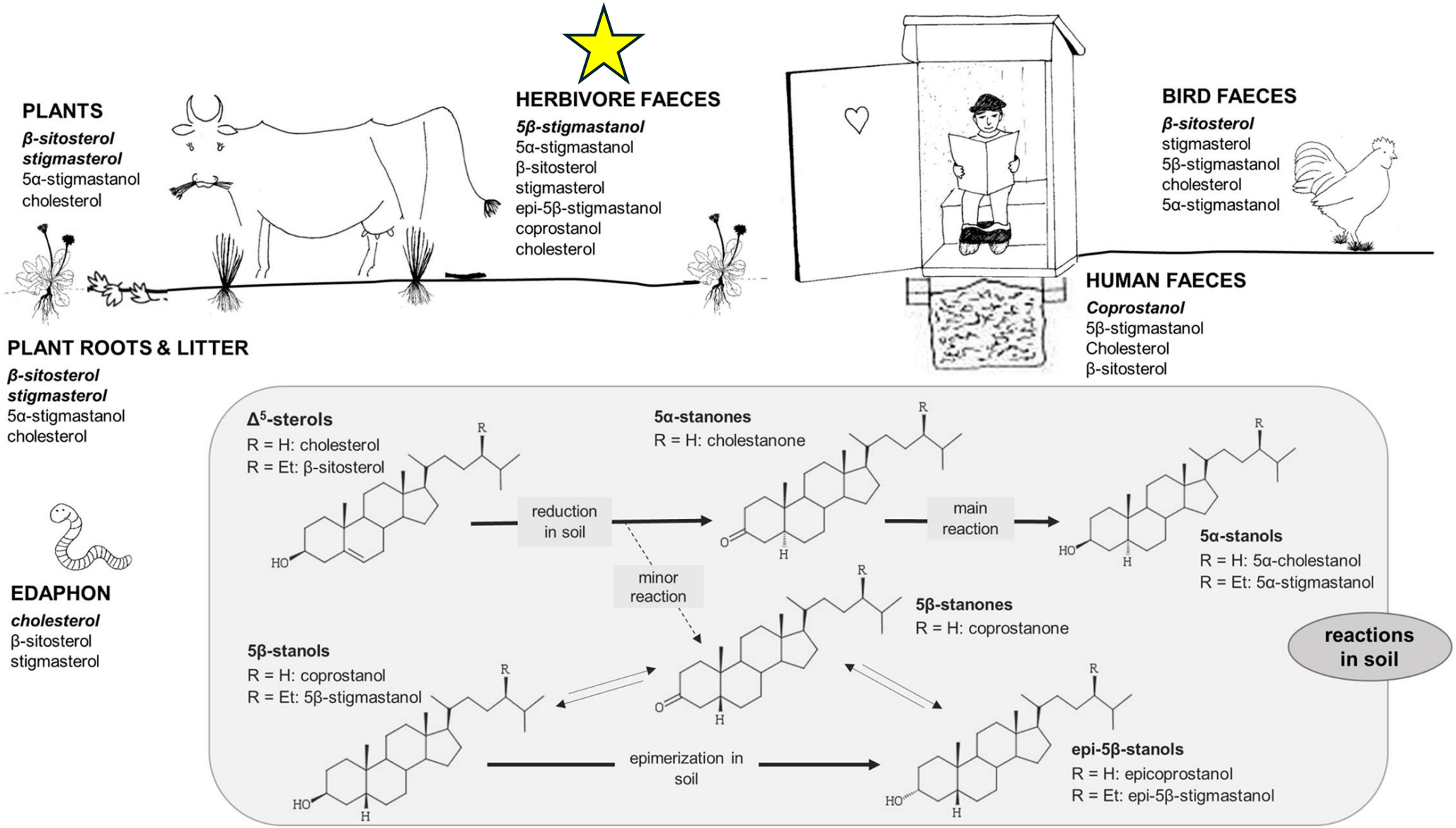
Compound-Specific Carbon Isotope Measurements of Fecal Steroids

A Potential Proxy for Herbivore Dietary Shifts

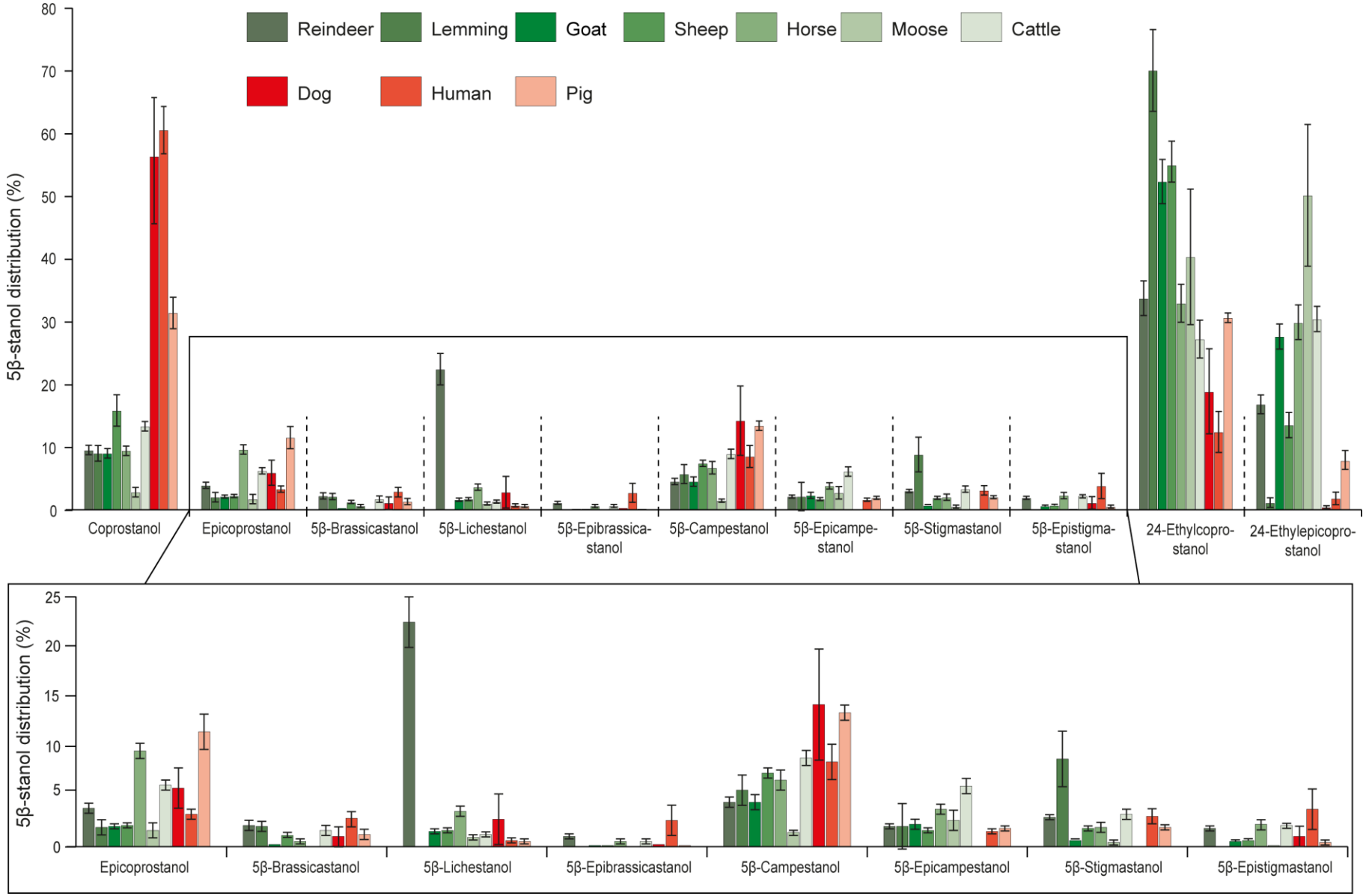
Alessandro A. Mauceri, Joel O. Abraham, James M. Russell, A. Carla Staver, Corli Coetsee, Tercia Strydom, and A. Tyler Karp

What are fecal steroids?



