Following pathways with isotopes and other applications with isotopes

Stephen Barnes

University of Alabama at Birmingham

sbarnes@uab.edu

Synopsis

- **Extra value in the M+2 isotope peak**
- **Using isotopes in tracing metabolic and physiologic pathways**
  - Historical
  - Fluxomics
  - Physiology
- **Isotopes and enhanced chemical detection of metabolites**
- **Disturbing energy levels in NMR**

Value of natural isotopes

- The natural abundance of isotopes enables the investigator to determine the charge state of an ion
- The principal contribution to $[M+H]^+$ or $[M-H]^-$ isotope ions comes from ^{13}C (~1.1% of all carbon atoms)
- The intensity of the $[M+H]^+$ or $[M-H]^-$ ^{13}C isotope ion increases relative to the number of carbon atoms
- There is often an observable $^{13}C_2$ isotope peak

Value of the [M+2] peak

- The mass difference due to a nominal increase in mass of 2 contains a lot of information
  - These are isotopic mass differences for each of the common elements
    - $^{1}H_2/^{2}H_2$ 2 x 1.006277 = 2.012554 (0.012%)
    - $^{12}C_2/^{13}C_2$ 2 x 1.003355 = 2.006710 (1.078%)
    - $^{14}N_2/^{15}N_2$ 2 x 0.997035 = 1.994079 (0.364%)
    - $^{16}O_2/^{17}O_2$ 2 x 1.004217 = 2.008434 (0.038%)
    - $^{16}O_2/^{18}O_1$ 1 x 2.004246 = 2.004246 (0.205%)
    - $^{32}S_2/^{33}S_2$ 2 x 0.999387 = 1.998774 (0.752%)
    - $^{32}S_2/^{34}S_1$ 1 x 1.995796 = 1.995796 (4.252%)
- Needs the highest possible mass resolution
  - FT-ICR

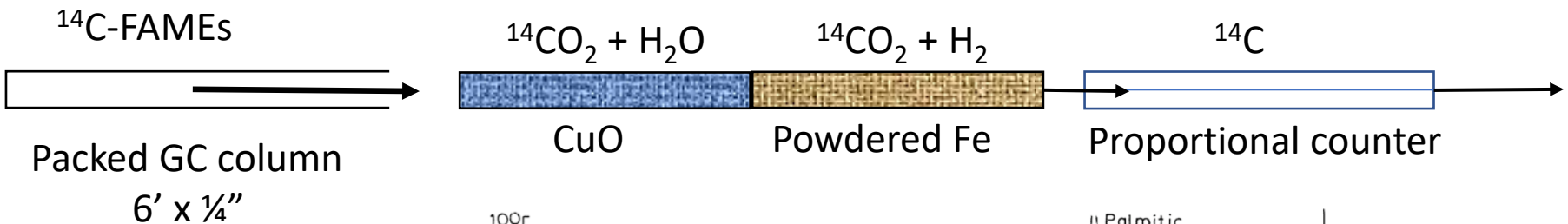
Using isotopes to trace a pathway

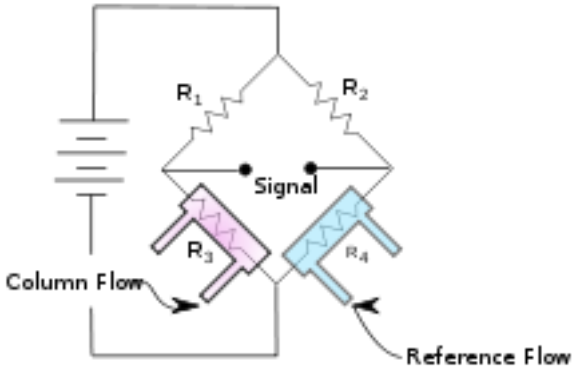
- Early studies (1930s) used ^{2}H, ^{13}C and ^{15}N labeling to map pathways
  - Limited to 1-200 *m/z* mass range
- 1950s/60s ^{14}C-radiotracers
  - 2D-Paper or thin layer chromatography
  - Radio gas chromatography
    - labeling of specific carbon atoms

