1	$C_{10}H_{16}NO_2^+$	7, 18-20	
Codeine	300.2	$C_{18}H_{22}NO_3^+$	152.1	$C_{10}H_{18}N^+$	7, 12, 22-24	Possible structure
			165.1	$C_{13}H_9^+$	4, 7, 9, 11, 12, 21, 23-25	
			215.1	$C_{14}H_{15}O_2^+$	4, 9, 17, 26, 27	
Morphine	286.1	$C_{17}H_{20}NO_3^+$	152.1	$C_{10}H_{18}N^+$	12, 21-24	Possible structure
			153.1	$C_{12}H_9^+$	7, 9, 25	
			165.1	$C_{13}H_9^+$	4, 7, 9, 11, 12, 17, 23, 24	
			201.1	$C_{13}H_{13}O_2^+$	4, 11, 17, 22, 26, 27	Multiple suggested
6-acetylmorphine	328.2	$C_{19}H_{22}NO_4^+$	165.1	$C_{13}H_9^+$	7, 9, 11, 12, 21-23, 25	
			193.1	$C_{14}H_9O^+$	4, 26, 27	
			211.1	$C_{14}H_{11}O_2^+$	7, 9, 11, 12, 22, 23, 25-27	
Hydrocodone	300.2	$C_{18}H_{22}NO_3^+$	128.1	$C_8H_{18}N^+$	7, 12, 23, 24	Possible structure
			171.1	$C_{12}H_{11}O^+$	21, 22, 25, 27	
			199.1	$C_{13}H_{11}O_2^+$	9, 12, 21-25	
Hydromorphone	286.1	$C_{17}H_{20}NO_3^+$	152.1	$C_{10}H_{18}N^+$	12, 24	Possible structure
			157.1	$C_{11}H_9O^+$	7, 9, 12, 21-23	
			185.1	$C_{12}H_9O_2^+$	7, 9, 12, 21-25, 27	
Oxycodone	316.2	$C_{18}H_{22}NO_4^+$	212.1	$C_{15}H_{18}N^+$	21, 25	Possible structure
			241.1	$C_{15}H_{13}O_3^+$	7, 9, 12, 21-24, 28	
			256.1	$C_{16}H_{18}NO_2^+$	12, 25-28	Possible structure
			298.1	$C_{18}H_{20}NO_3^+$	7, 9, 22-24, 26, 27, 29	Water loss

Analyte	Precursor (*m/z*)	Formula	Product (*m/z*)	Formula	Sources	Notes
Oxymorphone	302.1	$C_{17}H_{20}NO_4^+$	198.1	$C_{10}H_{16}NO_3^+$	9, 12, 21, 24	Possible structure
			227.1	$C_{14}H_{11}O_3^+$	7, 9, 12, 21-24, 28	
			242.1	$C_{15}H_{16}NO_2^+$	27, 28	Possible structure
			284.1	$C_{17}H_{18}NO_3^+$	7, 22, 23, 27	Water loss
Phencyclidine (PCP)	244.2	$C_{17}H_{26}N^+$	86.1	$C_5H_{12}N^+$	7, 12, 17	
			91.1	$C_7H_7^+$	9, 12, 17, 21	Tropylium ion
			159.1	$C_{12}H_{15}^+$	7, 9, 17, 21	
Amphetamine	136.1	$C_9H_{14}N^+$	65.0	$C_5H_5^+$	9	
			91.1	$C_7H_7^+$	7, 9-12, 17	Tropylium ion
			119.1	$C_9H_{11}^+$	7, 10-12, 17, 21	Loss of ammonia
Methamphetamine	150.1	$C_{10}H_{16}N^+$	65.0	$C_5H_5^+$	9, 12	
			91.1	$C_7H_7^+$	9-12, 17, 21	Tropylium ion
			119.1	$C_9H_{11}^+$	7, 10-12, 17, 21	
Methylenedioxy-methamphetamine (MDMA)	194.1	$C_{11}H_{16}NO_2^+$	105.1	$C_8H_9^+$	7, 9, 11, 12, 17	
			135.0	$C_8H_7O_2^+$	9, 10, 17, 21	
			163.1	$C_{10}H_{11}O_2^+$	7, 10-12, 17, 21	
Methylenedioxy-amphetamine (MDA)	180.1	$C_{10}H_{14}NO_2^+$	105.1	$C_8H_9^+$	9, 17	
			133.1	$C_9H_9O^+$	7, 12, 21	
			135.0	$C_8H_7O_2^+$	7, 9, 11, 12, 17, 21	
			163.1	$C_{10}H_{11}O_2^+$	11, 17	Loss of ammonia
Fentanyl	337.2	$C_{22}H_{29}N_2O^+$	105.1	$C_8H_9^+$	4, 9, 11, 12, 17, 21, 26, 27, 30	
			132.1	$C_9H_{10}N$	7	
			188.1	$C_{13}H_{18}N$	4, 7, 9, 11, 12, 17, 21, 26, 27, 30	