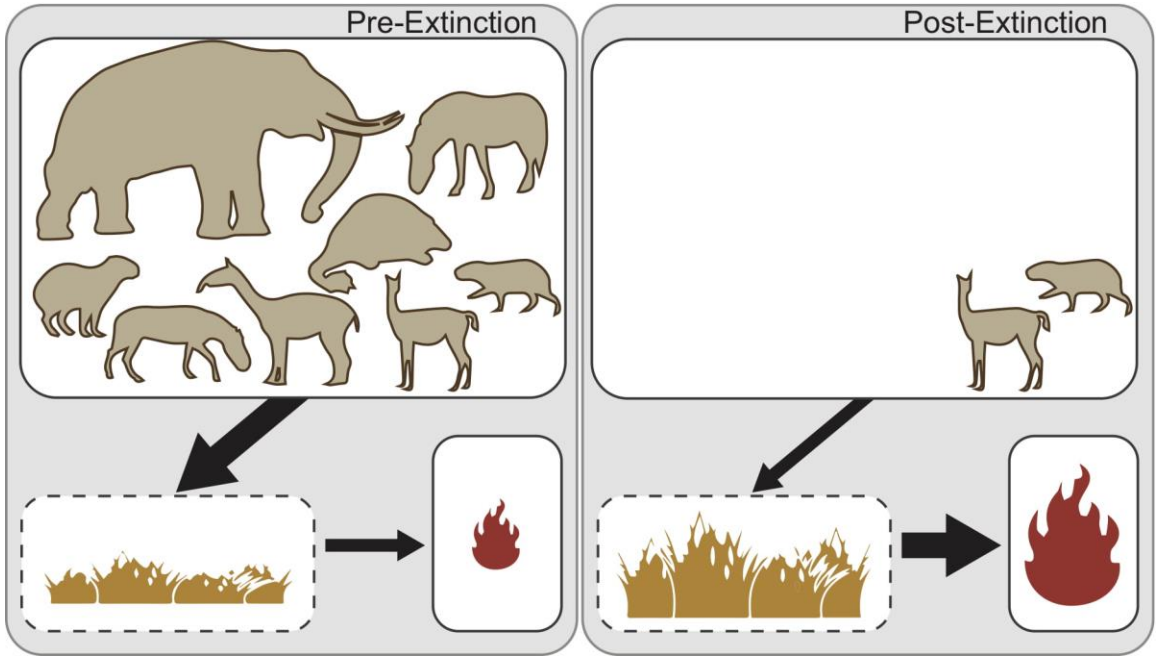
Prost et al. (2017), *PloS One*

Fecal steroid fingerprints differ across species



Harrault et al. (2019), *PloS One*

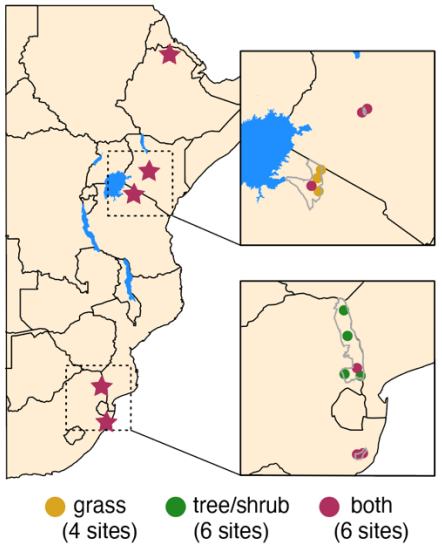
Herbivores: Grazers & Browsers

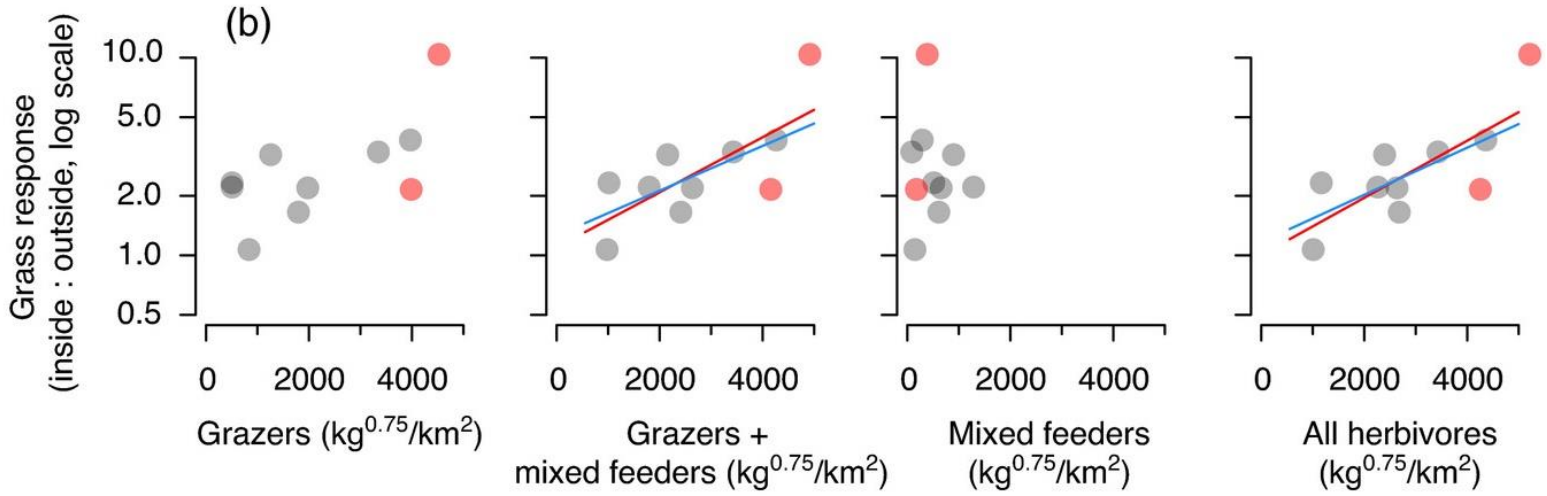


Left: Photo Credit to A. Tyler Karp
Top: Karp et al. (2021), *Science*

↑ Grazing Animals = ↑ Grass Impacts

Synthesis of exclusion experiments in East Africa

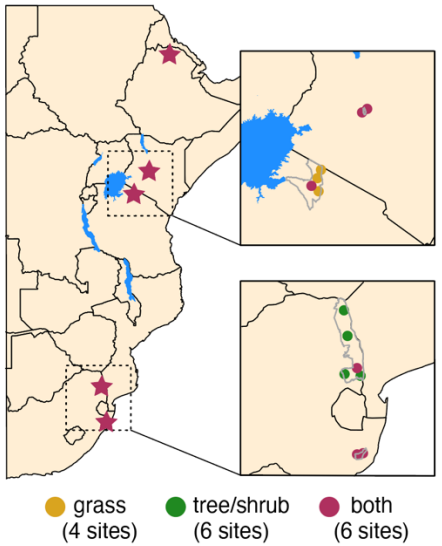


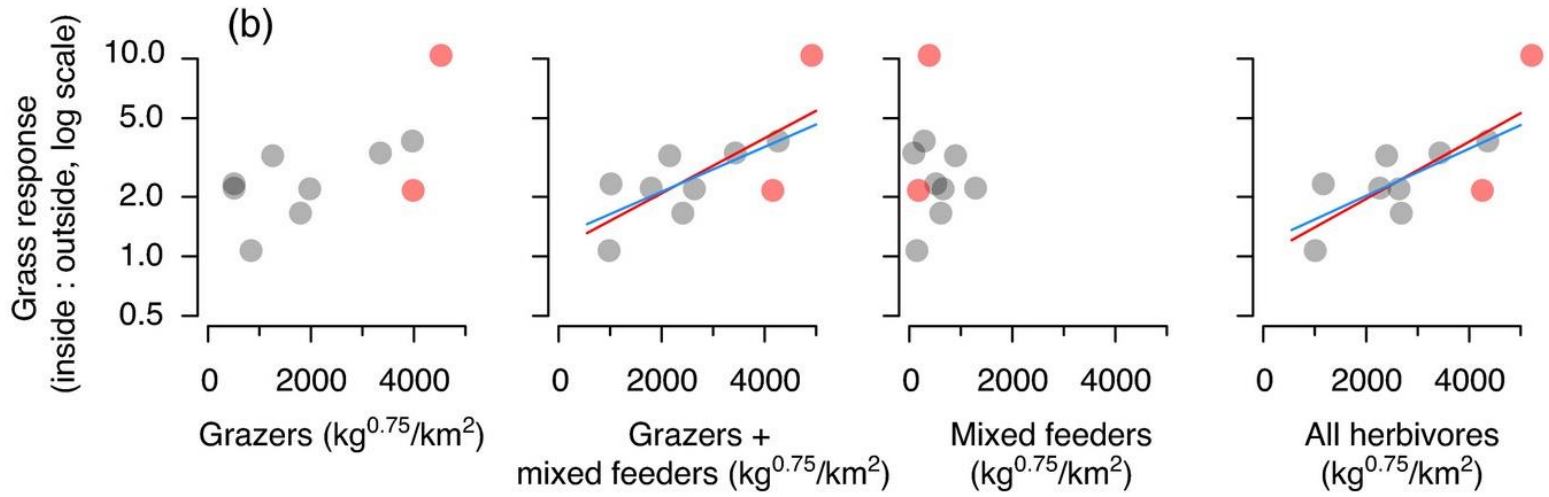


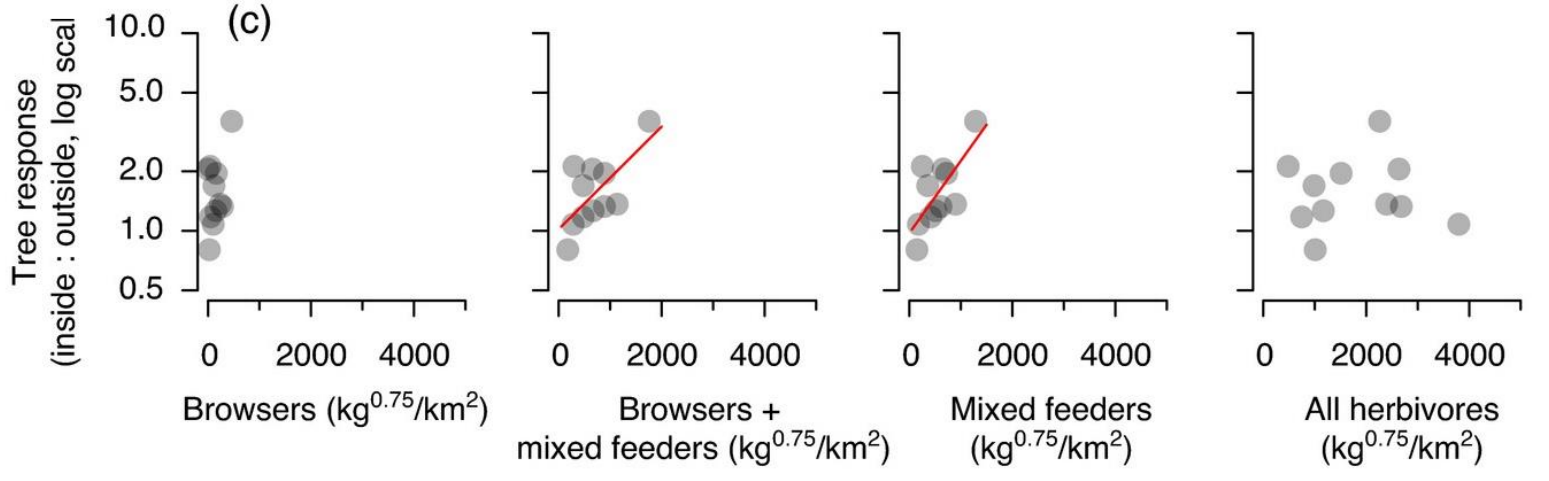
Staver et al. (2021), *Journal of Ecology*

↑ Grazing Animals = ↑ Grass Impacts
↑ Browsing Animals = ↑ Tree Impacts

Synthesis of exclusion experiments in East Africa

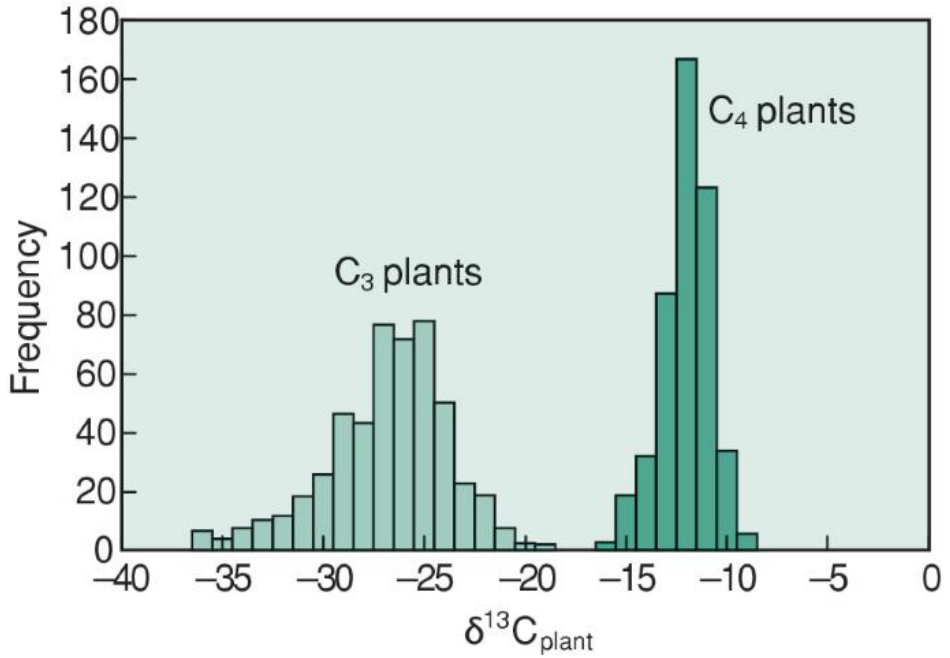






Staver et al. (2021), *Journal of Ecology*

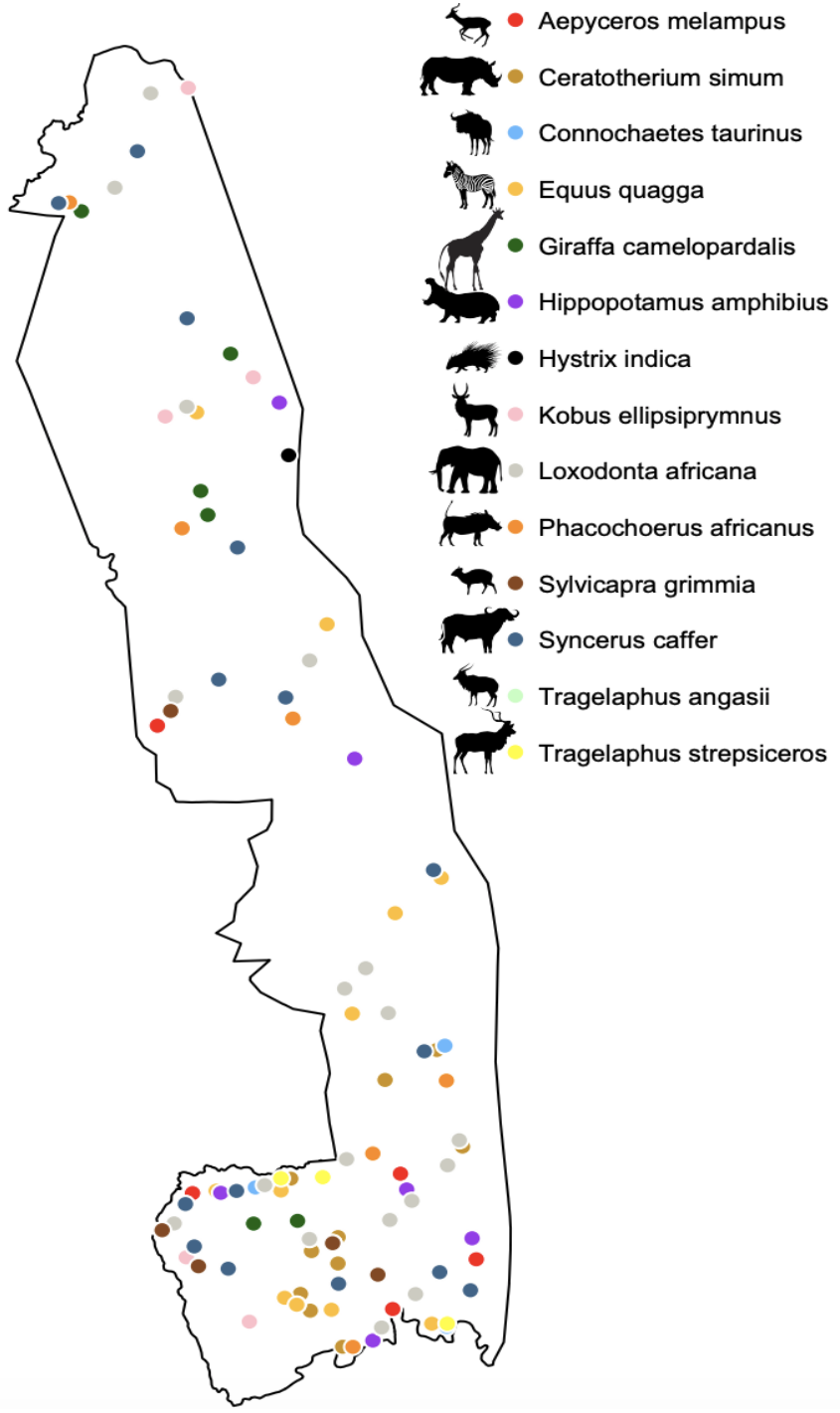
Impacts only related to herbivore that eats that type of vegetation!



Hoefs, (2021), *Stable Isotope Geochemistry*

Study Site:
Kruger National Park, ZA

- Species-rich community of large herbivores that represent a diversity of dietary habits.
- Opportunistic sampling along roads and from within vegetation monitoring sites. Only fresh, wet samples were collected.
- Provisional dung identification was performed in field via context morphology and other visual characteristics → provenance confirmed via DNA metabarcoding.



