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Nota de aplicación

The Application of MS/MS-Directed Purification to the Identification of Drug Metabolites in Biological Fluids

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Abstract

This application note shows how tandem quadrupole mass spectrometry has been employed for the isolation of the metabolites of common pharmaceuticals from urine. The application of different modes of data acquisition, including scan, MRM, constant neutral loss, and precursor ion is demonstrated. We also demonstrate how the use of MS/MS directed purification facilitates the combination of samples from several chromatographic runs.

Introduction

The identification of drug metabolites following animal or human volunteer studies is essential to drug discovery and development as well as the regulatory submissions process. Traditionally, this has been achieved by the use of liquid or gas chromatography coupled to mass spectrometry.[1,2] More recently, the use of hyphenated techniques such as LC/NMR and LC/NMR/MS have become more commonplace in the drug metabolism

laboratory, allowing a more precise identification of the site of metabolism.[3,4]

While LC/NMR and LC/NMR/MS are extremely powerful tools, they are typically low throughput and limited in sensitivity. The capacity of analytical columns restricts the amount of material that can be loaded onto the column before the column exhibits either volume or mass overloading effects and the chromatographic resolution is lost. Thus, LC/NMR is less attractive for the analysis of highly potent compounds dosed at low levels or those compounds that undergo extensive metabolism. In such cases, it is often necessary to perform a pre-concentration step, such as SPE or liquid/liquid extraction, both of which are time consuming and run the risk of losing of valuable information.

The use of MS-directed purification, using semi-preparative scale columns (typically 19 mm I.D.), is now commonplace within the pharmaceutical industry, especially to support lead candidate purification. This approach has also been applied to the isolation of drug metabolites with some success.5 The extra sensitivity and selectivity of MS/MS mass spectrometry allows for more precise selection of drug metabolites. Furthermore, the use of neutral loss and precursor ion scanning detection modes facilitates the collection of drug metabolites without the need for prior knowledge of compound metabolism. The Application of MS/MS Directed Purification to the Identification of Drug Metabolites in Biological Fluids Paul Lefebvre, Robert Plumb, Warren Potts, and Ronan Cleary Waters Corporation, Milford, MA, USA.

Figure 1. The Alliance HT System with the Quattro micro Mass Spectrometer.

Experimental

A Waters Alliance HT System was used with a SunFire C_{18} 5 µm 4.6 x 100 mm Column at 40 °C. Eluent flow was split 1:20 with a Valco tee. 95% of the flow passed the 2996 Photodiode Array (PDA) Detector to the Fraction Collector III. The other 5% of the flow was routed directly to the Quattro micro Mass Spectrometer equipped with an ESCi multi-mode ionization source.

Caffeine Metabolites Methods

Separation

Water/acetonitrile in 0.1% formic acid, 1.25 mL/min total flow gradient. 0 to 5 min: 0%; 5 to 35 min: 0% to 10% B; 35 to 35.5 min: 10% to 95% B; 35.5 to 39.5 min: 95% B; 39.5 to 40 min: 95% to 5% B; 45 minutes end.

MS Detection

Electrospray positive, 3 kV capillary voltage, 30 V cone voltage, 20 V collision energy (for MS/MS experiments).

Metabolites of Interest

Figure 2 shows a portion of the caffeine metabolism pathway by demethylation.6 Target metabolites maintain the methyl group in position 1. They also have a common fragment ion, *m/z* 57.