Analyte	Precursor (*m/z*)	Formula	Product (*m/z*)	Formula	Sources	Notes
Norfentanyl	233.2	$C_{14}H_{21}N_2O^+$	56.0	$C_3H_6N^+$	4, 7, 12, 30	
			84.1	$C_5H_{10}N^+$	4, 7, 12, 21, 26, 30	
			177.1	$C_{11}H_{17}N_2^+$	26, 30	

Note: Only product ions used by at least two publications are included, except where needed to provide at least three product ions per analyte.

Method development

After identifying the transitions to use, the next step is usually to optimize them to increase method performance. This includes using exact masses and optimizing analyte-specific and global parameters.

Defining precursor and product ion masses

If we disregard the isotopes, the mass of a drug molecule can be defined very precisely, but there is an uncertainty in the measurement. If you zoom in on a mass spectrum, you see that the mass intensities are not bars (as typically depicted) but are instead peaks created by this uncertainty. Quadrupole and ion trap instruments typically generate flat-topped peaks with a width of about 0.7 Da, which would be sufficient to separate *m/z* 243.0 from *m/z* 244.0, for example.

This uncertainty of measurement also generates a mass error when the masses are determined empirically, such as when using vendor optimizing software. The errors are usually small (0–0.2 Da), but replacing them with masses calculated from the molecular formula of the precursor and product ion (known as exact masses) removes the bias of the instrument calibration and will be beneficial as the method is used over time and on multiple instruments.

Given a peak width of around 0.7 Da, defining the precursor and product ion masses using one decimal is sufficient to capture the whole mass peak. This improves sensitivity and reduces the impact of mass calibration drift.

Optimizing settings

Mass spectrometer settings to consider for optimization include those of the ion source and the collision cell. However, it is difficult to give specific suggestions because the configuration of these components varies between mass spectrometer vendors.

Transition-specific settings (e.g., fragmentor voltage, collision energy) can be changed very quickly from transition to transition. A good place to start is to use the data from the vendor optimizing software, which typically makes suggestions for each transition.

Conversely, global parameters (e.g., ion source temperatures and gases) are set for the whole analysis. A good starting point is the vendor standard settings or the settings in an application note for similar drugs. If needed, the settings can then be optimized specifically for an analyte to increase sensitivity.