Sample Information

- 28 dung samples representing 13 herbivore species → browsers, mixed feeders, and grazers.
- Bulk $\delta^{13}C$ measurements informed preliminary samples for CSIA analysis.

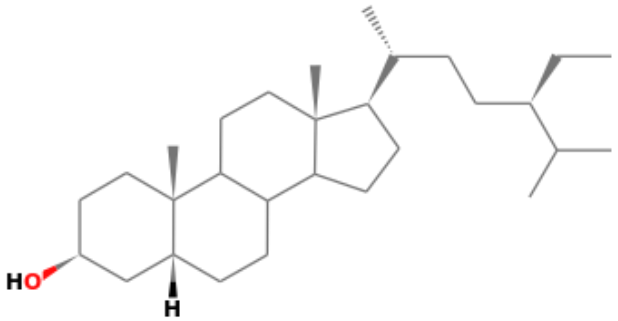
Objectives

- Optimize a GC-C-IRMS method to achieve chromatographic separation and compound-specific $\delta^{13}C$ analysis of fecal steroids.
- Determine whether fecal steroid $\delta^{13}C$ values accurately reflect C_3, C_4, and mixed-feeding herbivore diets.
- Evaluate the feasibility of developing an isotope-based fecal steroid proxy for reconstructing past herbivore dietary shifts from sedimentary archives.

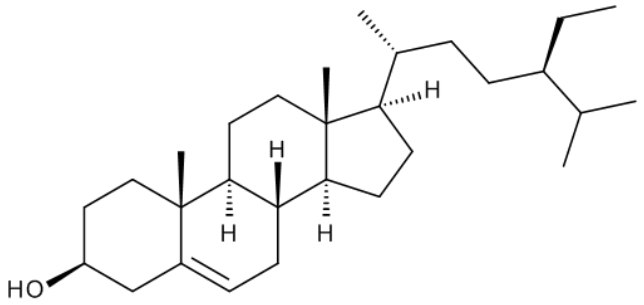
Target Compounds

- Fecal Steroids:
  - Epistigmastenol (24-ethyl-5ß-cholest-22-en-3α-ol)
  - 5ß-Stigmastanol (24-ethyl-5ß-cholestane-3ß-ol)
  - Epistigmastanol (24-ethyl-5ß-cholestane-3α-ol)
- Plant Steroids:
  - ß-Sitosterol
  - 5α-Stigmastanol (24ß-ethyl-5α-cholestan-3ß-ol)

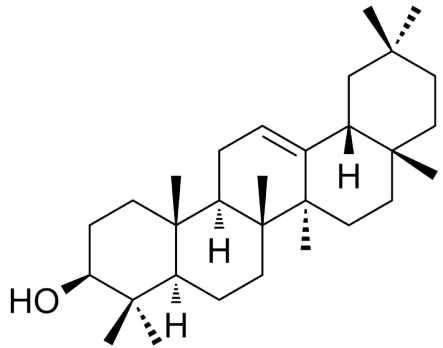
5ß-Stigmastanol



ß-Sitosterol

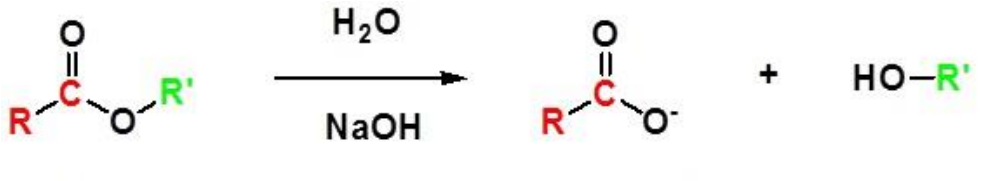


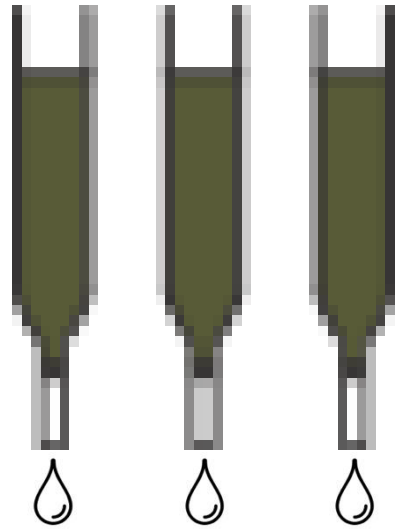
ß-Amyrin



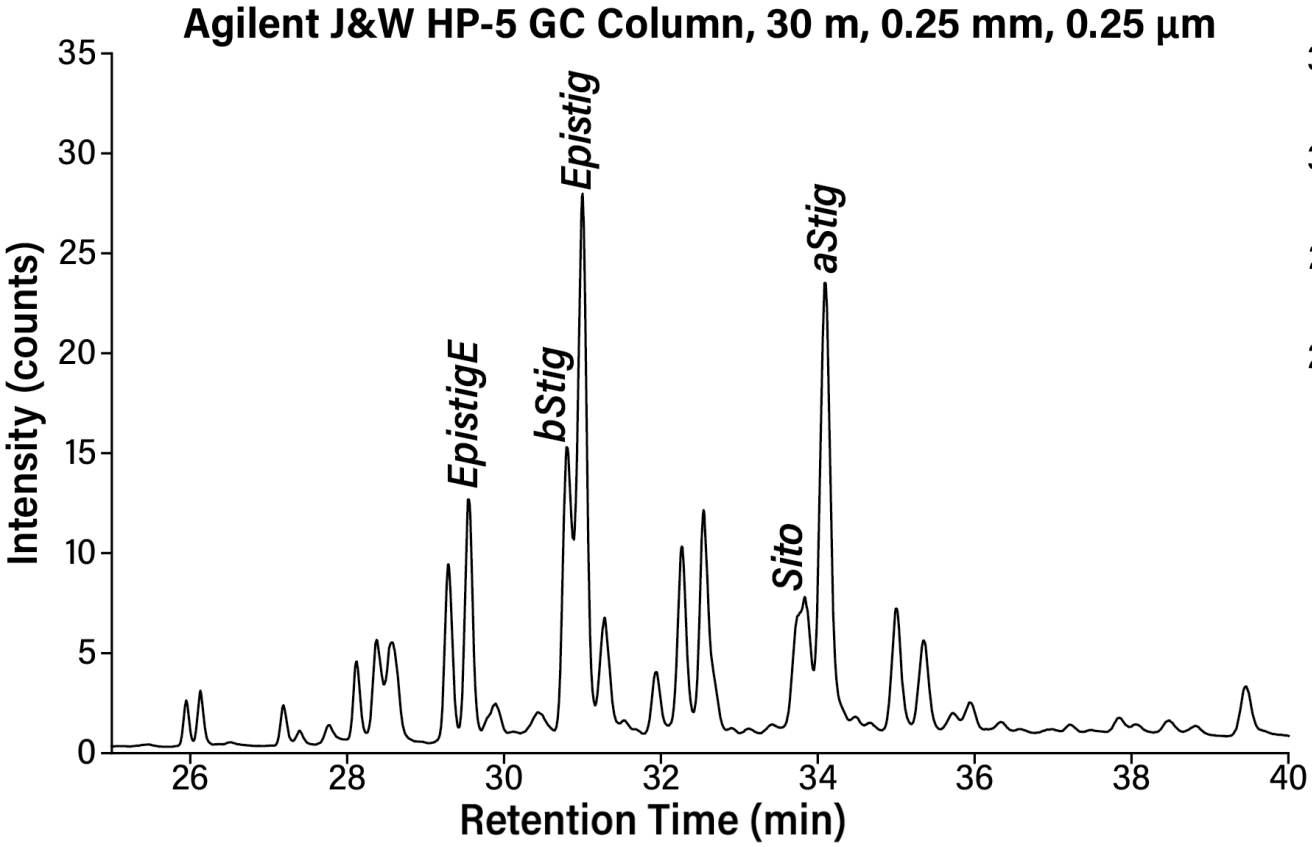
Extraction, Saponification, & Purification

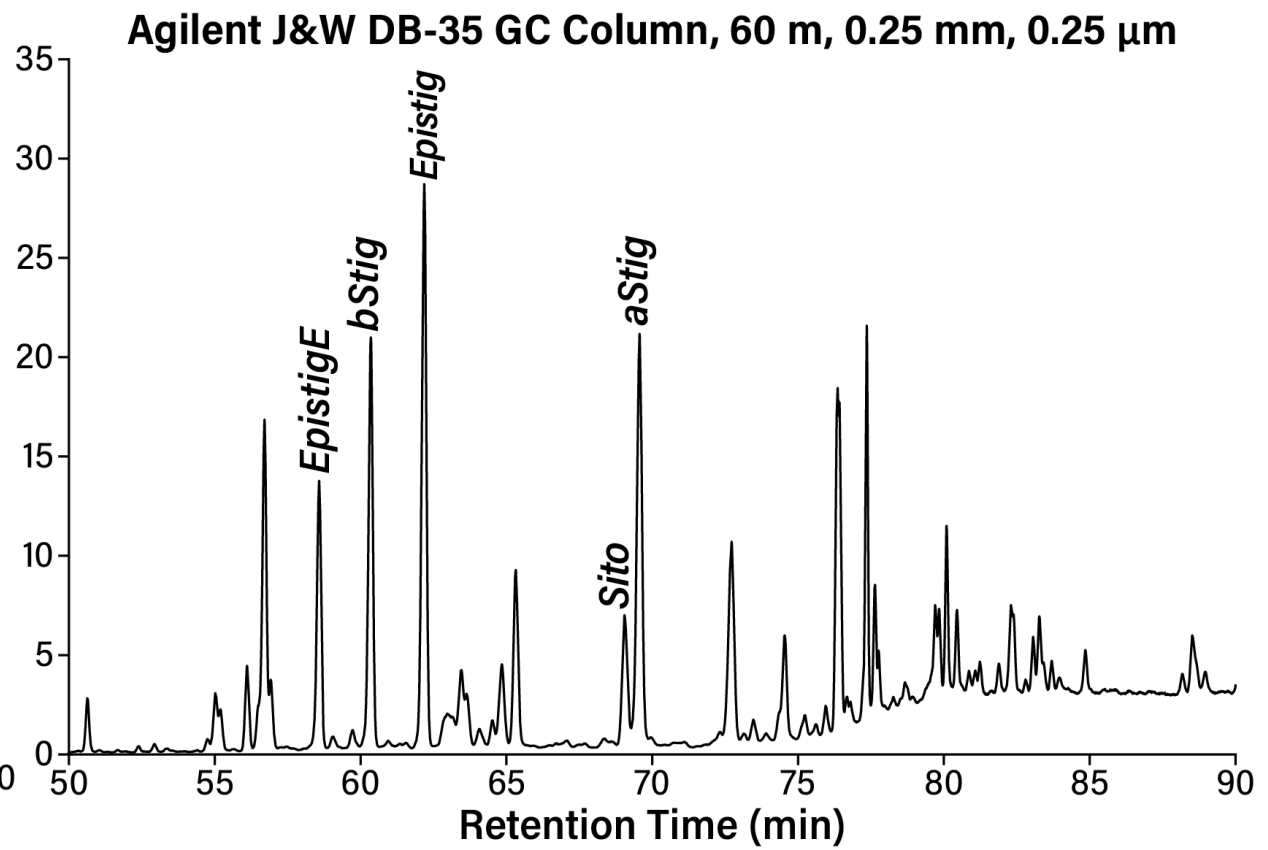
- Extraction via Dionex™ ASE350 using 9:1 DCM:MeOH.
- TLEs were dried and saponified with a 1M KOH solution in 95:5 MeOH:H_2O for three hours at 65°C → $NaSO_4$ columns to remove residual water.
- Three-fraction silica gel flash columns for purification, using hexane, DCM, and MeOH.