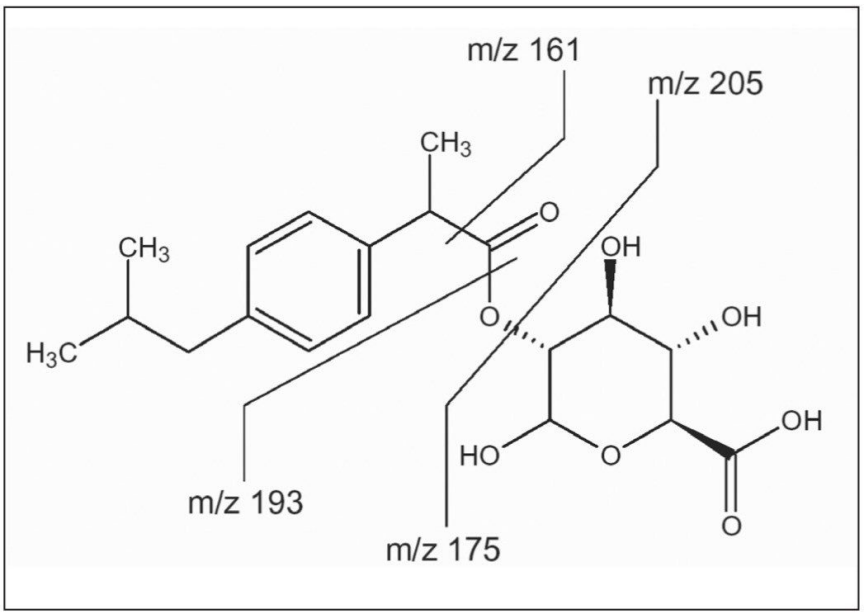

Figure 2. Metabolism of caffeine by demethylation: metabolites that maintain the methyl group in position 1 have a common fragment ion, m/z 57.

Ibuprofen Metabolites

Separation

Water/acetonitrile/10 mM ammonium formate, 1.25 mL/min total flow gradient. 0 to 5 min: 5%; 5 to 35 min: 5% to 60% B; 35 to 35.5 min: 60% to 95% B; 35.5 to 39.5 min: 95% B; 39.5 to 40 min: 95% to 5% B; 45 minutes end.

MS Detection

Electrospray negative, 3 kV capillary voltage, 30 V cone voltage, 20 V collision energy.

Results and Discussion

Metabolites of Interest

Figure 3 shows the fragmentation patterns of the ibuprofen gluceronide metabolite.[7]

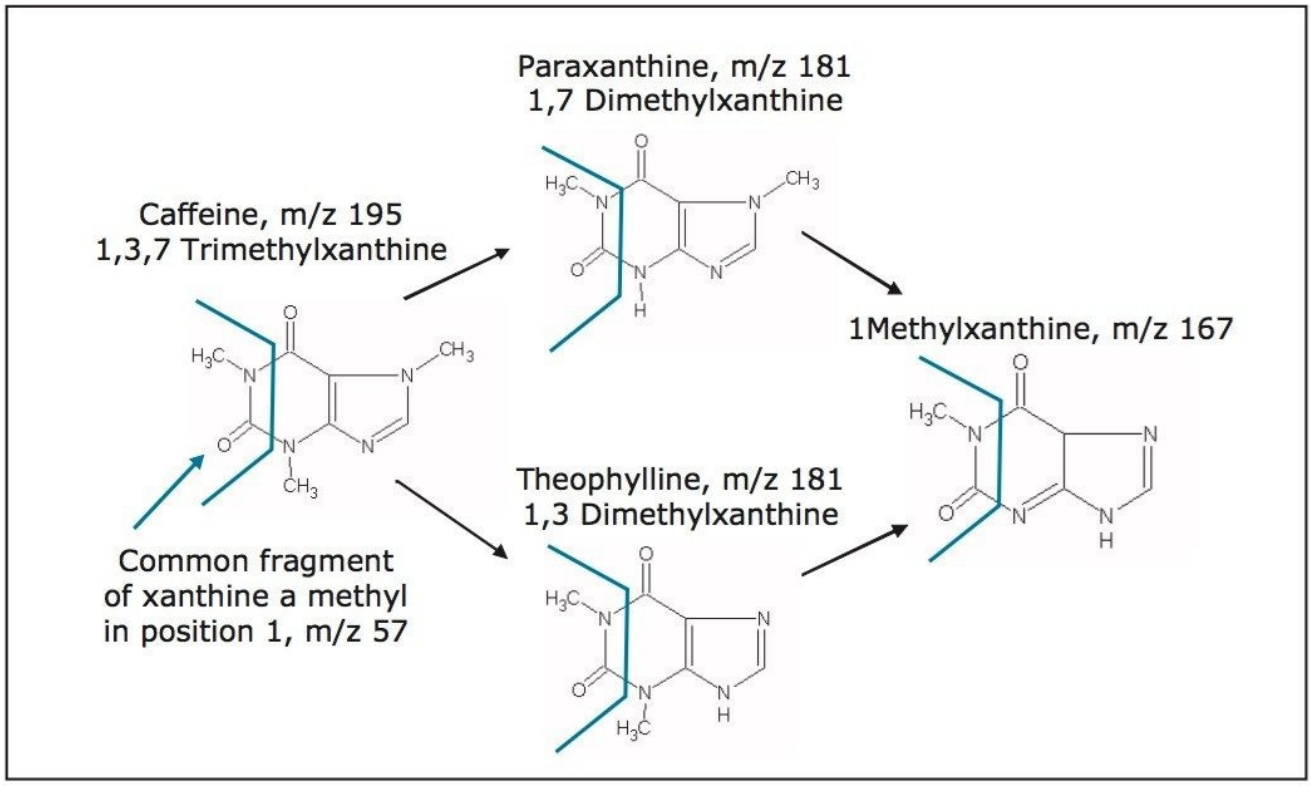

Figure 3. Ibuprofen gluceronide metabolite with a common product ion of m/z 193.