Product ions of cannabinoids

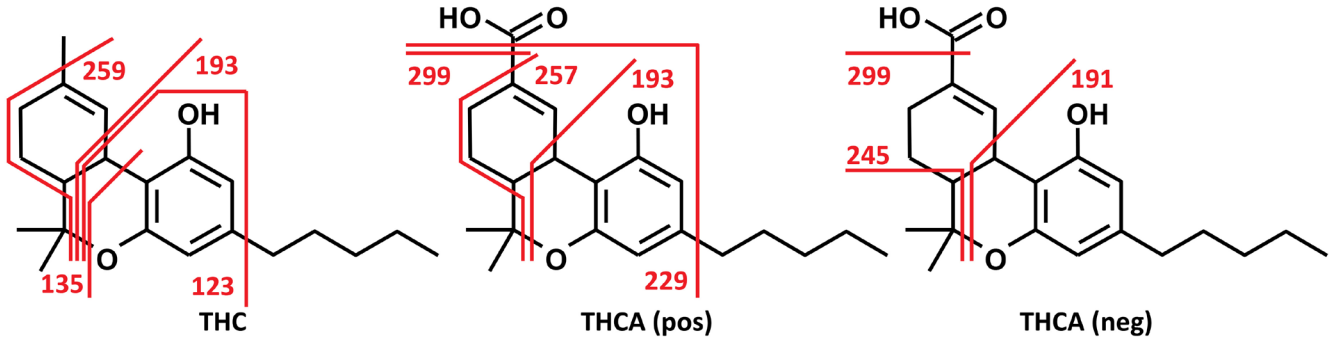

Exhibit 2. Product ions of cannabinoids

THC-COOH is the only NLCP analyte commonly detected in negative mode, generating different product ions than in positive mode. Several product ions are formed through the cleavage of multiple bonds, particularly in the ring system, yielding complex fragmentation and making it difficult to assign structures to the product ions. In particular, the structure assigned to *m/z* 245 for THC-COOH (negative mode) is an acceptable option; however, alternative acceptable product ions exist. Product ions *m/z* 327 and 325 correspond to the loss of water and *m/z* 299 to the trivial loss of carbon dioxide and should be avoided. Lelario et al. is a good reference on cannabinoid fragmentation.[31] Both Δ^9- and Δ^8-tetrahydrocannabinol (THC) generate several identical product ions, and this is assumed to also be true for the THC-COOH isomers. One study used different product ions for the two isomers,[8] which could be one way to minimize interference between them.

Suggested product ions of cocaine and metabolites

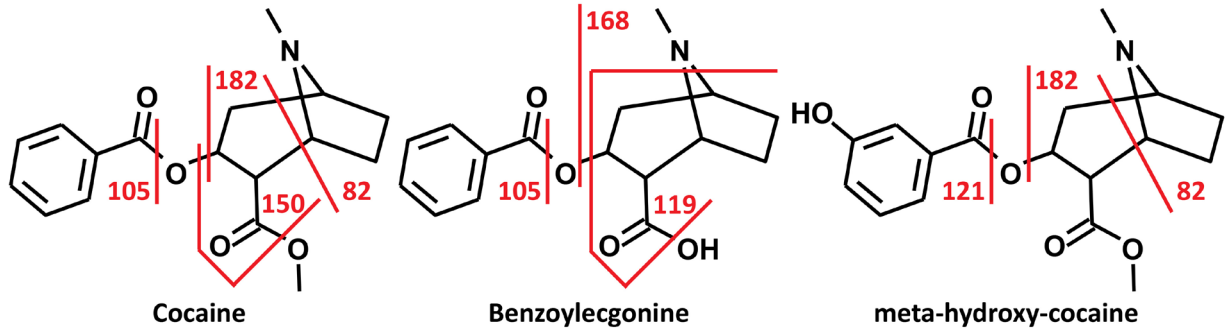

Exhibit 3. Product ions of cocaine and metabolites

The mass spectra of cocaine and its metabolites are dominated by *m/z* 182 (or the equivalent *m/z* 168 for benzoylecgonine), whereas other product ions typically show less intensity (<20%). The *m/z* 182 product ion is formed through loss of the benzoyl moiety. Smaller product ions such as *m/z* 82, 150, and 119 are likely formed from the *m/z* 182 product ion. Wang & Bartlett is a good reference on the fragmentation of cocaine and its metabolites.[32] Although *m/z* 119 and 150 were not observed in methods for any of the three analytes, they have been reported as product ions.[32] Para-hydroxy-cocaine is expected to have the same product ions as meta-hydroxy-cocaine.