David Rittenberg

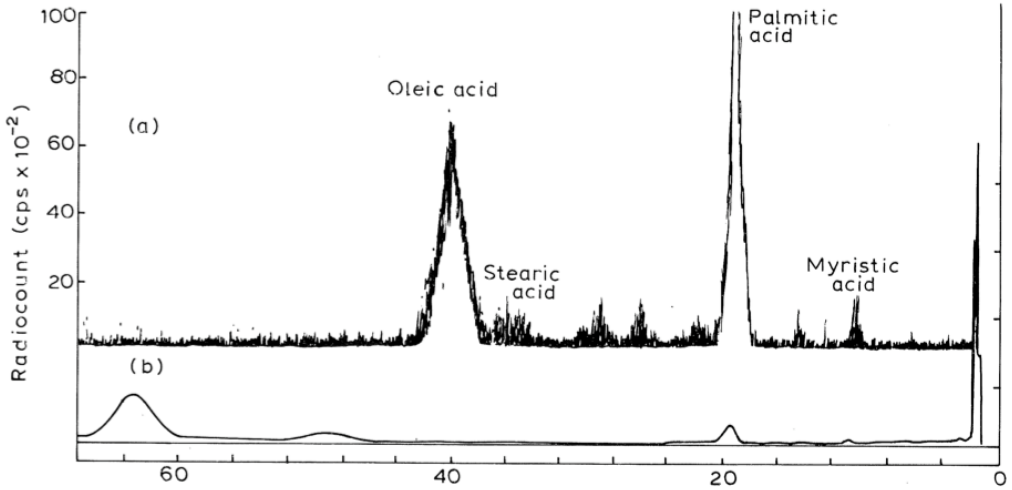
Radio-gas chromatography of FAMEs

In Tony James' lab





Katharometer to detect H_2 by thermal conductivity



We were studying an isomer of palmitoleic acid – from β-oxidation of oleic acid, or direct desaturation?
Collected the 16:1 peak using a gas density balance (no degradation) in **ether-soaked cotton wool**.
Subjected to oxidation with permanganate-periodate – identified C_{11} monobasic acid and C_7 dibasic acid, i.e., $16{:}1\Delta^7$

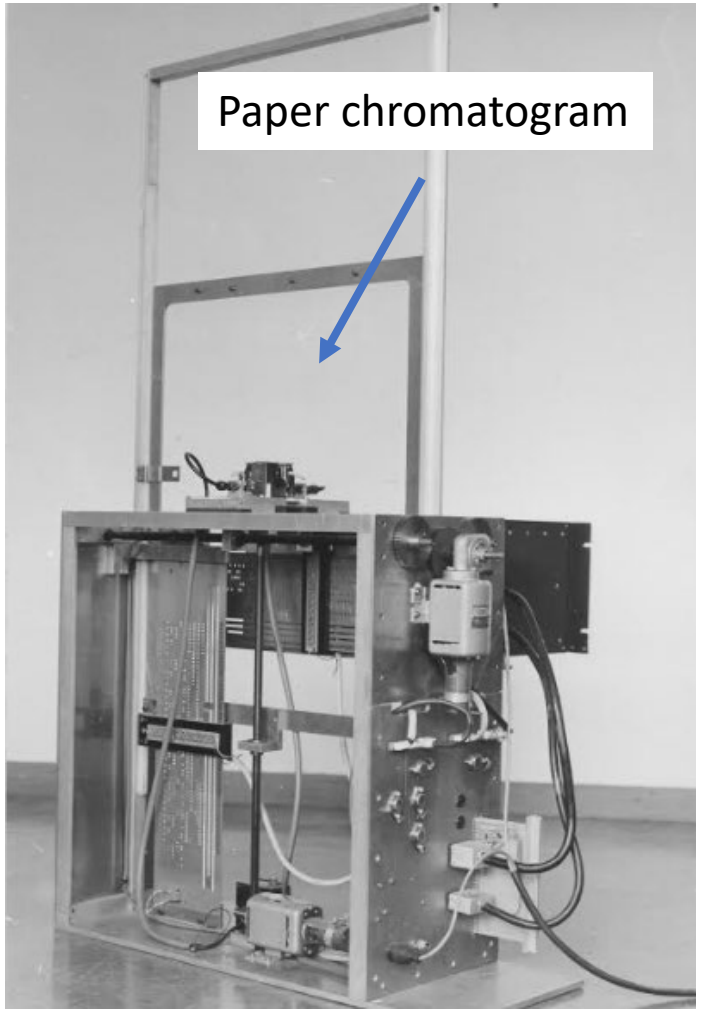
Early beginnings of metabolomics in London

- **Sir Ernst Chain (1945 Nobel Laureate – the biochemist who characterized penicillin)**
  - **Also renown for his work on microanalysis**