Single Quadrupole Directed Purification

With single quadrupole directed purification, all ions generated in the source are passed through the quadrupole and detected. This is possible on the Quattro micro Mass Spectrometer by using the scan mode of acquisition. Only MS1 is scanned and there is no collision energy or scanning of Q3.

Because all of the ions generated are detected in this mode, complex mixtures can contain numerous isobaric interferences. Consequently, multiple fractions can be generated from a single *m/z* value. Figure 4 shows the collection of the caffeine metabolites with *m/z* 167 and 181 detected using only the first quadrupole. There are eight fractions collected for *m/z* 167 and five fractions collected for *m/z* 181, with additional analysis required to determine the fraction of interest.

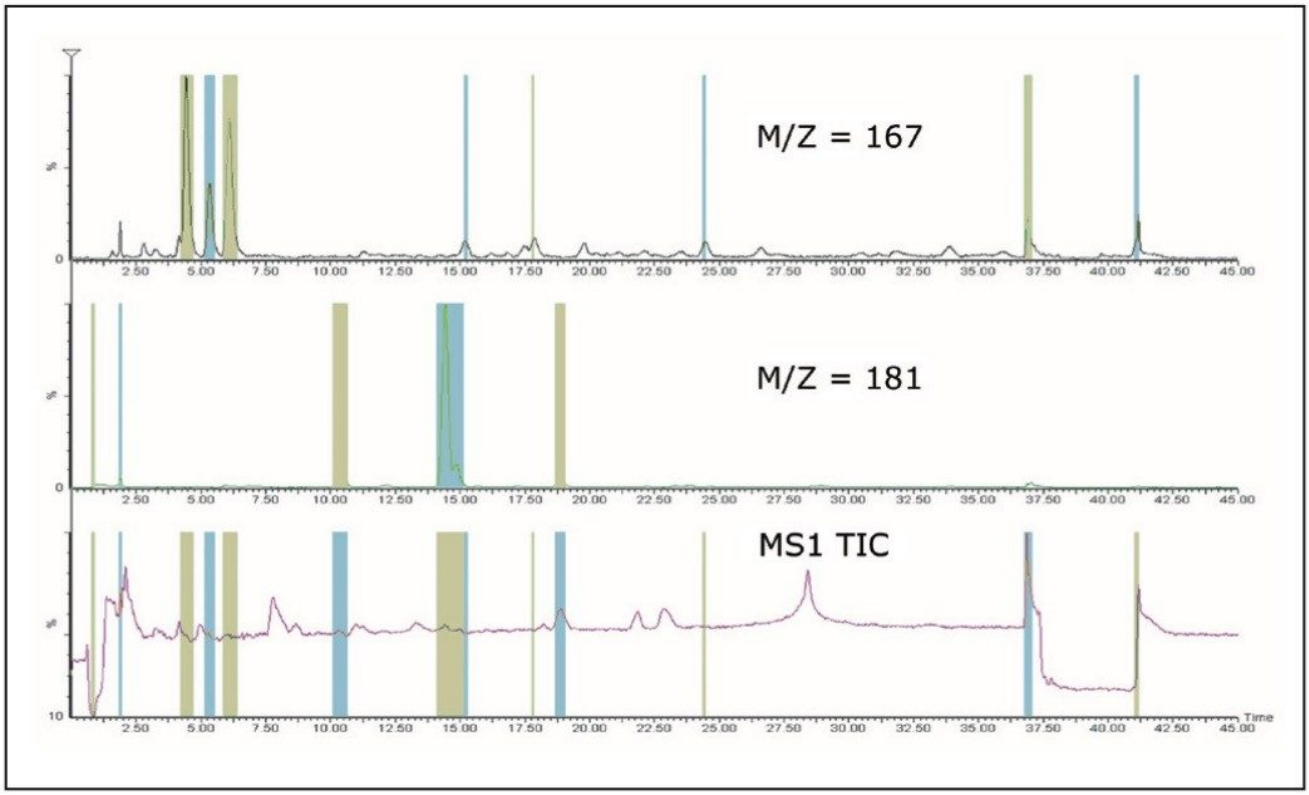

Figure 4. Fractionation based only on scanning the first quadrupole.