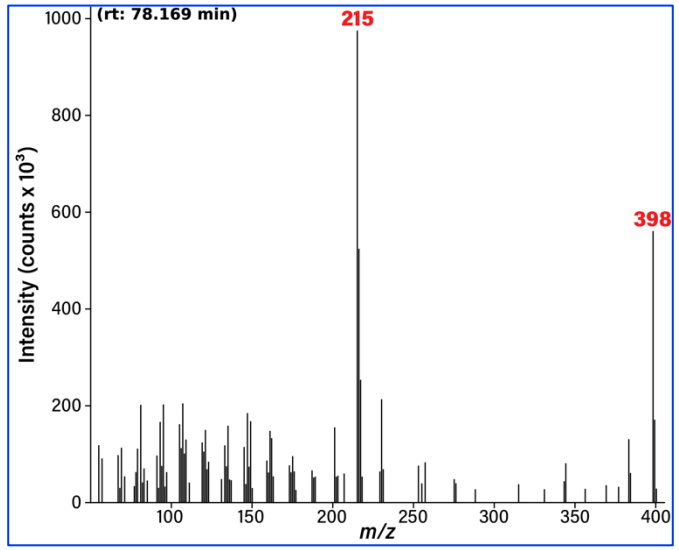


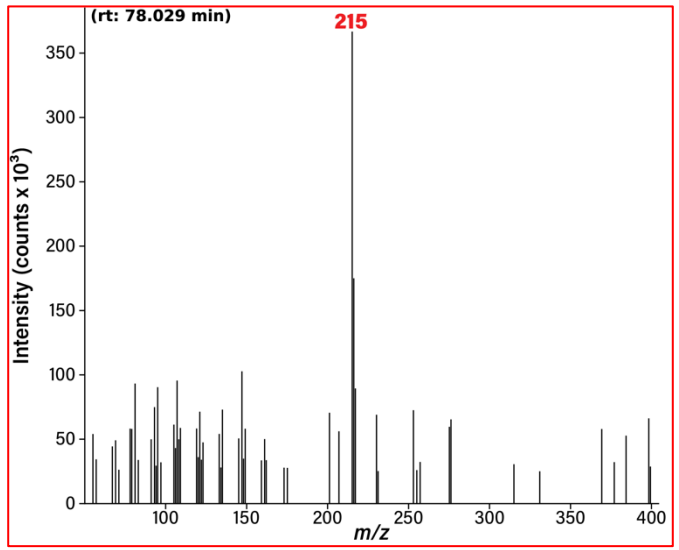
DB-35 column successfully separates fecal steroids of interest

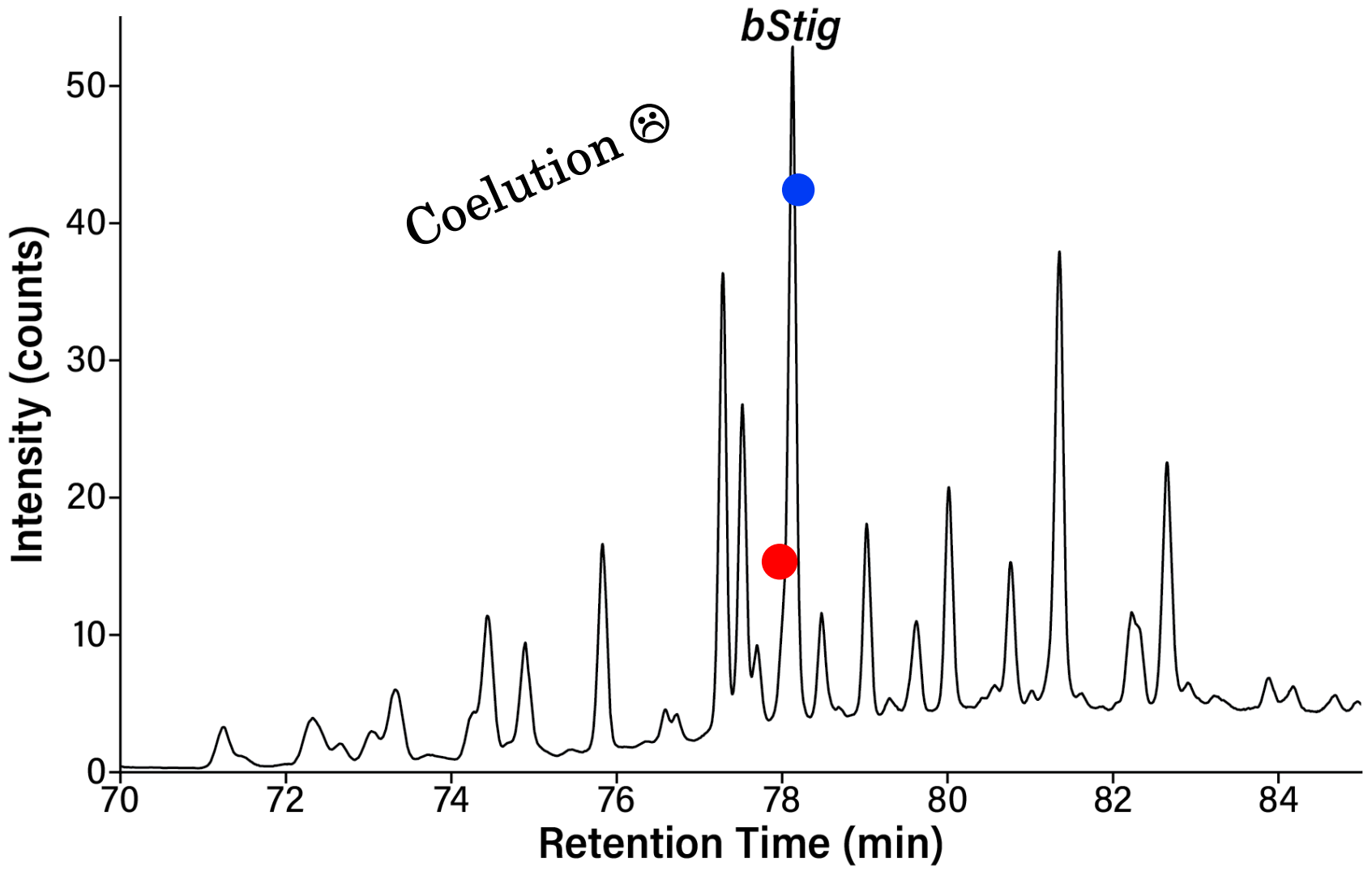




Steroid acetylation not feasible for CSIA







Instrumentation

Trace 1610 GC

↓

Isolink II

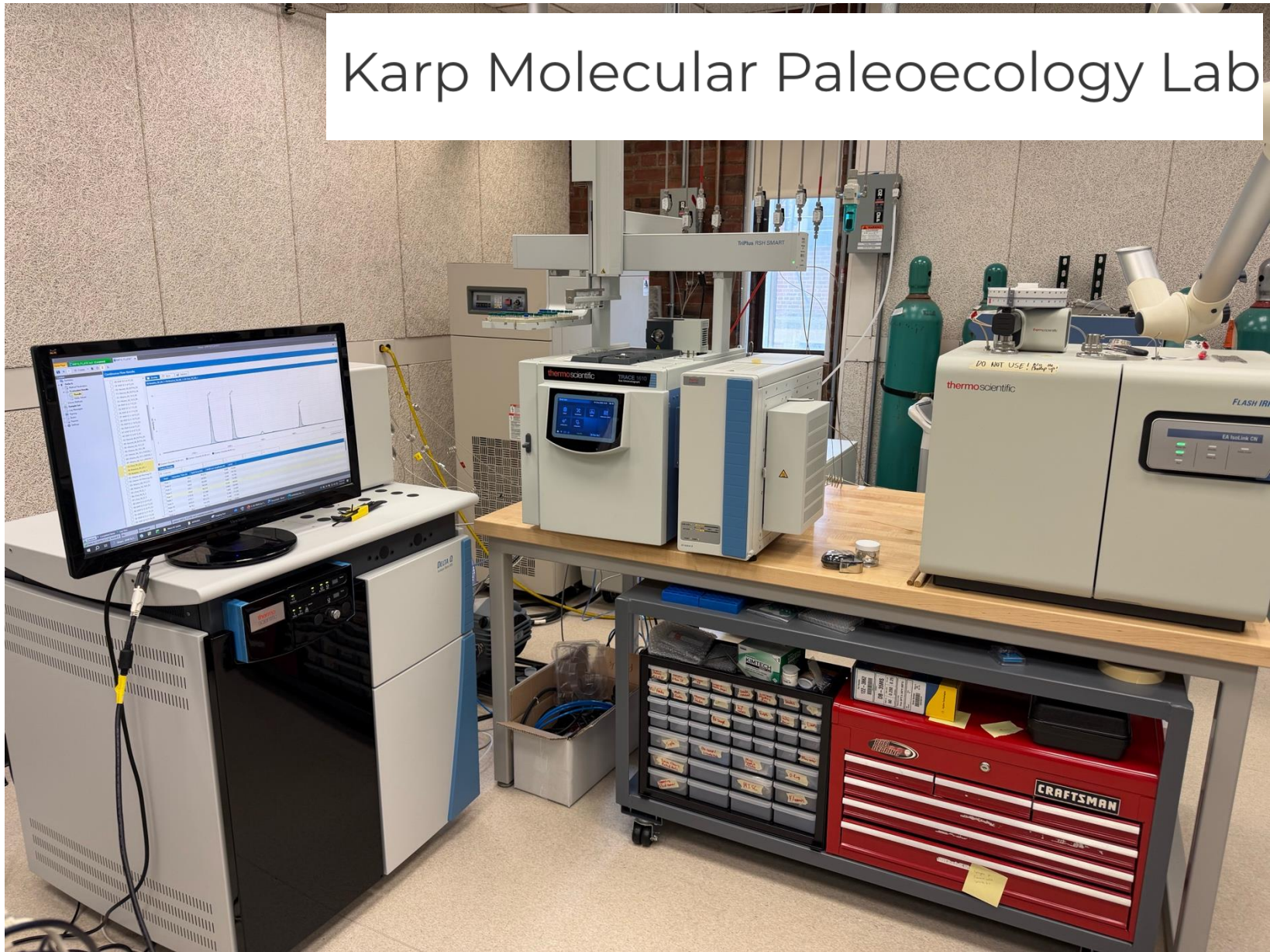
↓

Conflo IV

↓

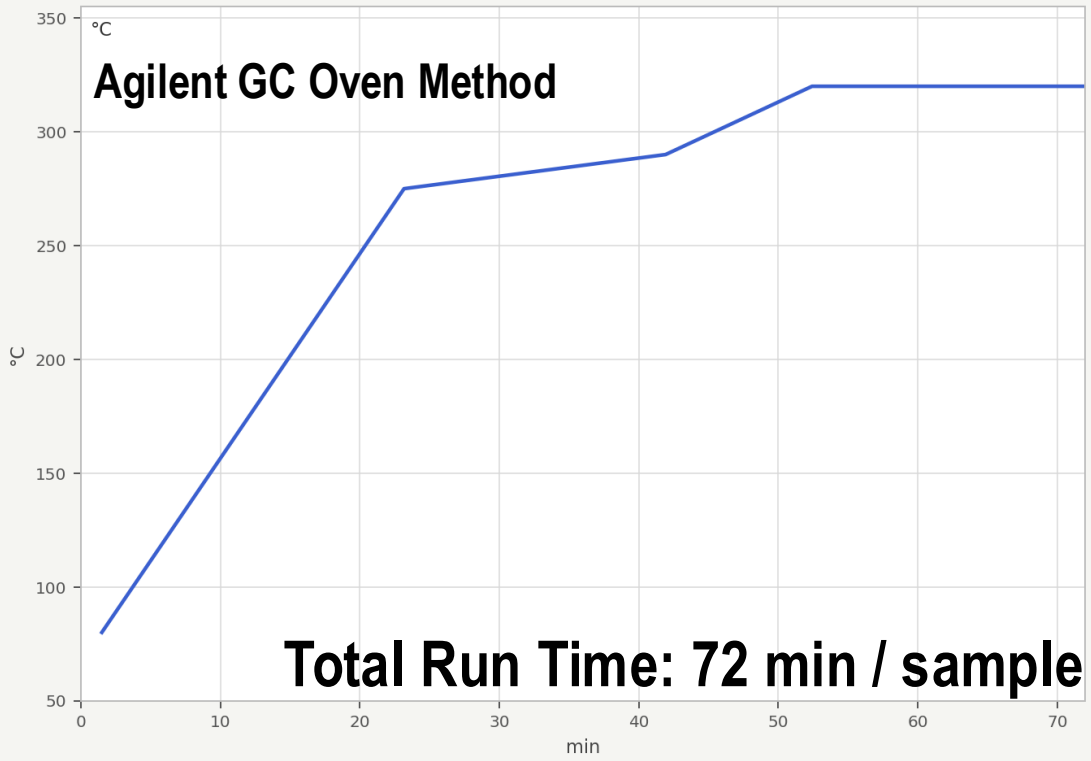
Delta Q IRMS

QTEGRA



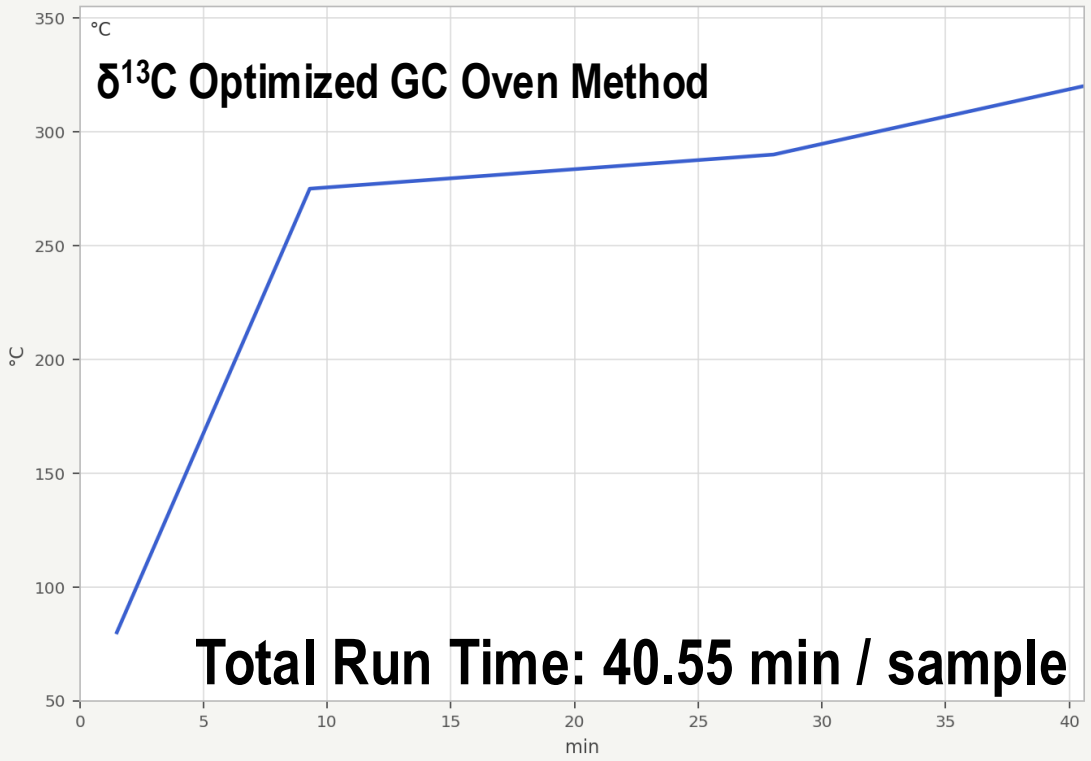
Optimization: GC Oven Ramp

Old Oven Ramp



No	Retention time [min]	Rate [°C/min]	Target value [°C]	Hold time [min]
1	0.000	Run		
2	1.500	0.00	80.0	1.50
3	23.167	9.00	275.0	0.00
4	41.917	0.80	290.0	0.00
5	52.417	12.00	320.0	8.00
6	72.000	StopRun		