Suggested product ions of opiates and semi-synthetic opioids

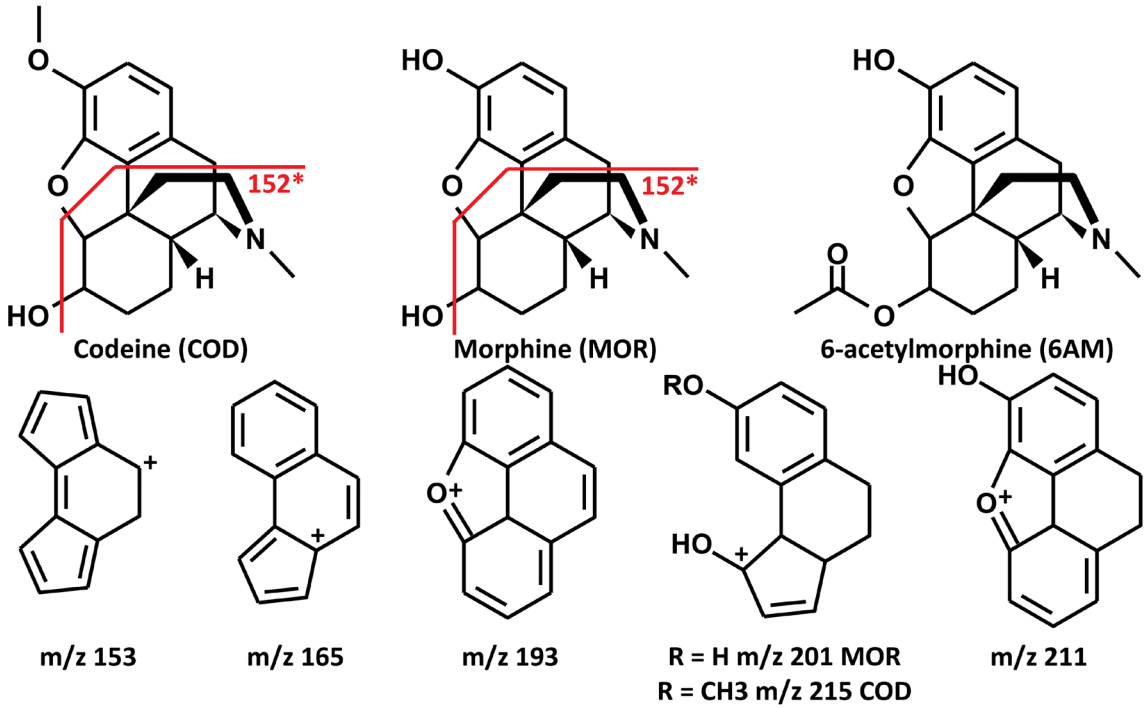

Exhibit 4. Product ions of opiates. **Structure is a possibility but not supported by literature.*

Given their extended ring systems, the fragmentation of opiates and semi-synthetic opioids is more complicated relative to the other drug classes in this article because it involves multiple cleavages and rearrangements across the rings. Most of the structures in **Exhibits 4** and **5** are based on the works of Bijlsma et al.[3] and Poeaknapo et al.[33]

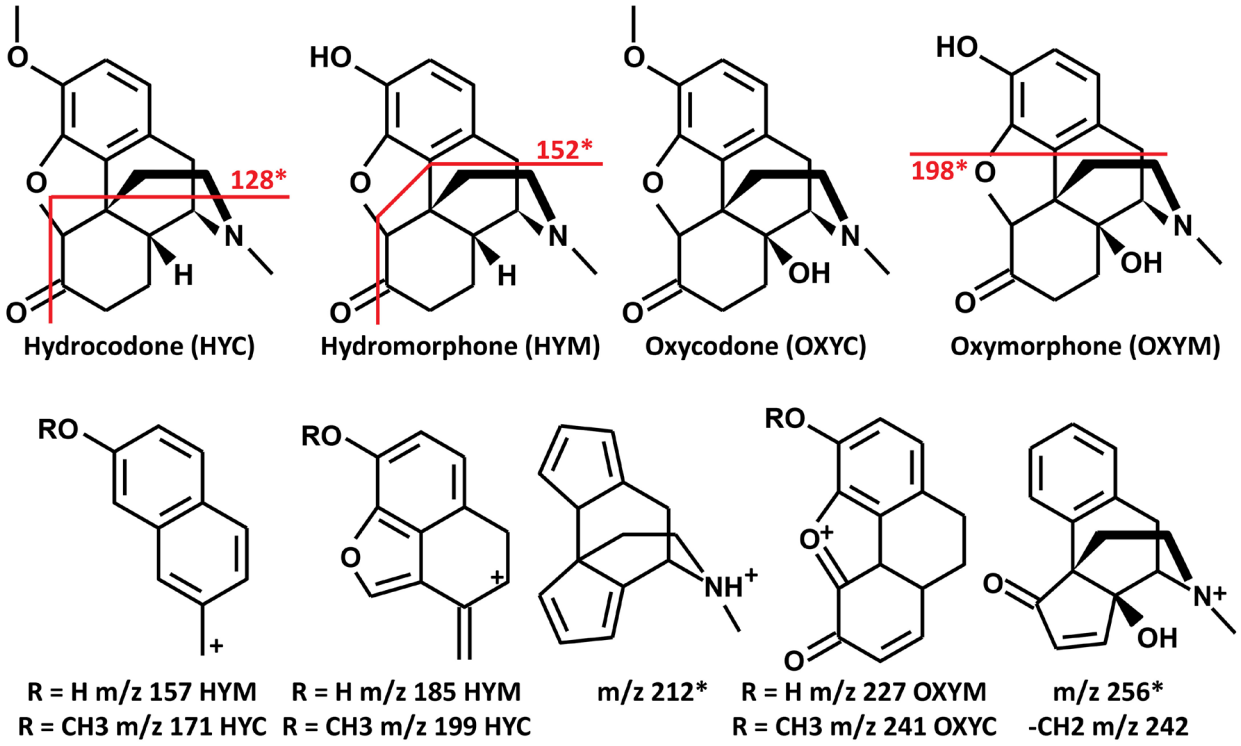

Exhibit 5. Product ions of semi-synthetic opioids. **Structure is a possibility but not supported by literature.*

Product ions that contain a nitrogen, as indicated by an even nominal mass (e.g., *m/z* 152 and 212), are poorly understood. Although commonly used in LC-MS/MS methods, the formulas and structures provided here should be interpreted as minimally acceptable plausible suggestions. Commonly used product ions *m/z* 284 and *m/z* 298 for oxymorphone and oxycodone, respectively, are poor choices as they represent a water loss from the parent.