Tandem Quadrupole Directed Purification:

MRM Collection

With multiple reaction monitoring (MRM) data acquisition, MS1 is pre-selected on the precursor mass and MS2 is preselected on a specific product ion, as illustrated in Figure 5.

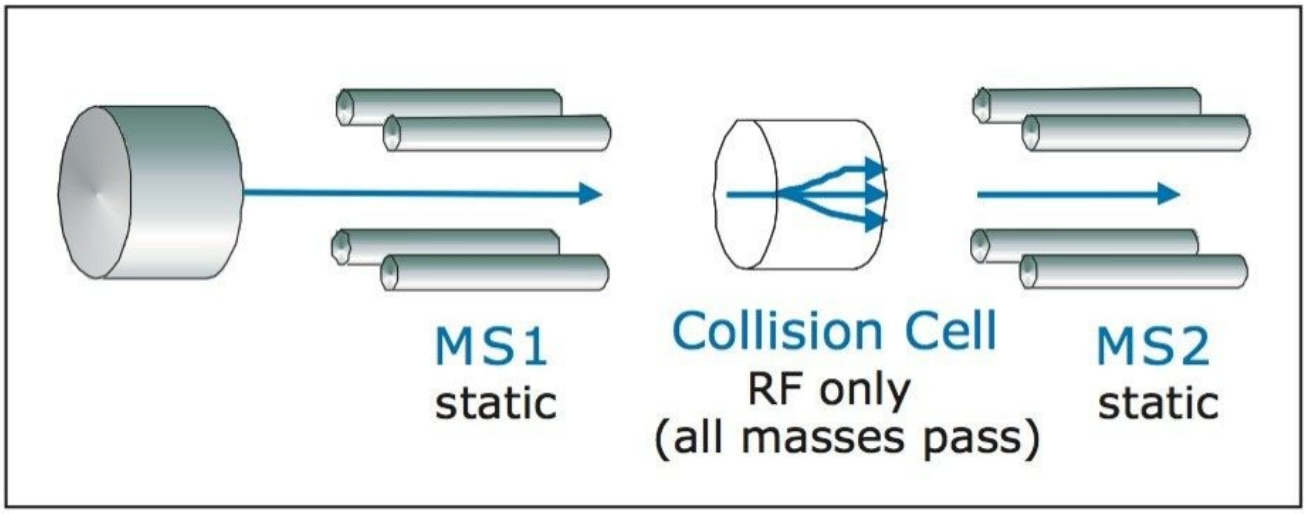

Figure 5. MS/MS MRM data acquisition.

By selectively detecting a product ion, the signal-to-noise ratio is optimized, thus reducing the isobaric interference and allowing only the target to be collected. This mode of acquisition requires previous knowledge of the exact precursor and the exact product ions before purification.

Figure 6 shows the MRM acquisition and collection of the caffeine metabolites. The metabolites of interest for isolation have the transitions of 181 to 134, and 167 to 110.

