IROA TECHNOLOGIES
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# IROA-based metabolomics

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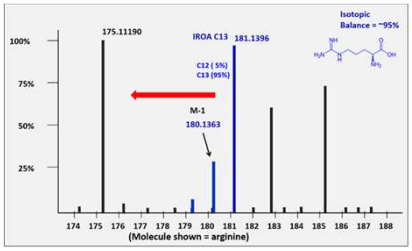
## What is IROA?

**The IROA (Isotopic Ratio Outlier Analysis) protocols embed specific chemical characteristics into the mass spectral data stream in the form of mathematically definable isotopic patterns.**

**This information is used to retrieve higher quality data, with reduced error, and lowest possible experimental variance when the IROA peak is a constant or Internal Standard.**

Phenotypic IROA peak ->



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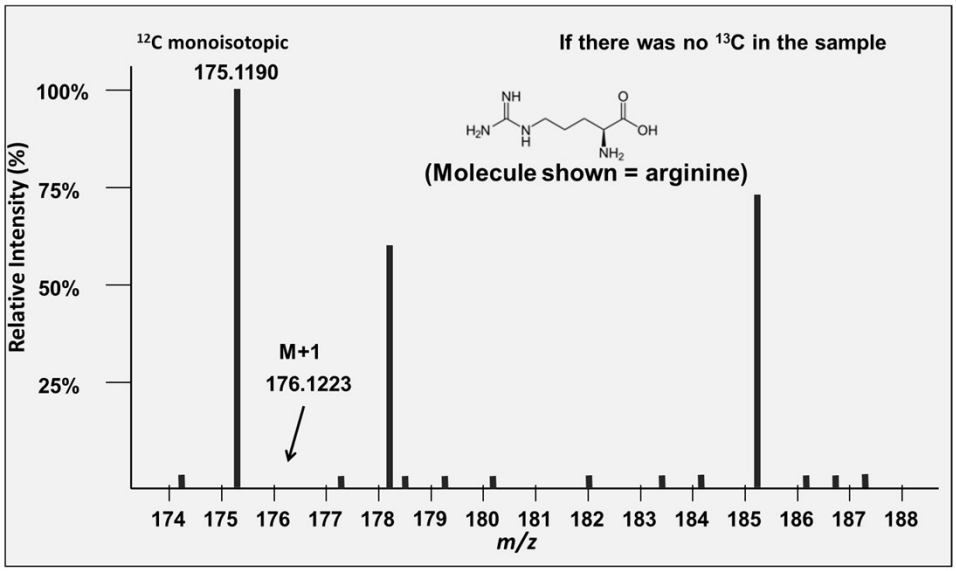
# Creating Isotopic Patterns

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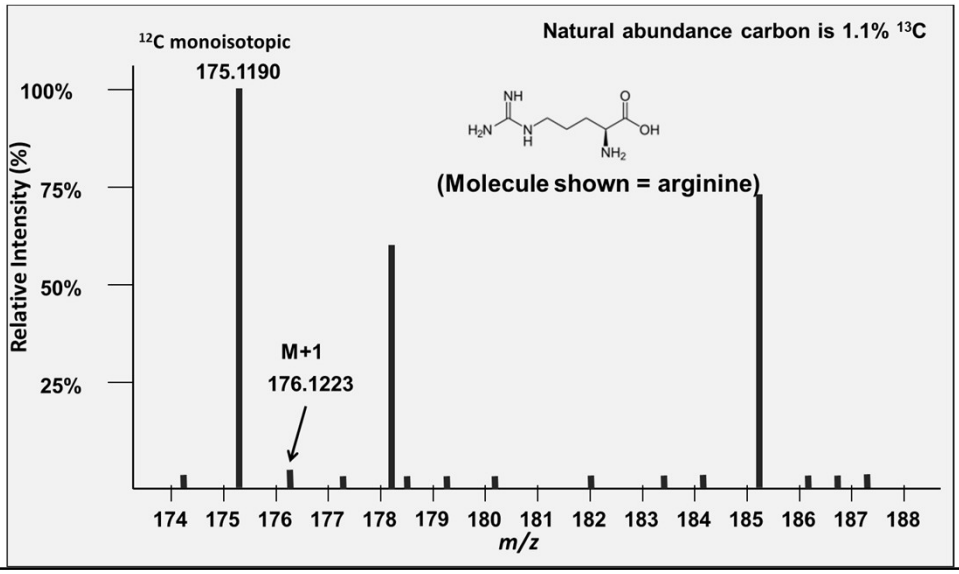
# Creating Isotopic Patterns