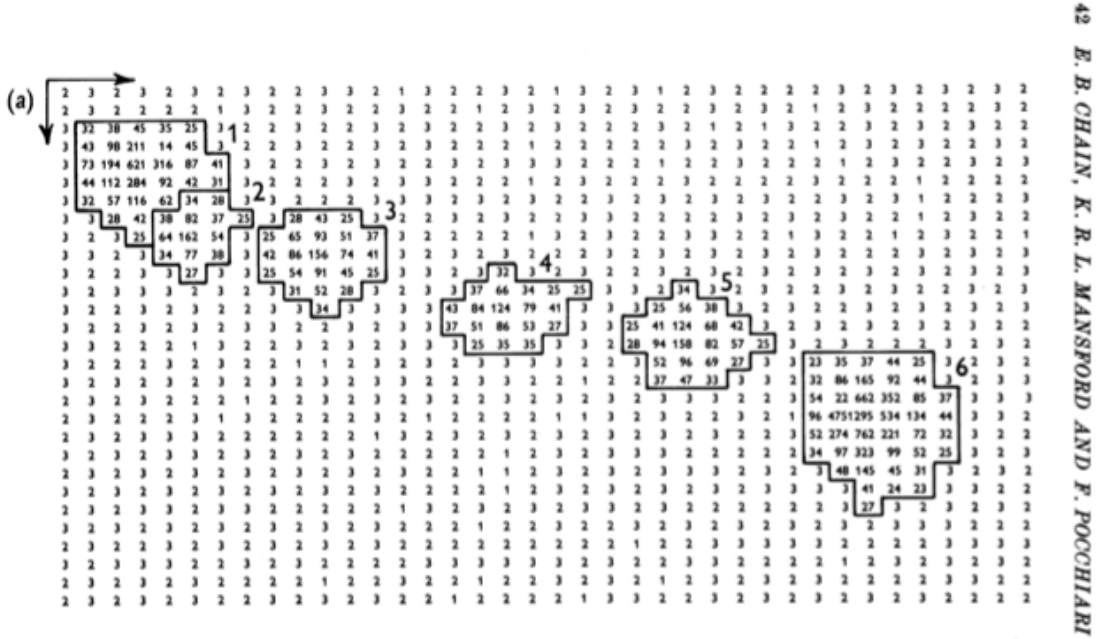
- **Used 2D-paper chromatography to resolve glycolytic, Krebs cycle and amino acids derived from ^{14}C-glucose**
  - **Geiger counter mounted on a typewriter frame**
  - **Digitized the collected data and prepared computer-generated figures**

METABOLOMICS

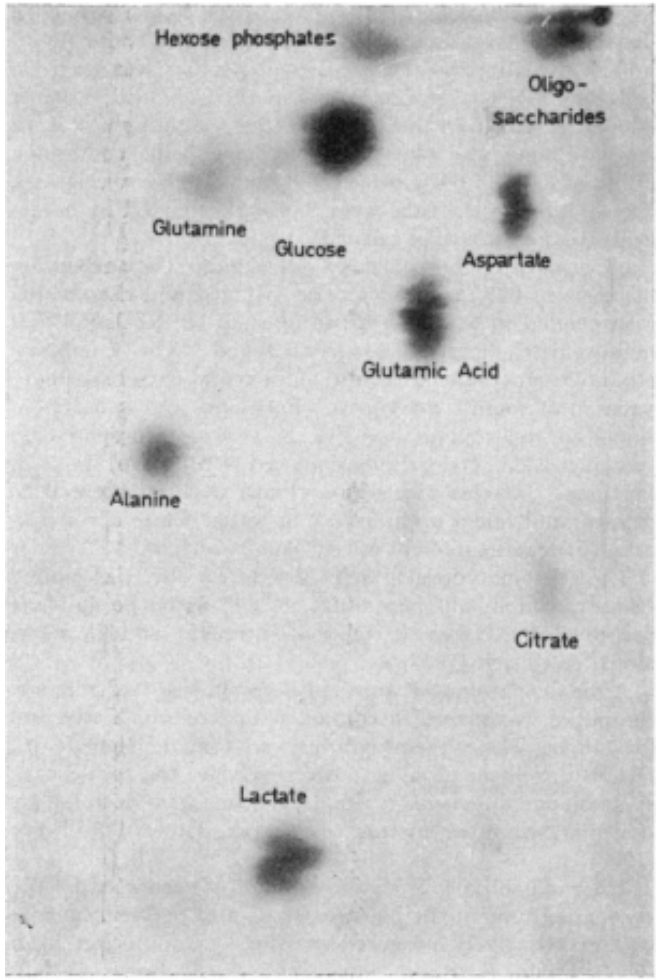
Keith Mansford



Radiochromatography examples



J Physiol (1960) 154:39
E.B. Chain, K.R.L. Mansford and F. Pocchiari



Autoradiogram of ^{14}C-glucose metabolites from an isolated perfused Langendorff rat heart preparation. The metabolites were separated by 2D-paper chromatography.