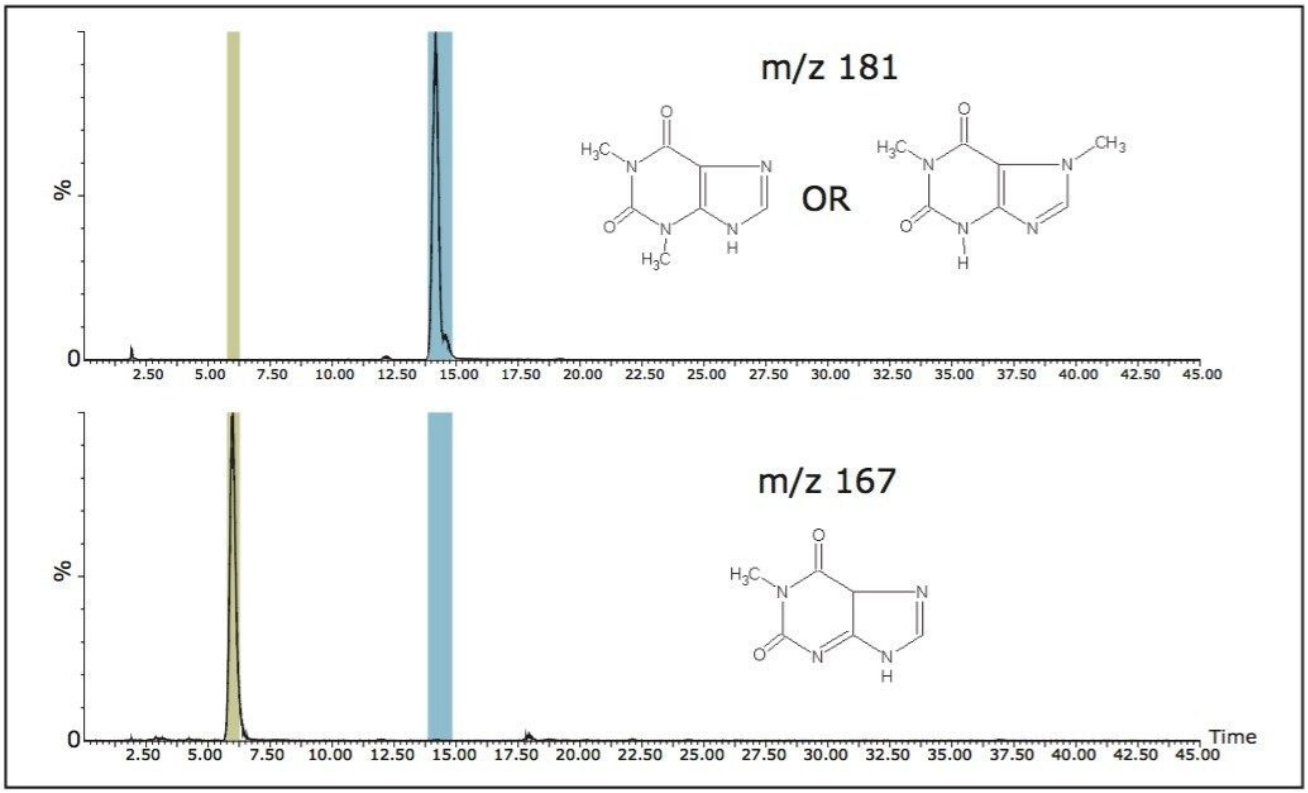

Figure 6. Fractionation based on MRM acquisition.

For a peak to be present in the MRM chromatogram, both the specific precursor and the specific product ion need to be detected. For each target, only one fraction was collected.

Constant Neutral Loss Collection

A second possible mode of fraction triggering is from constant neutral loss acquisition. Here both MS1 and MS2 are scanned in synchronization, as illustrated in Figure 7. When MS1 transmits a specific precursor ion, MS2 looks for a product that is the precursor minus the neutral loss value. If the correct product is present, it registers at the detector. The constant neutral loss spectrum shows only the masses of all the precursors that lose the specific mass.

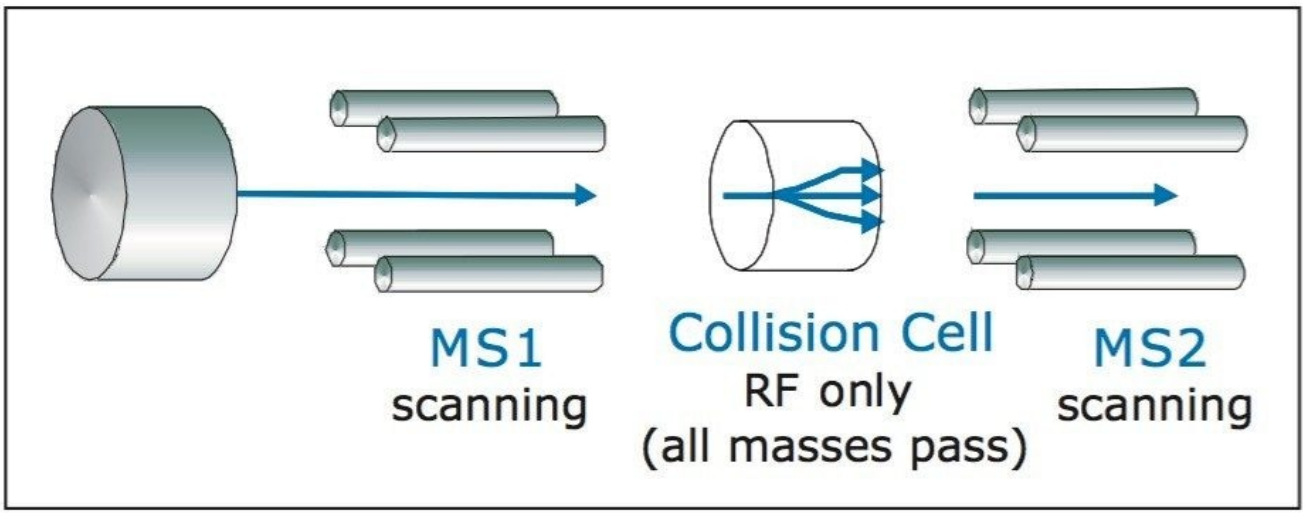

Figure 7. MS/MS constant neutral loss data acquisition.