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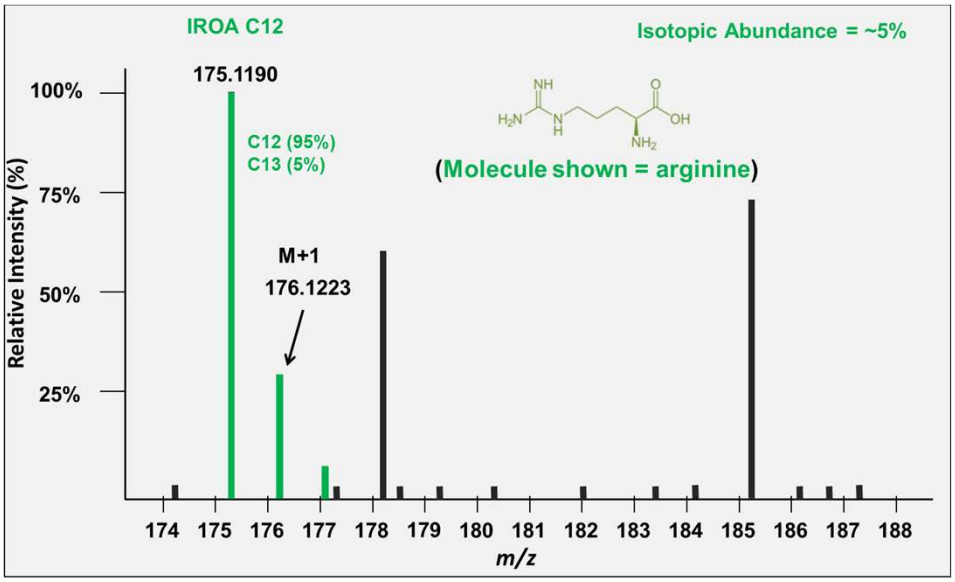
# Creating Isotopic Patterns – C13 (5%)

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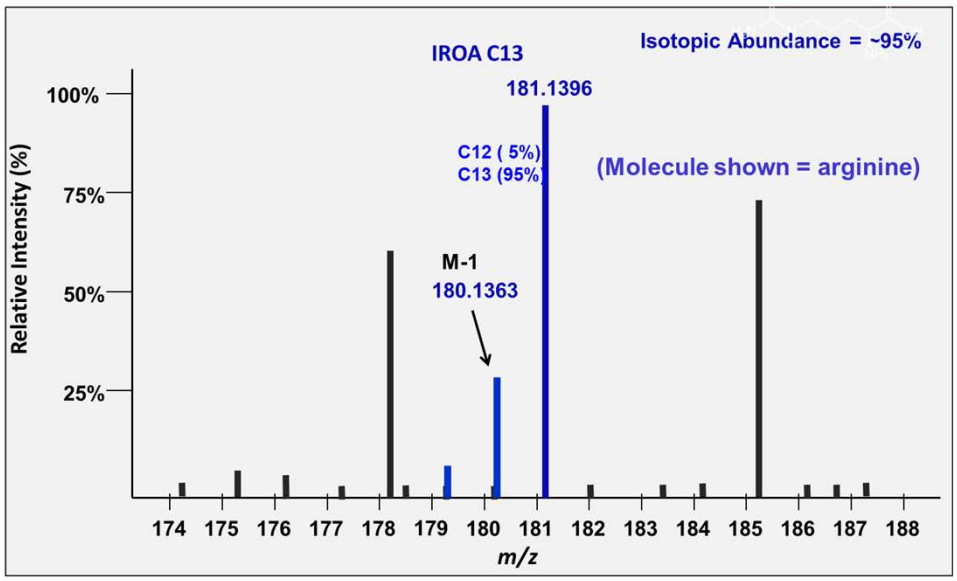
# Creating Isotopic Patterns - C13 (95%)



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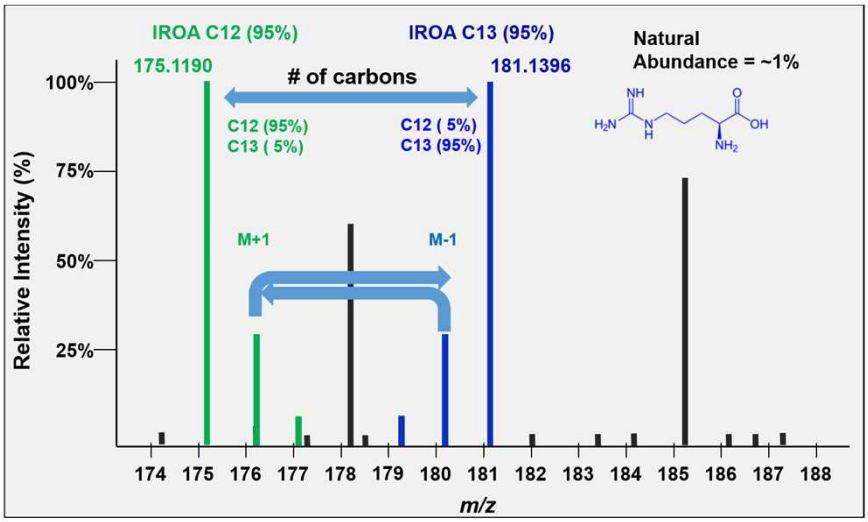
## The IROA Peaks