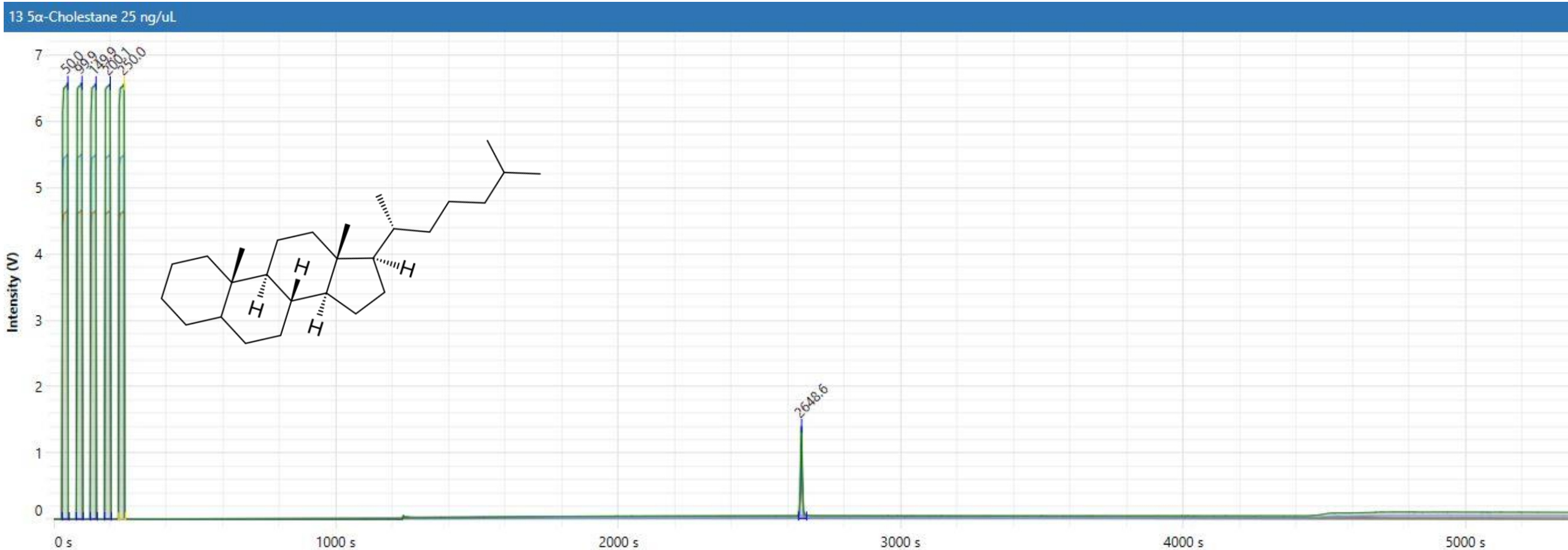
New Oven Ramp



No	Retention time [min]	Rate [°C/min]	Target value [°C]	Hold time [min]
1	0.000	Run		
2	1.500	0.00	80.0	1.50
3	9.300	25.00	275.0	0.00
4	28.050	0.80	290.0	0.00
5	40.550	12.00	320.0	10.00
6	40.550	StopRun		

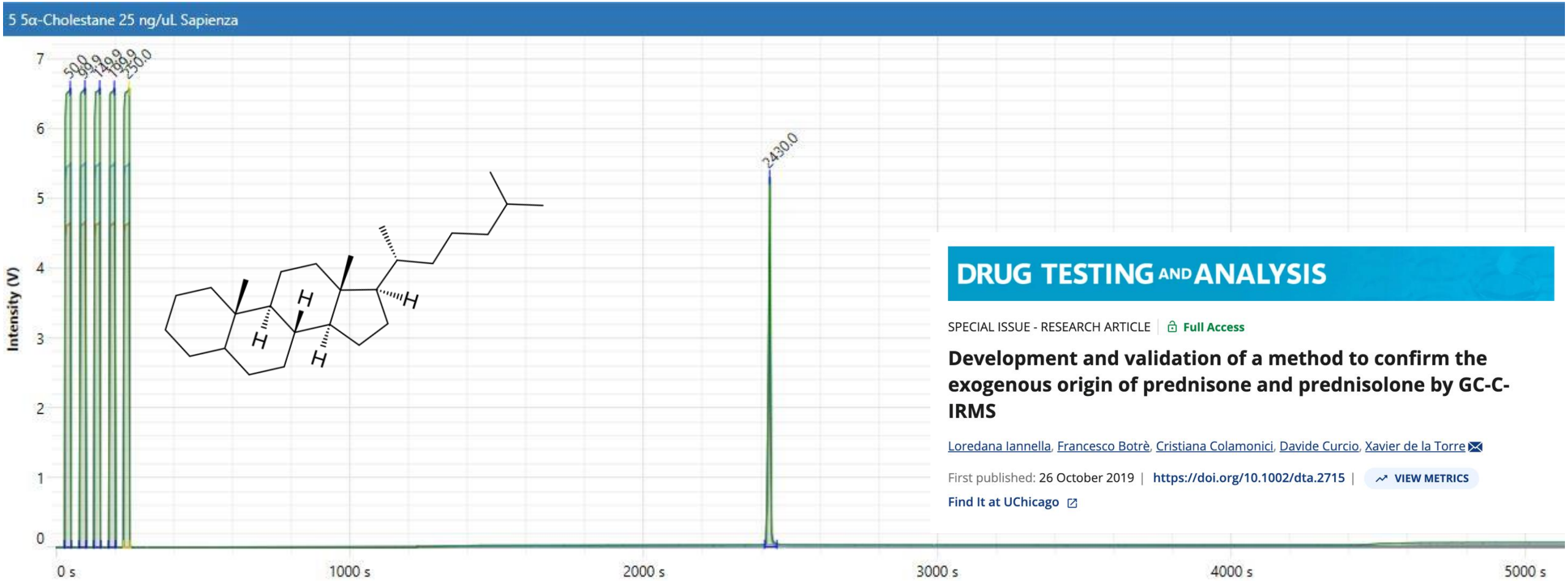
Optimization: Inlet Type (SSL)

- 5α-cholestane test injections
- SSL injections (splitless) resulted in low-amplitude peaks → large volume injections?



Optimization: Inlet Type (PTV)

Followed large volume PTV injection method of study that performs CSIA of steroidal compounds in athlete urine → much better!



DRUG TESTING AND ANALYSIS

SPECIAL ISSUE - RESEARCH ARTICLE | Full Access

Development and validation of a method to confirm the exogenous origin of prednisone and prednisolone by GC-C-IRMS

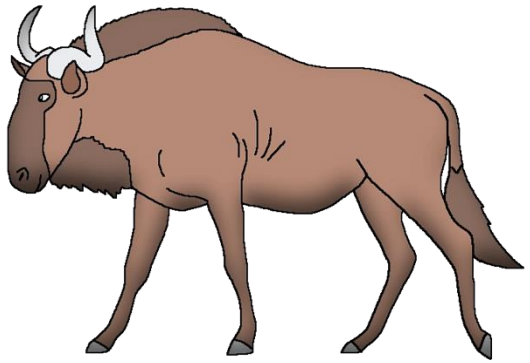
Loredana Iannella, Francesco Botrè, Cristiana Colamonici, Davide Curcio, Xavier de la Torre

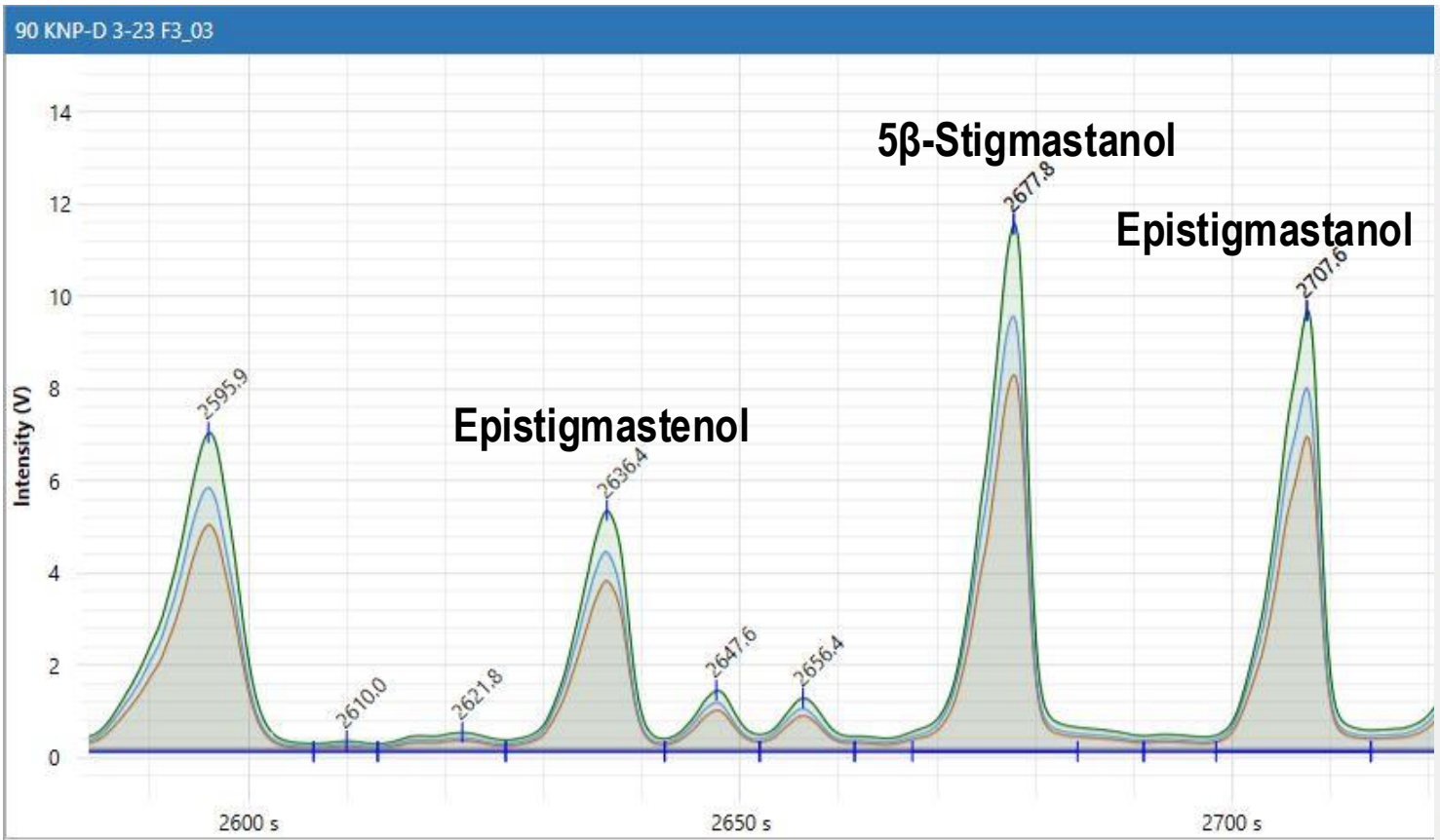
First published: 26 October 2019 | https://doi.org/10.1002/dta.2715 | VIEW METRICS