Figure 8 displays the constant neutral loss of 57 acquisition and collection of the caffeine metabolites with *m/z* 167 and 181. It shows that two fractions are collected, one for each mass. These fractions contain the target mass and have the specific neutral loss.

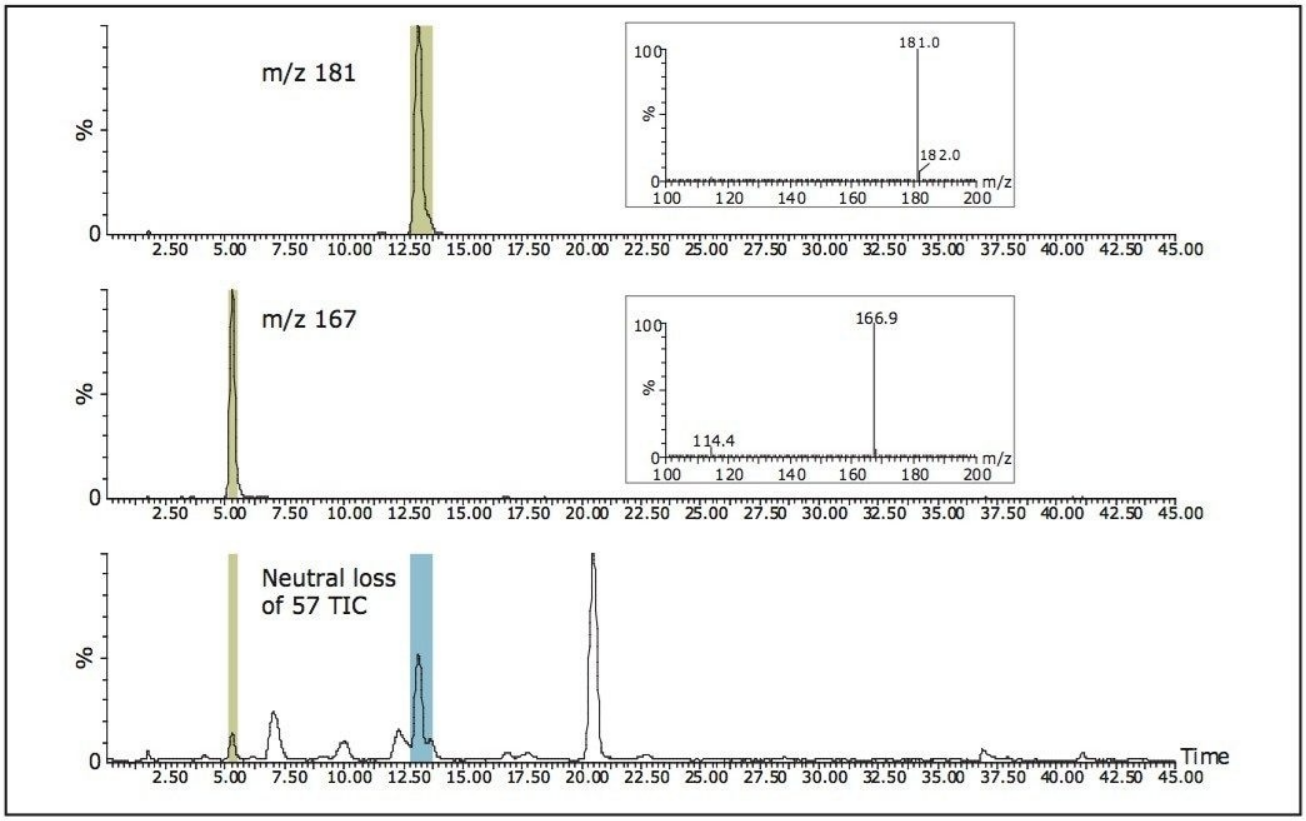

Figure 8. Fractionation based on constant neutral loss acquisition.

Applications for Fraction Collection from Constant Neutral Loss Acquisition

Mass Triggered Collection

With constant neutral loss acquisition, the only peaks detected are the ones with the loss of the specific mass, in this case, 57. Depending on the specificity of the loss, numerous ions can be detected. This leads to complex total ion chromatograms. Therefore, when triggering by a specific mass, the collected target must contain the precursor of interest and have a specific neutral loss.

Collection Triggered on TIC

When using this mode of acquisition and collection, all the peaks with a specific neutral loss are collected. This functionality is valuable when the metabolites have a specific loss related to the drug's structure. It could also be used for isolating a class of metabolites with a generic loss (e.g., sulfates (–80) or glucuronides (–176)). The precursor mass for each fraction can then be extracted and used to aid in the identification of the metabolites.