**This comprises a triply-redundant information system.**

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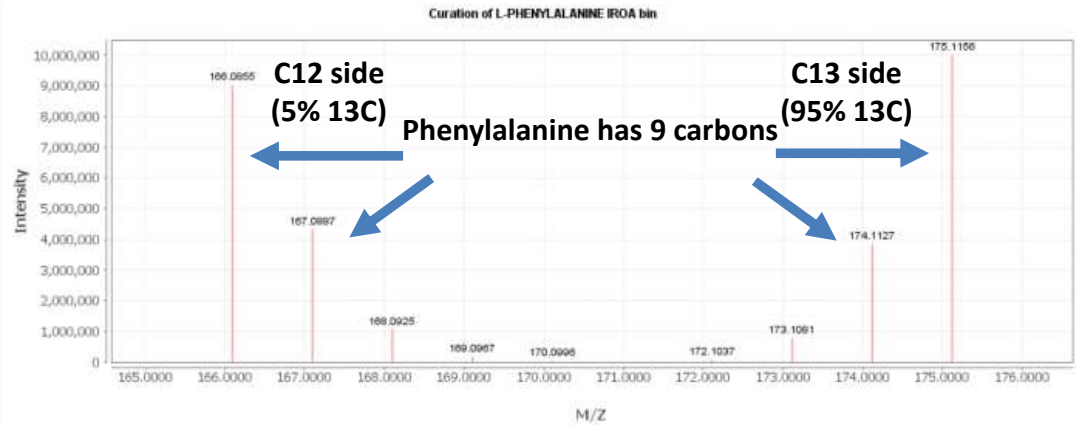
## Anatomy of the IROA Signal




**The IROA signal is made up of two halves.**
**Together they make a complete picture.**
**<u>Only a molecule of biological origin can show this signal.</u>**

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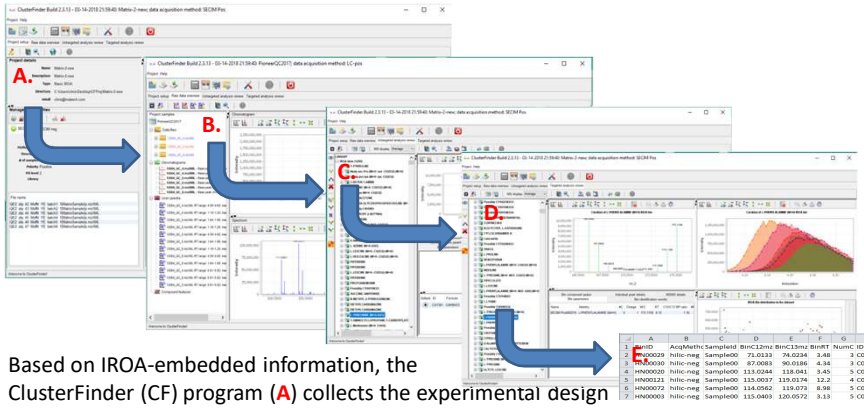
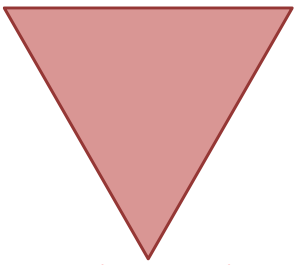
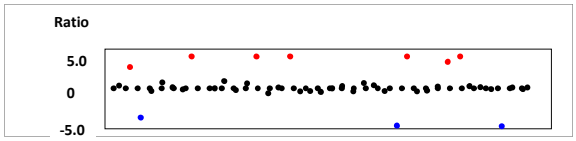
# Automated Data Reduction




Based on IROA-embedded information, the ClusterFinder (CF) program (**A**) collects the experimental design and data, (**B**) provides tools to examine the spectra, performs an automated non-targeted analysis (**C**), and a targeted analysis (**D**), and produces a detailed output (**E**).

CF handles variance control, data reduction, noise removal, data definition, formula assignment, quantitation (by area, height, and ratio), and peak correlation of adducts and fragments.