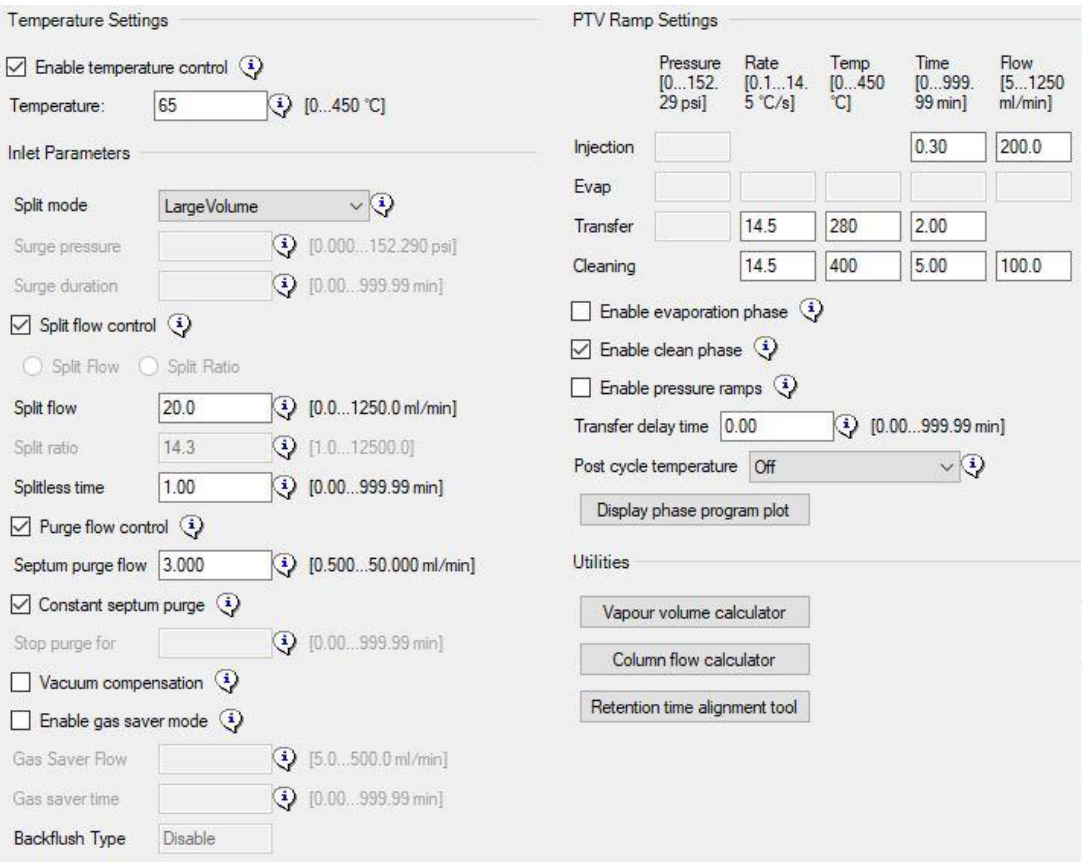
Find It at UChicago

Sample Chromatography: KNP-D 3-23

- 5β-Stigmastanol triplicate injection $\delta^{13}C$ precision: 0.30‰
- Good first step for optimizing these measurements, but some issues…

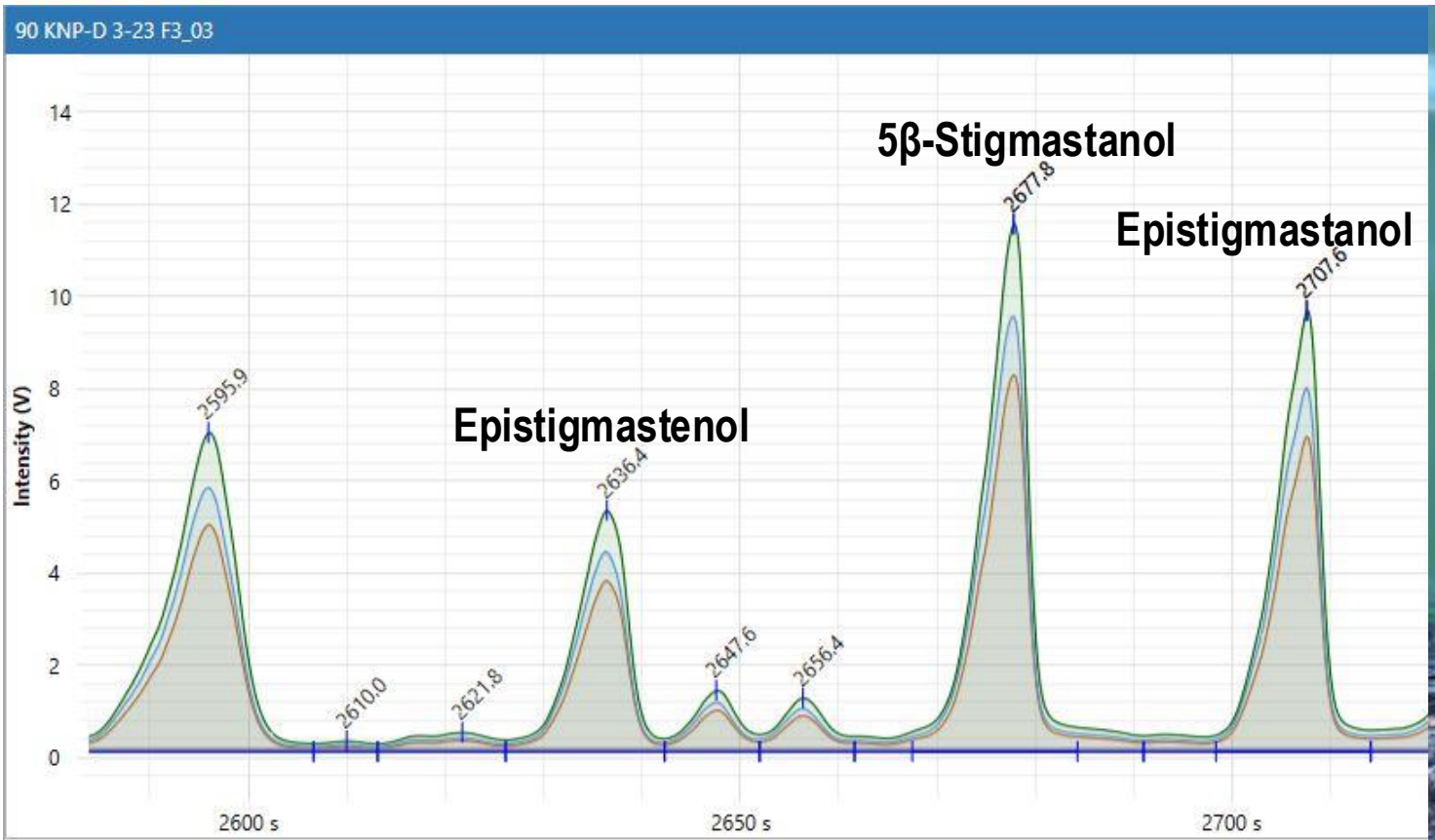






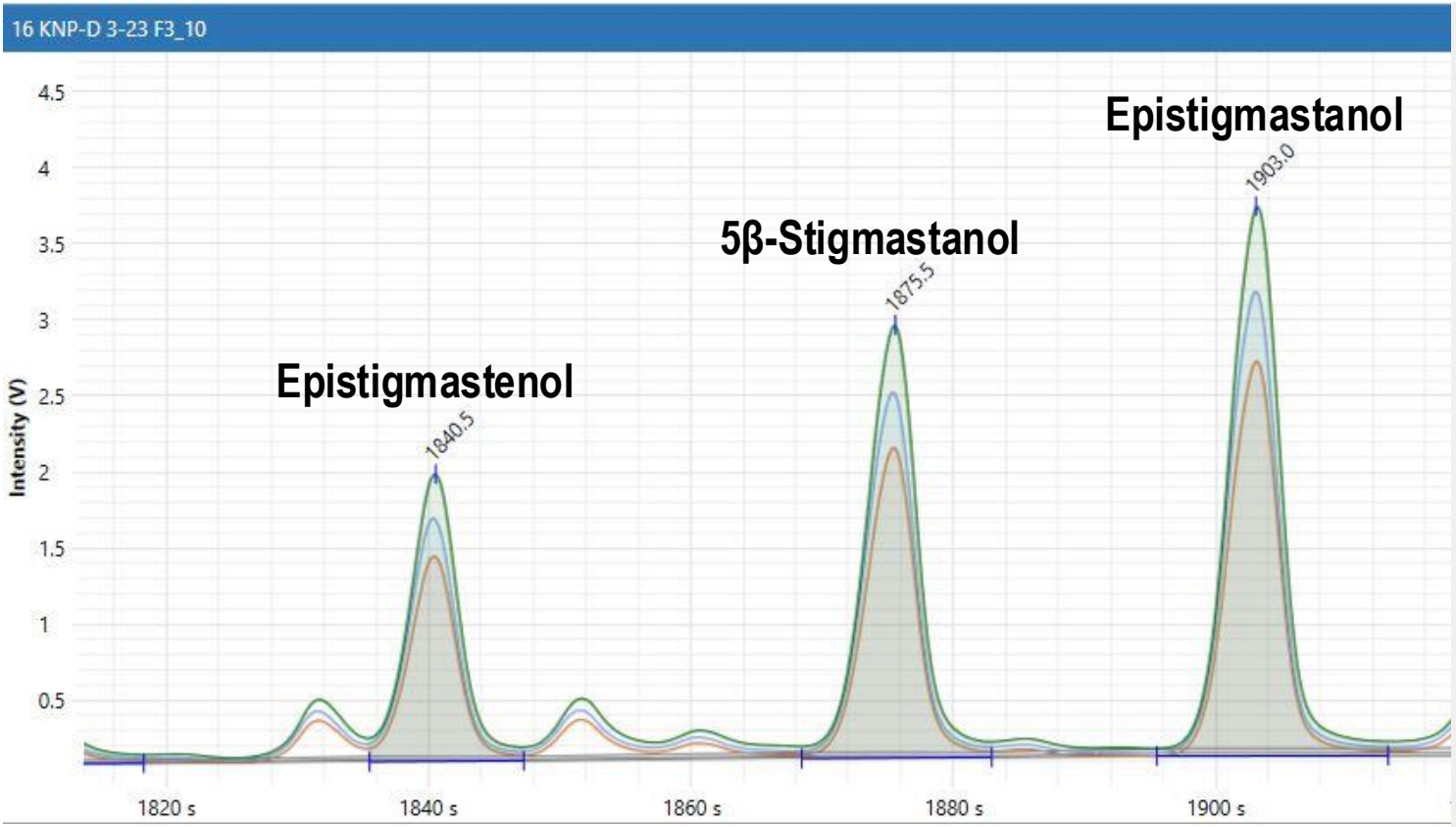
Issues

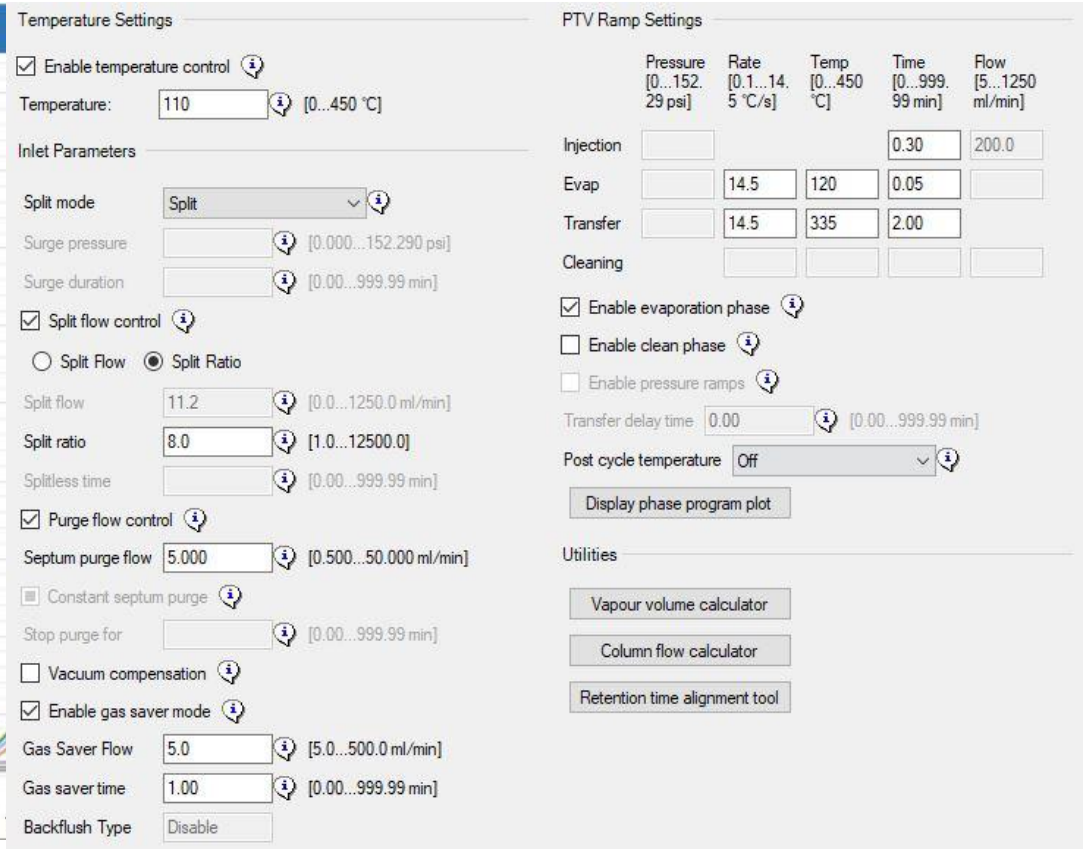
- Too much O_2 from default seed conditioning parameters (5 min) → "shark fin" peak shape.
- Decreased seed oxidation interval to every ~15 samples and changed seed to 2 min.
- Injection parameters were also inefficient…