In the constant neutral loss example shown, collection could also have been triggered from the total ion chromatogram (TIC). All peaks in the –57 TIC would be collected and then additional analysis or data review would be required to find the desired fractions.

Precursor Ion Collection

A third mode of fraction triggering is from precursor ion acquisition, as illustrated in Figure 9. Here, MS1 is scanning and MS2 is fixed on a specific product ion. If the specific product ion is observed, it is registered at the detector. The spectrum only shows the masses that have that specific product.

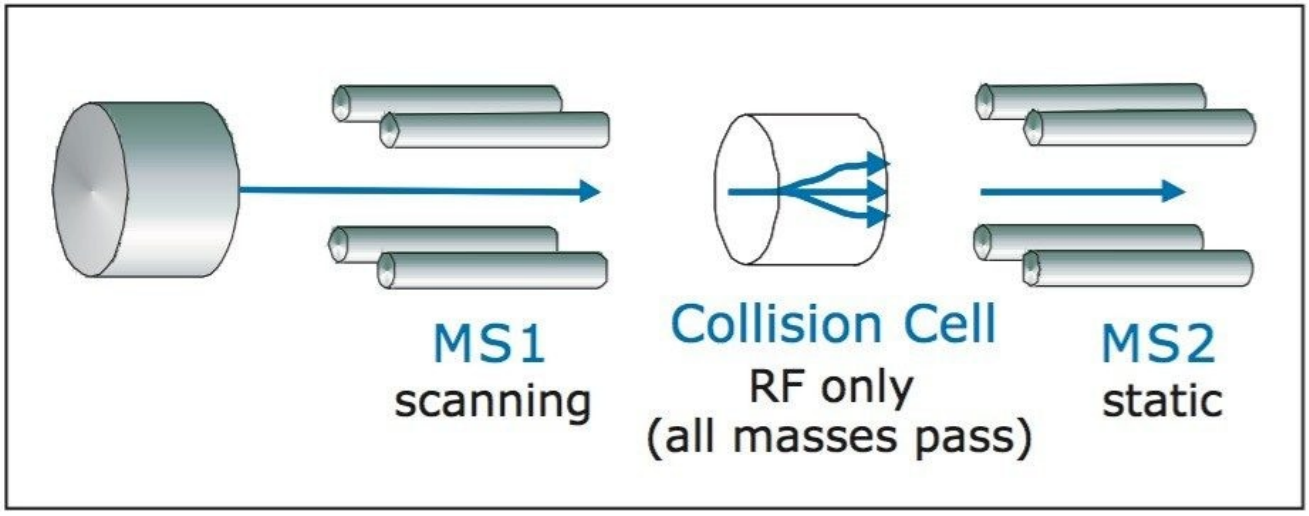

Figure 9. MS/MS precursor ion data acquisition.

Fraction collection from a precursor ion acquisition has to be from the TIC, since the precursor mass is unknown. This mode of fraction collection is valuable when the metabolites are unknown, but there is a common fragment of the core compound that can be detected.

To illustrate the common fragment ion collection capability, Figure 10 shows the glucuronic acid conjugates collected from the ibuprofen urine samples using the precursor ion scan mode of *m/z* 193. There are three fractions that are collected, *m/z* 273 (not drug-related), *m/z* 397 (hydroxyglucuronide conjugate), and *m/z* 381 (glucuronide conjugate).