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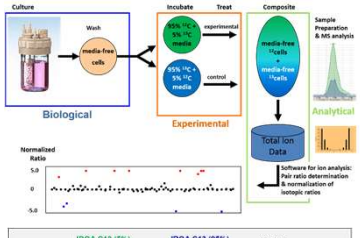
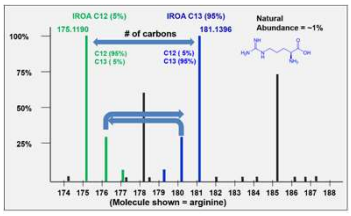
# Three IROA Protocols

## The Basic IROA Protocol

For R&D: labeled isotopomer-paired control and experimental samples; untargeted analysis

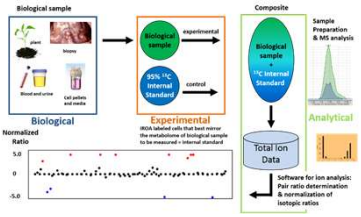


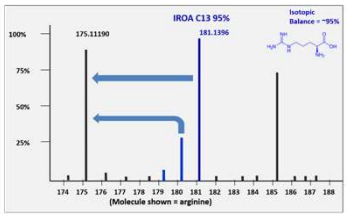




Triply redundant peak info

## The Phenotypic IROA Protocol

For clinical and molecular diagnostics: labeled Internal Standards; targeted analysis for 100s of compounds

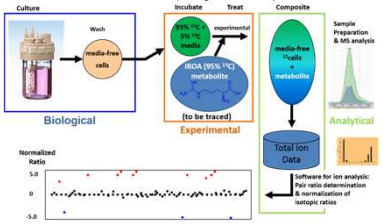


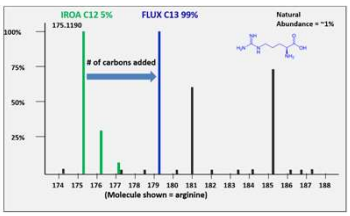




Redundant peak info

## The Fluxomic IROA Protocol

For medical flux studies; labeled experimental samples and tracer metabolites; targeted analysis




Nuclear mass spacing

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# IROA TruQuant IQQ

## Identify, Quantify, Qualify

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## What is TruQuant/IQQ

IROA TruQuant/IQQ is a high-quality quantitation system for making accurate biological measurements on several hundred biochemicals simultaneously in small quantities of biological samples.

This is achieved by spiking a complex IROA Internal Standard (**IROA-IS**) into a biological sample and 1) quantifying all of the biochemicals in the sample relative to their counterparts in the IROA-IS, 2) suppression-correction of each compound, and 3) normalization of sample to sample variances are determined through the **IROA-IS**.

Day-to-day reproducibility (QA/QC), and chemical identification are determined using a Long-Term Reference Standard (**IROA-LTRS**) that is always the same and is used assure reproducible instrument performance.

The system is completely automated using IROA ClusterFinder software.

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## IROA TruQuant/IQQ

**The IROA TruQuant / IQQ workflow uses 2 Standards: IROA-LTRS and IROA-IS**

**IROA-LTRS is a "Long Term Reference Standard" - run daily every 10 to 12 injections**

- **Yeast extract fully-labeled at both 95% C13 and 5% C13; mixed 1:1**
- ***Provides the "I" and "Q" of IQQ; assured Identity, and Quality (complete QA/QC)***

**IROA-IS is an "Internal Standard" - spiked into experimental samples**

- **Same fully-labeled 95% C13 (only) yeast extract as the LTRS, at the same concentration**
- **Provides the 2nd "Q" of IQQ; Quantitation**

**The IROA TruQuant workflow combines 2 Protocols**

**Protocol # 1 is the IROA Basic Protocol - used with the IROA-LTRS Standard**

**Protocol # 2 is the IROA Phenotypic Protocol - used with samples with added IS**

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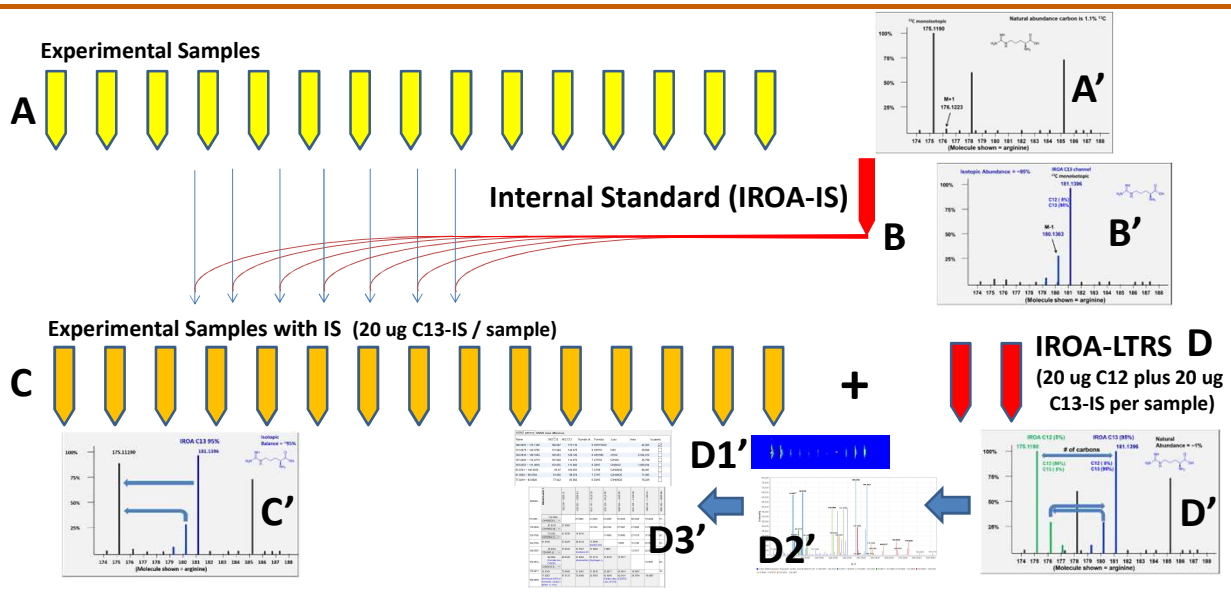
## IROA-based Workflow