Sample Chromatography: KNP-D 3-23

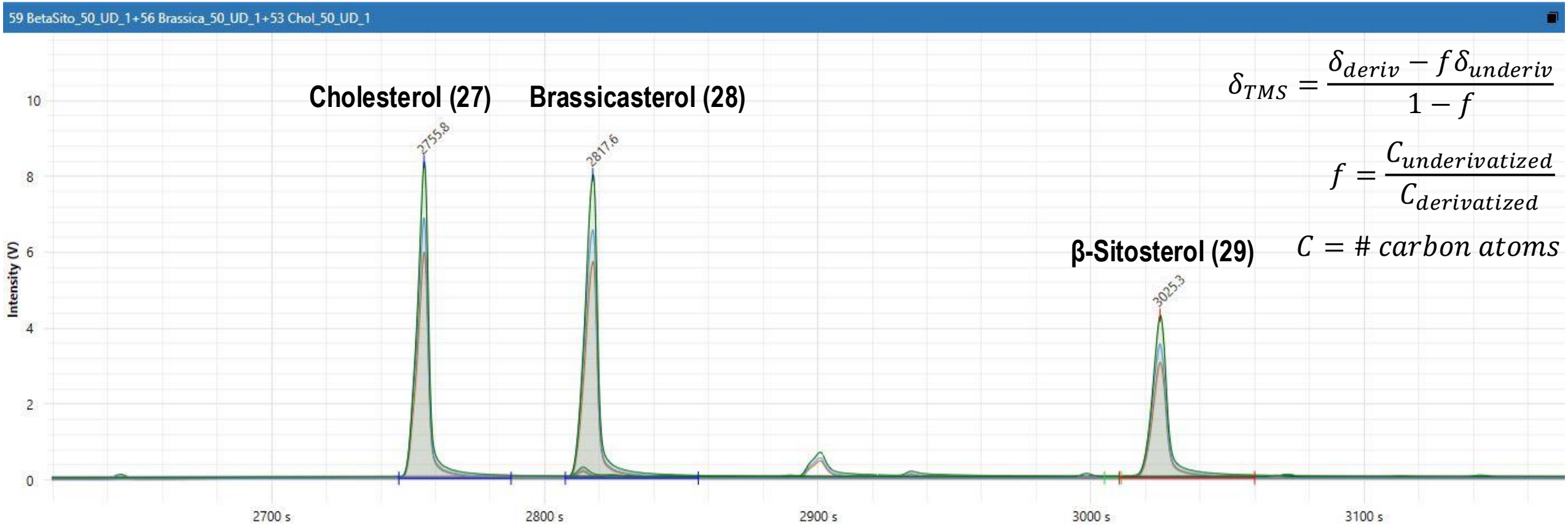
- 5β-Stigmastanol triplicate injection $\delta^{13}C$ precision: 0.02‰
- Improved chromatography after changing split mode, split ratio, injection temperature, PTV ramp settings, and purge flow.





Determining $\delta^{13}C$ of TMS Group

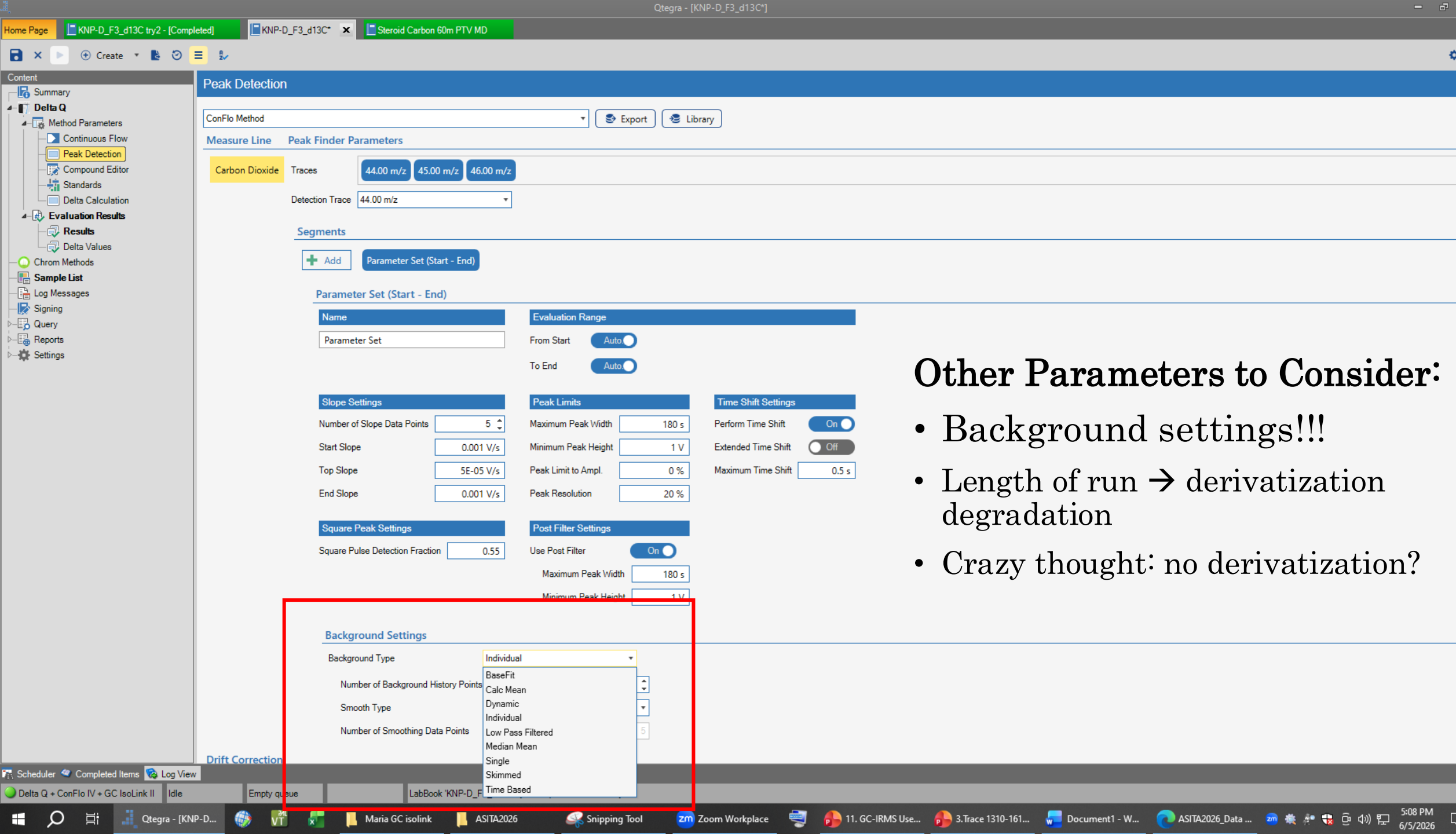
- TMS group added to steroids contributes three additional carbon atoms to each compound.
- $\delta^{13}C$ of the TMS group determined by measuring $\delta^{13}C$ of underivatized and derivatized cholesterol, brassicasterol, and β-sitosterol
- Once underivatized standards were grounded, $\delta^{13}C_{TMS}$ determined for each new batch of BSTFA:TMS.



$$\delta_{TMS} = \frac{\delta_{deriv} - f\,\delta_{underiv}}{1 - f}$$

$$f = \frac{C_{underivatized}}{C_{derivatized}}$$

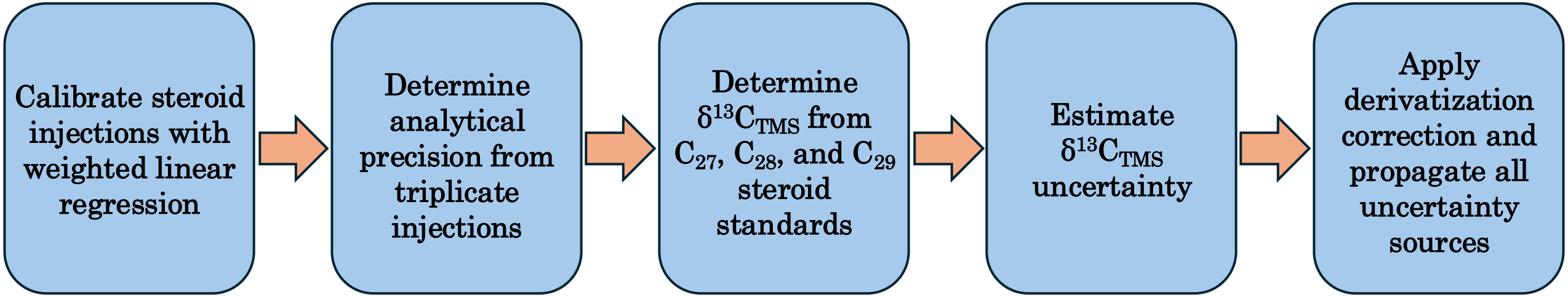
$$C = \#\ carbon\ atoms$$



Other Parameters to Consider:

- Background settings!!!
- Length of run → derivatization degradation
- Crazy thought: no derivatization?

Data Processing & Error Propagation



$$\delta_{calibrated} = mx + b$$

$$\sigma_{\delta calibrated} = \sqrt{x^2\sigma_m^2 + \sigma_b^2 + m^2\sigma_x^2}$$

$$\sigma_{TMS} = \sqrt{\left(\frac{\sigma_{deriv}}{1-f}\right)^2 + \left(\frac{f\sigma_{underiv}}{1-f}\right)^2}$$

$$\sigma_{deriv} = \sqrt{x^2\sigma_m^2 + \sigma_b^2 + m^2\sigma_x^2}$$

$$\sigma_{underiv} = \sqrt{x^2\sigma_m^2 + \sigma_b^2 + m^2\sigma_x^2}$$

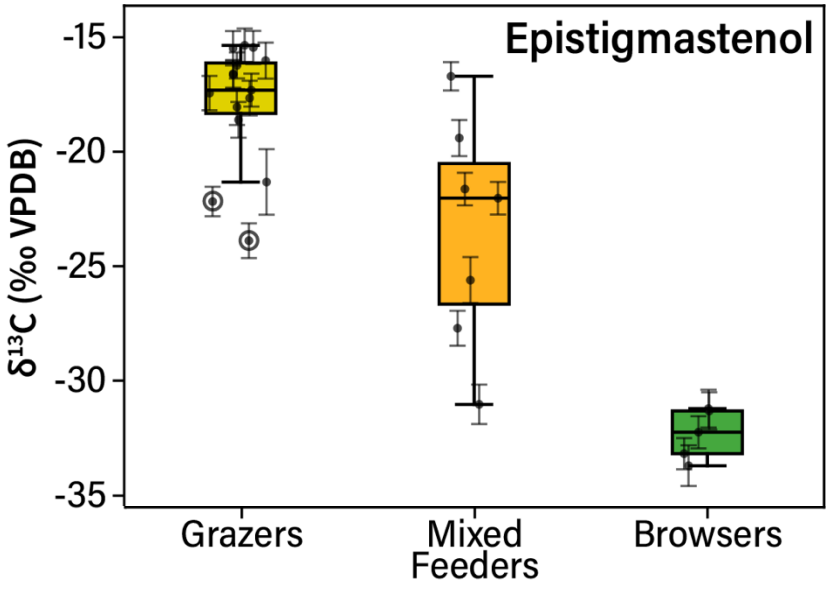
$$SE_{TMS,final} = \sqrt{\left(\frac{SD_{empirical}}{\sqrt{n}}\right)^2 + \left(\frac{\overline{\sigma_{analytical}}}{\sqrt{n}}\right)^2}$$