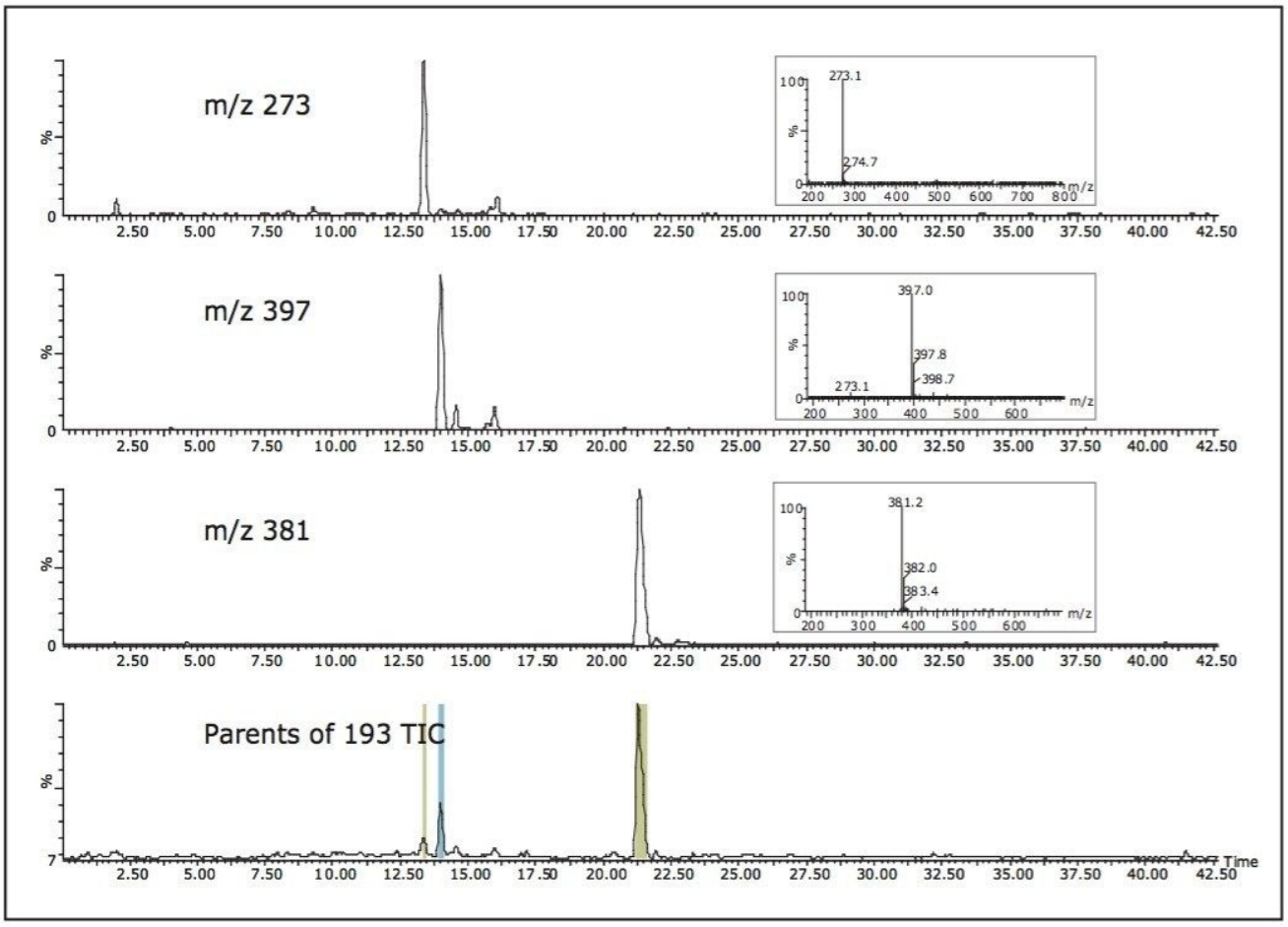

Figure 10. Fractionation based on the precursor ions of the m/z 193 TIC acquisition.

Additional Collection Options

The ESCi multi-mode ionization source enables both ESI +/- and APCI +/- acquisition to occur within the same run. This allows for fraction collection to be triggered from any of the acquisition channels, thus proving useful if the metabolites require different ionization modes. Prior to this enabling technology, the only options for collection would be to split the sample and run in different modes, or rely upon time-based fractionation and then analyze all the fractions by both modes to determine the targets.

The selectivity of the ESCi-enabled fraction collection process can be further enhanced by the use of mixed triggers. This approach uses Boolean logic strings to trigger collection from multiple data traces (e.g., collection can occur only when Mass A is present and Mass B is not, or a peak has to be present in two different traces at

the same time for fractionation).

Conclusion

Fraction collection with a tandem quadrupole mass spectrometer is now possible using four different modes of data acquisition: scan, MRM, constant neutral loss, and precursor ion, which enables improved versatility for triggering options.

- Scan mode has the potential to increase the number of isobaric inferences detected and collected.
- MRM mode is the most selective because it only monitors a specific precursor/product ion transition and greatly reduces the isobaric interferences, but requires previous knowledge of the transition.
- Constant neutral loss mode can be used for collecting a class of compounds with a target-specific loss or a generic group loss for a broader study, or can be used as a second filter where the target has to have a specific mass and the neutral loss.
- Collection in precursor ion mode allows for all the precursors with a specific product ion to be collected, which is valuable when the metabolites are unknown, but there is a common fragment of the core compound that can be detected.

Thus, these different modes of collection add value to a wide variety of applications previously accomplished with more laborious, time consuming, and less specific methodologies.

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