Suggested product ions of phencyclidine

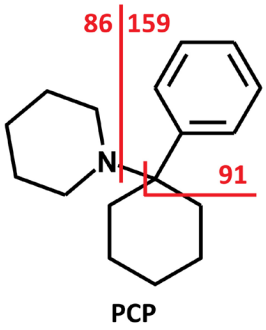

Exhibit 6. Product ions of PCP

PCP consists of three rings, and the product ions are produced from one (*m/z* 86 and 91) or two (*m/z* 159) of the rings. Product ion *m/z* 91 is likely the tropylium ion, which is a very stable and common product ion from benzoyl groups (phenyl group next to an aliphatic carbon).

Suggested product ions of amphetamines

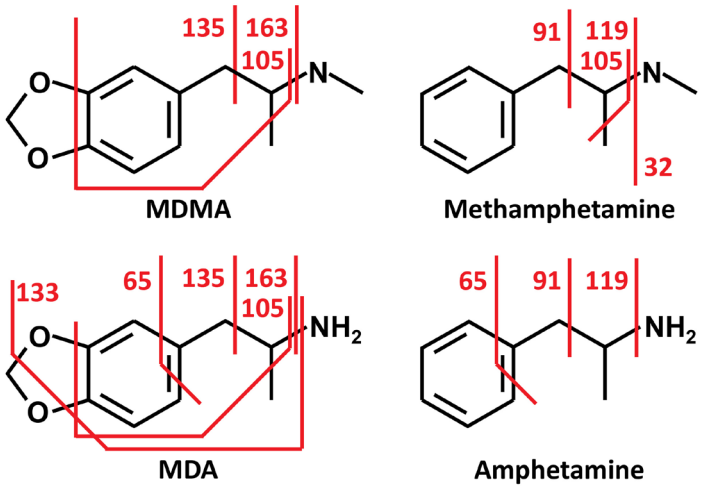

Exhibit 7. Product ions of amphetamines

The most commonly used product ions for amphetamine and methamphetamine are *m/z* 91 and 119, which are formed through losses on the aliphatic part of the molecule. MDA and MDMA undergo the same fragmentation, but because of the methylenedioxy-group, the masses are now *m/z* 135 and 163. Product ion *m/z* 133, observed for MDA, is likely formed from product ion *m/z* 163 through the loss of CH2O from the methylenedioxy moiety.[3]

Suggested product ions of fentanyl and norfentanyl

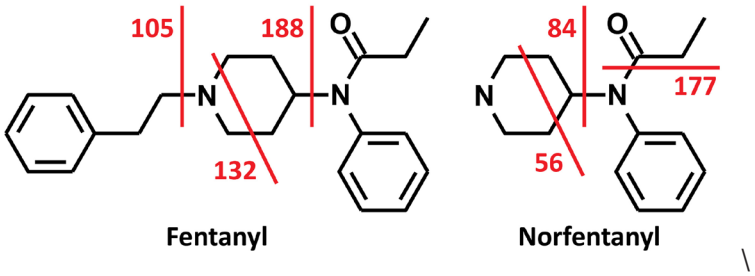

Exhibit 8. Product ions of fentanyl and norfentanyl

Fentanyl typically fragments close to the nitrogen atoms, producing the very characteristic product ions *m/z* 105 and 188.[34] Although these are the most commonly used product ions in LC-MS/MS, there are also others, including *m/z* 132, which is likely formed through a cleavage of the piperidine ring. Norfentanyl product ion *m/z* 84 (corresponding to *m/z* 188 in fentanyl) and *m/z* 56 are likely formed through the cleavage of the piperidine ring. The *m/z* 56 product ion provides less structural information because it represents only part of the piperidine ring. Product ion *m/z* 177 has been suggested to avoid interference from a bupivacaine metabolite product ion *m/z* 84[30] but is also expected to be formed from the normetabolites of several fentanyl isomers.

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