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# TruQuant IQQ

## IDENTIFICATION* IS BASED ON THE IROA-LTRS

***using both IROA ms characteristics, and secondary IROA (ms/ms, IMS, etc.) assures identity is correct.**

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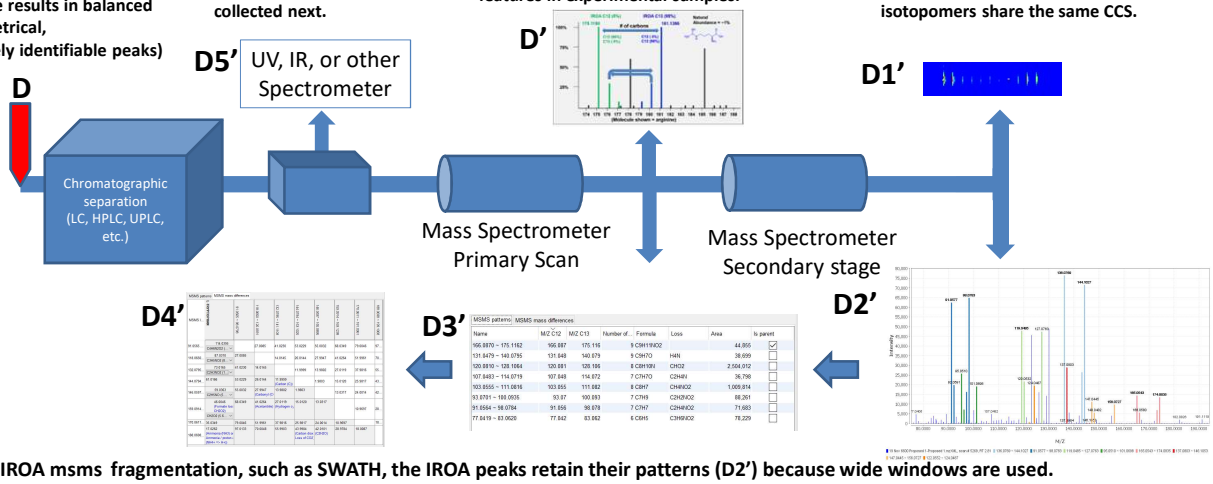
## Compound Identification in the IROA Matrix

**IROA-LTRS**
**(Contains 20 ug 5% C13 plus 20 ug 95% C13-IROA-IS per sample results in balanced symmetrical, uniquely identifiable peaks)**

**The basic chemical attributes lends support to the mass spectral characteristics collected next.**

**The triply redundant IROA Basic peak in ms provides formula, retention time, and all of the basic spectral features needed to find the same features in experimental samples.**

**In IROA Ion Mobility the IROA IM peaks retain their patterns perfectly because all IROA isotopomers share the same CCS.**




**In IROA msms fragmentation, such as SWATH, the IROA peaks retain their patterns (D2') because wide windows are used. Since all fragments retain their IROA character, their formulae (D3') and the relationships between them (D4') are determinable.**

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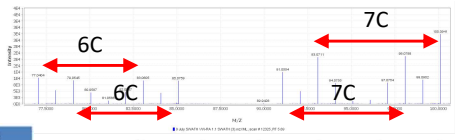
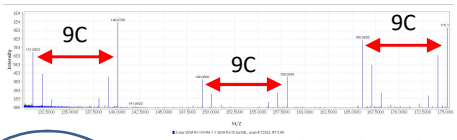
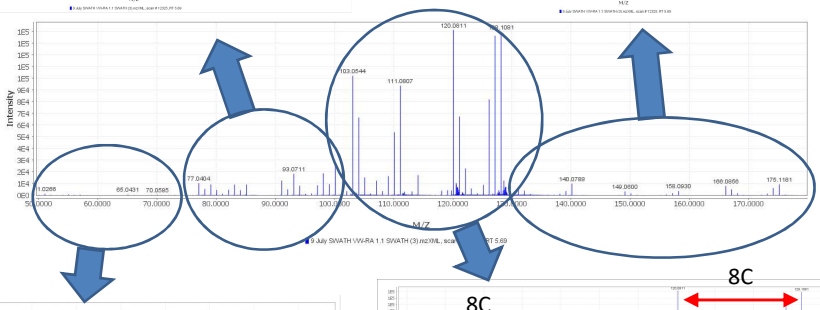
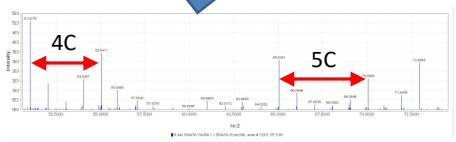
# Paired Peaks (ms)