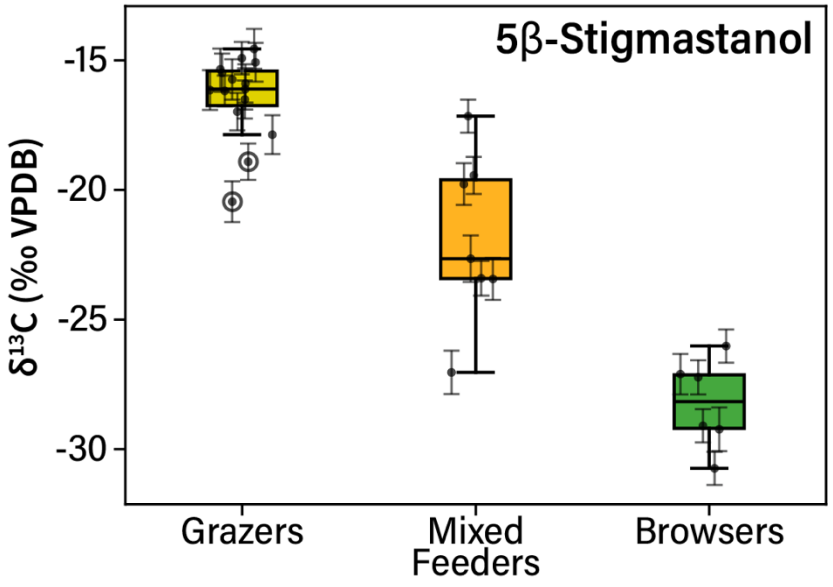
$$\sigma_{final} = \sqrt{\left(\frac{\sigma_{deriv}}{f}\right)^2 + \left(\frac{(1-f)\sigma_{TMS}}{f}\right)^2}$$

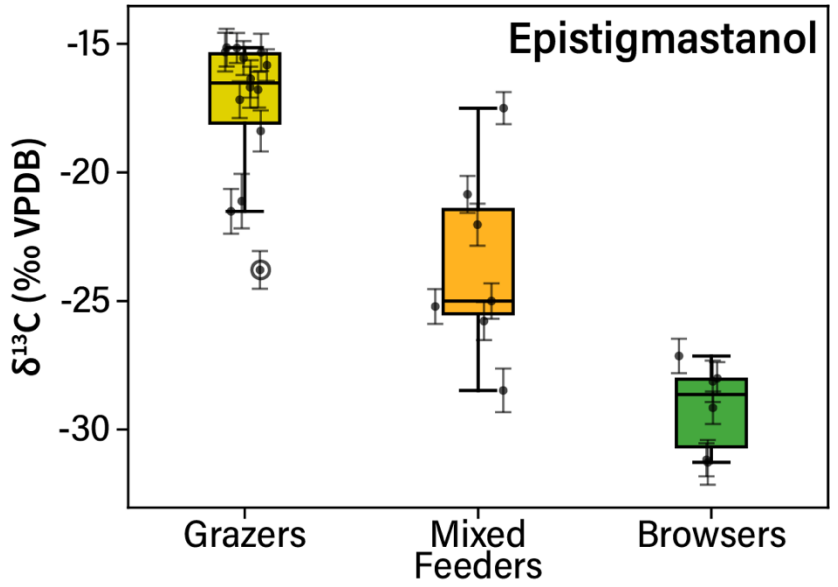
Preliminary Results

Fecal Steroid $\delta^{13}C$ Reflects Herbivore Diets!

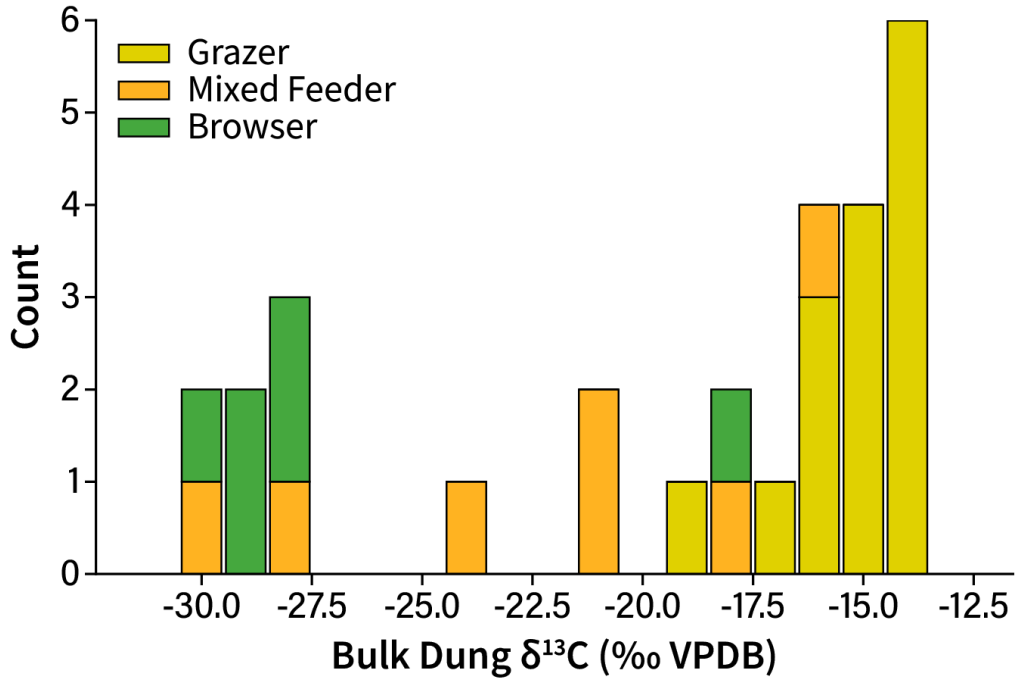
Fecal steroid $\delta^{13}C$ reflects dietary preference. Strong C_4 signal for grazers, and C_3 signal for browsers, and intermediate $\delta^{13}C$ values for mixed feeders.

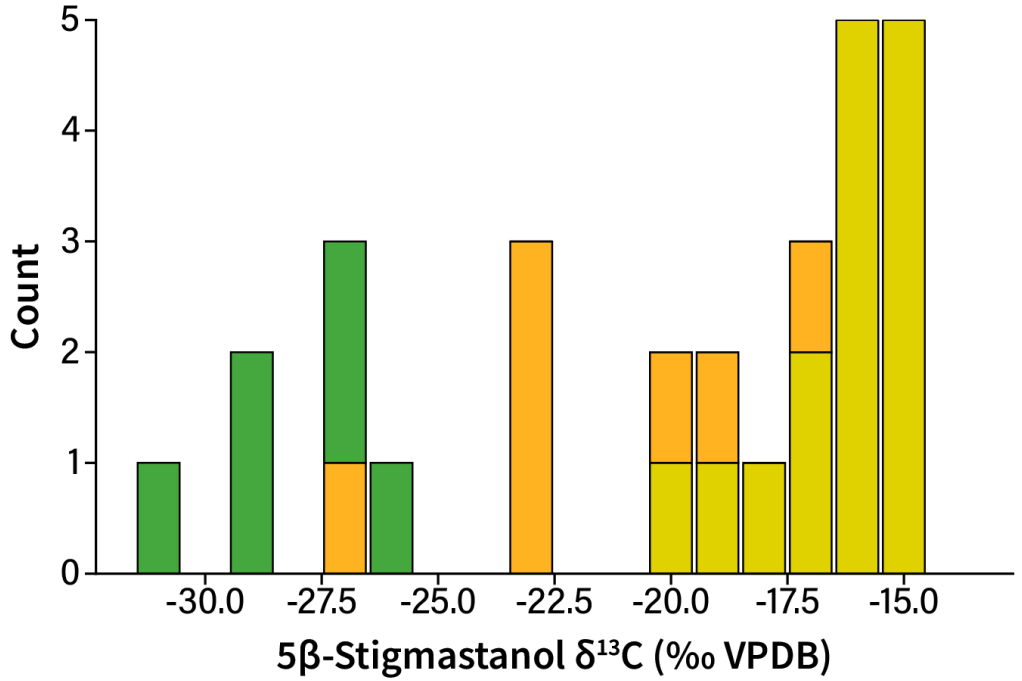




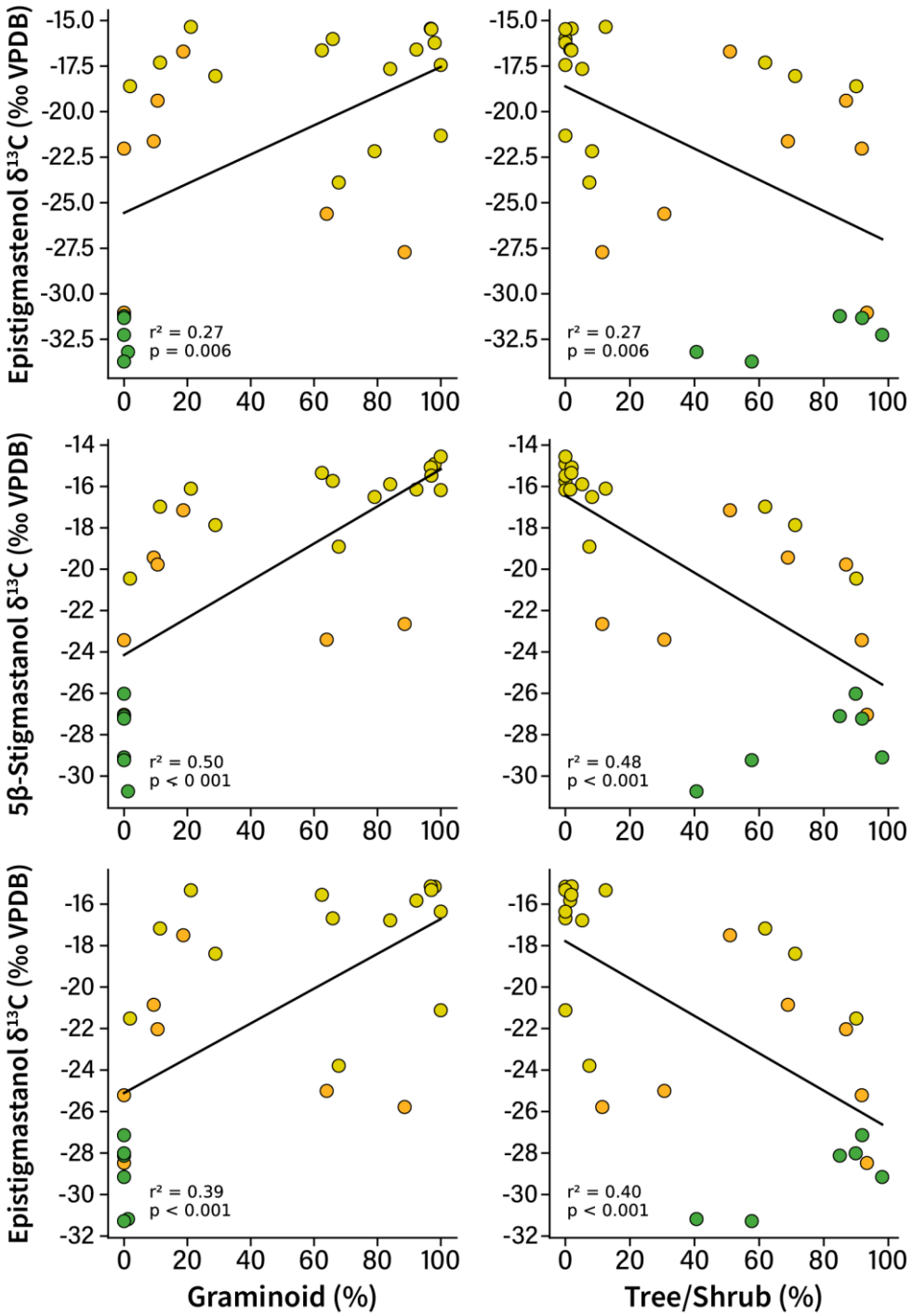


5ß-Stigmastanol $\delta^{13}C$ reflects $\delta^{13}C_{bulk}$, but with slightly better discrimination between browsers & grazers.



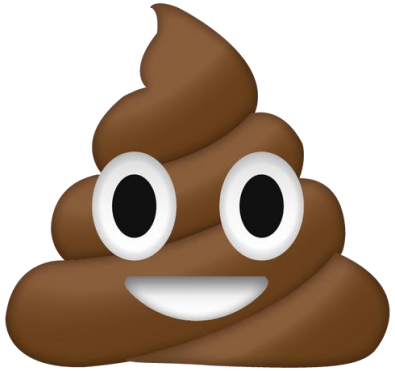


Somewhat statistically significant relationships between fecal steroid $\delta^{13}C$ and DNA-derived plant type %



Future Work

- Streamline and optimize $\delta^{13}C$ analysis workflow—open to any and all suggestions!
- Offline separation of sterols and stanols via $AgNO_3$ silica gel columns → specifically for separation of ß-sitosterol and α-stigmastanol.
- Constrain ε_{bio} of fecal steroid $\delta^{13}C$ and phytosterol $\delta^{13}C$ → potential differences between fermentation type (i.e., foregut ruminants, foregut non-ruminants, and hindgut colon)?
- Extract, process, and measure an additional 112 dung samples, representing fourteen different herbivore species.



Acknowledgments

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- **Dr. Thomas Piper** — Center for Preventive Doping Research, German Sport University Cologne
- **Dr. Frank Hülsemann** — Manfred Donike Institute for Doping Analysis, German Sport University Cologne
- **Dr. Brett Davidheiser, Taylor Graham, and Mark Belmont** — Thermo Fisher Scientific

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