| IROA pair | Number of carbons | Formula |
|---|---|---|
| 166.0855 – 175.1181 | 9 | C9H11NO2 |
| 149.0600 – 158.0930 | 9 | C9H8O2 |
| 131.0503 – 140.0805 | 9 | C9H6O |
| 120.0824 – 128.1092 | 8 | C8H9N |
| 107.0511 – 114.0746 | 7 | C7H6O |
| 103.0561 – 111.0829 | 8 | C8H6 |
| 93.0717 – 100.0952 | 7 | C7H8 |
| 91.0561 – 98.0796 | 7 | C7H6 |
| 79.0545 – 85.0746 | 6 | C6H6 |
| 77.0414 – 83.0615 | 6 | C6H4 |
| 65.0431 – 70.0599 | 5 | C5H4 |
| 51.0276 – 55.0276 | 4 | C4H2 |





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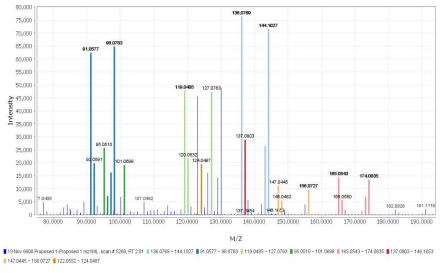
# ms/ms Compound Identification




**In IROA msms fragmentation, such as SWATH, the IROA peaks retain their patterns because wide windows are used.**

**Since all fragments retain their IROA character, their formulae and the relationships between them are determinable, supporting both identification and structure elucidation. Noise peaks are excluded.**

MSMS patterns | MSMS mass differences

| Name | M/Z C12 | M/Z C13 | Number of... | Formula | Loss | Area | Is parent |
|---|---|---|---|---|---|---|---|
| 166.0870 ~ 175.1162 | 166.087 | 175.116 | 9 | C9H11NO2 | | 44,855 | ☑ |
| 131.0479 ~ 140.0795 | 131.048 | 140.079 | 9 | C9H7O | H4N | 38,699 | ☐ |
| 120.0810 ~ 128.1064 | 120.081 | 128.106 | 8 | C8H10N | CHO2 | 2,504,012 | ☐ |
| 107.0483 ~ 114.0719 | 107.048 | 114.072 | 7 | C7H7O | C2H4N | 36,798 | ☐ |
| 103.0555 ~ 111.0816 | 103.055 | 111.082 | 8 | C8H7 | CH4NO2 | 1,009,814 | ☐ |
| 93.0701 ~ 100.0935 | 93.07 | 100.093 | 7 | C7H9 | C2H2NO2 | 88,261 | ☐ |
| 91.0564 ~ 98.0784 | 91.056 | 98.078 | 7 | C7H7 | C2H4NO2 | 71,683 | ☐ |
| 77.0419 ~ 83.0620 | 77.042 | 83.062 | 6 | C6H5 | C3H6NO2 | 78,229 | ☐ |

MSMS patterns | MSMS mass differences

| MSMS I... L-TRYPTOPHAN | 91.0565 ~ 98.0798 | 118.0650 ~ 125.0831 | 132.0795 ~ 141.1160 | 144.0794 ~ 153.1325 | 146.0597 ~ 155.1296 | 159.0914 ~ 168.1381 | 170.0611 ~ 181.1563 | 188.0698 ~ 199.1683 |
|---|---|---|---|---|---|---|---|---|
| 91.0565 ... | 114.0395 C4H5N2O2 (... | | 27.0085 | 41.0230 | 53.0229 | 55.0032 | 68.0349 | 79.0046 | 97... |
| 118.0650... | 87.0310 C3H5NO2 (8... | 27.0085 | | 14.0145 | 26.0144 | 27.9947 | 41.0264 | 51.9961 | 70... |
| 132.0795... | 73.0165 C2H3NO2 (1... | 41.0230 | 14.0145 | | 11.9999 | 13.9802 | 27.0119 | 37.9816 | 55... |
| 144.0794... | 61.0166 | 53.0229 | 26.0144 | 11.9999 (Carbon (C)) | | 1.9803 | 15.0120 | 25.9817 | 43... |
| 146.0597... | 59.0363 C2H5NO (5... | 55.0032 | 27.9947 (Carbonyl (O | 13.9802 | 1.9803 | | 13.0317 | 24.0014 | 42... |
| 159.0914... | 46.0046 (Formate loss CH2O2) CH2O2 (5.6... | 68.0349 | 41.0264 (Acetonitrile) | 27.0119 (Hydrogen c) | 15.0120 | 13.0317 | | 10.9697 | 28... |
| 170.0611... | 35.0349 | 79.0046 | 51.9961 | 37.9816 | 25.9817 | 24.0014 | 10.9697 | | 18... |
| 188.0698... | 17.0262 (Ammonia (NH3) o Ammonia / proton ( (NH4+ => H+)) | 97.0133 | 70.0048 | 55.9903 | 43.9904 (Carbon dioxi Loss of CO2 | 42.0101 (C2H2O) | 28.9784 | 18.0087 | |

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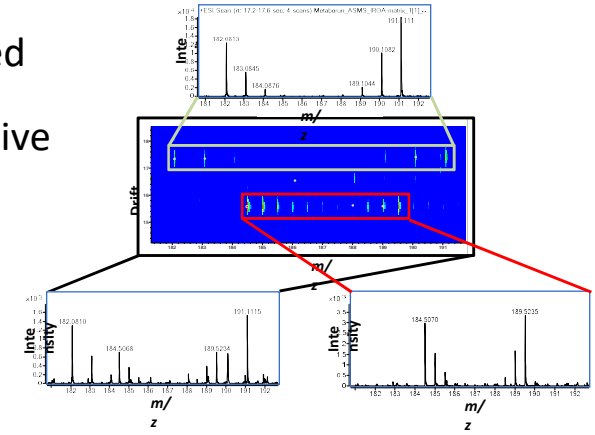
## IM Compound Identification

- Because all of the isotopomeric peaks in an IROA cluster share the same CCS they have identical Ion Mobility behavior
- Coeluting IROA peaks are separated
- Each compound retains its distinctive CCS which confirms the IROA MS-determined identity

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# IQQ

## QA/QC* IS BASED ON THE IROA-LTRS

***The reproducibility of the LTRS may be measured many ways.**

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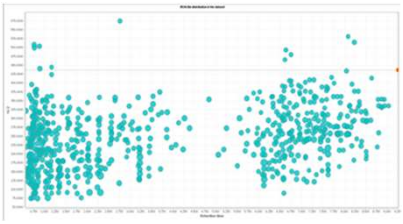
## Characterizing IROA-LTRS - Qualification

- The **IROA-LTRS** is injected (randomly) every 10 to 12 injections.
- Measurements made in every **IROA-LTRS** sample relative to instrument performance include:
  - Number of IROA paired peaks seen ✔ QA – sensitivity
  - Retention time for each compound ✔ QA – chromatography
  - Relative strength of signal/compound ✔ QA - in-source fragmentation activity
  - Total signal found for all compounds ✔ QA - injection accuracy

The distribution of standard compounds in the LTRS ->



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# IQQ

## QUANTITATION* IS BASED ON THE INTERNAL STANDARD IROA-IS

*** As a phenotypic sample, the quality of the measurement benefits from the redundancy of the IROA peaks in the IS. Measurements may be made either on the raw data, suppression-corrected data, or fully normalized data.**

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# Proof of Normalization and Suppression-correction

In order to prove the ability to correct for suppression and then use suppression-corrected values to normalize samples against one another the following experiment was defined:

- A large homogeneous single extract of plasma was made.
- Varying quantities of the extract were delivered as individual samples (in triplicate).
- Samples were dried and resolvated in a constant volume of the **IROA-IS**.
- Samples (and **IROA-LTRS**) were MS analyzed and the IROA peaks examined.

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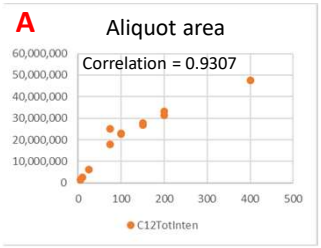
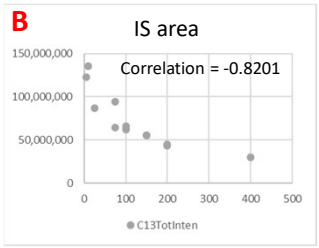
# IROA Ratios Overcome Suppression

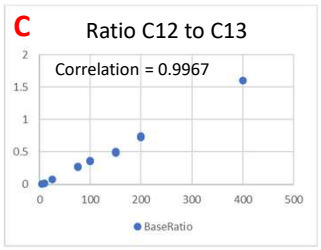
Variable aliquots (ranging from 5 ul to 400 ul) of a plasma sample were dispensed and dried. Each aliquot was resolubilized in an equal quantity of Internal Standard (IS). Aliquots were prepared in triplicate.

A strong suppression is seen for the compound (A) is shown (and its IROA-IS isotopologue-B). Both are non-linear, indicating any quantitation based on these measurements will be inaccurate.

However, the C12/C13 (area of sample/area of IS) ratio (C) for the compound present is perfectly linear.



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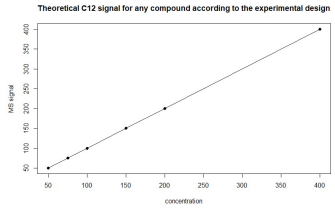
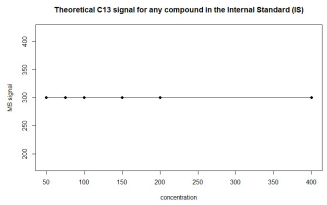
# Suppression (expected vs. observed)

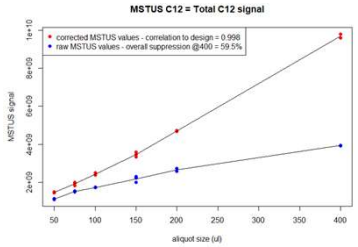
**A = analytes expected**



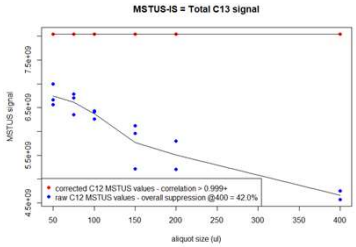

**B = standards expected**




**C = analytes seen (blue) and corrected (red)**




**D = standards seen (blue) and corrected (red)**

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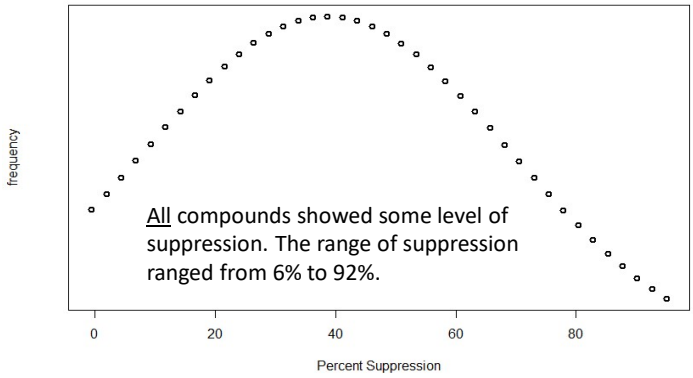
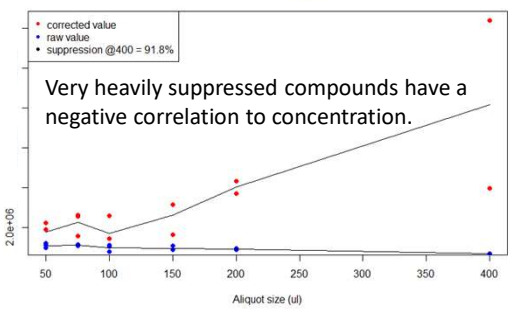
# All Compounds are Suppressed

**Distributions of suppression seen in this experiment**




**C9H16N2O5 [266] SECIM.Pos000428**



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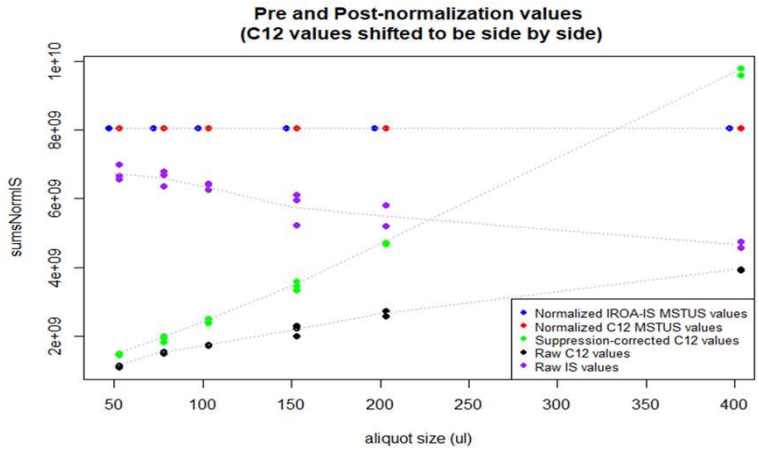
# Normalization (following suppression-correction)

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# Summary

- IROA TruQuant provides a robust system for reproducible peak identification, peak quantitation, and a daily QA/QC for instrument performance and process control.
- The **IROA-LTRS** is a Long Term Reference Standard that directly provides continuous run-time performance characteristics.
- The **IROA-LTRS** allows both inter- and intra- cross-platform comparisons.
- The **IROA-LTRS** is the basis of a validated chemical identification system for between 500 and 1000 compounds.
- The **IROA-IS** contains all of the compounds in the **IROA-LTRS** at exactly the same concentration, and provides for reproducible quantitation.
- The **IROA-IS** provides a mechanism for suppression-correction, and sample-to-sample normalization.

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# Thank You!

# Any Questions?