The conditions were:
1st dimension: butan-l-ol-acetic acid-water (40:11:25, by vol.) for 16hr.;
2nd dimension: (-) phenol-aq. NH_3 (sp.gr. 0.88)-water (80:1:20, by vol.) for 24hr.

Biochem. J. (1969) 115, 537
E.B. Chain, K.R.L. Mansford and L.H. Opie

Radio-GC analysis

metabolomics in its infancy

Radio gas-liquid chromatography with digitization of collected data

Developed this for my PhD work (1967-1970) to study glucose metabolism in acellular slime mold, *Physarum polycephalum*

Physarum polycephalum

The many headed slime mold

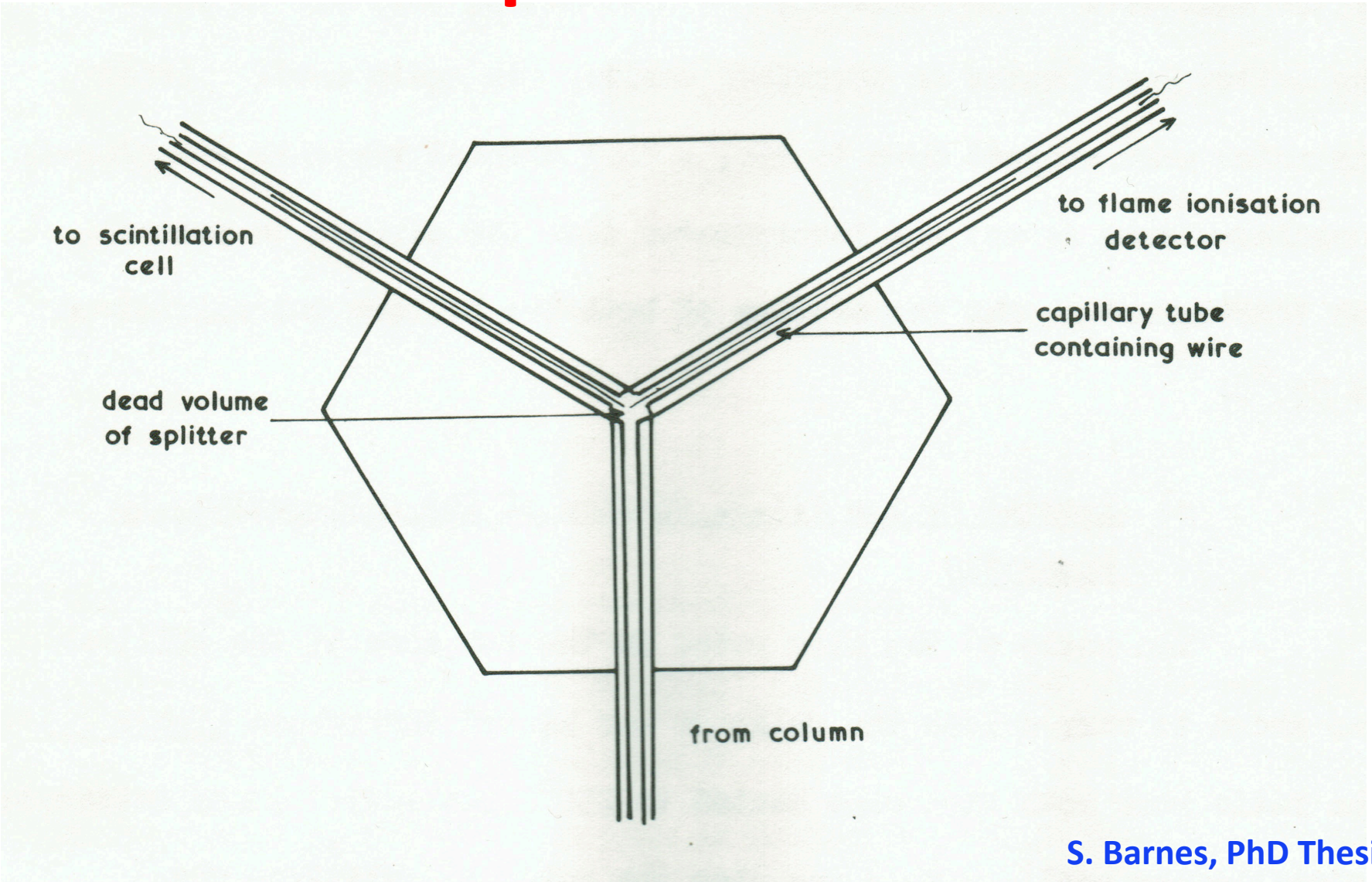
This is a single cell that spreads out to cover a petri dish

The cytoplasm is pushed to one end and back over a 30-45 s period

The cytoplasm has properties of a liquid and a solid

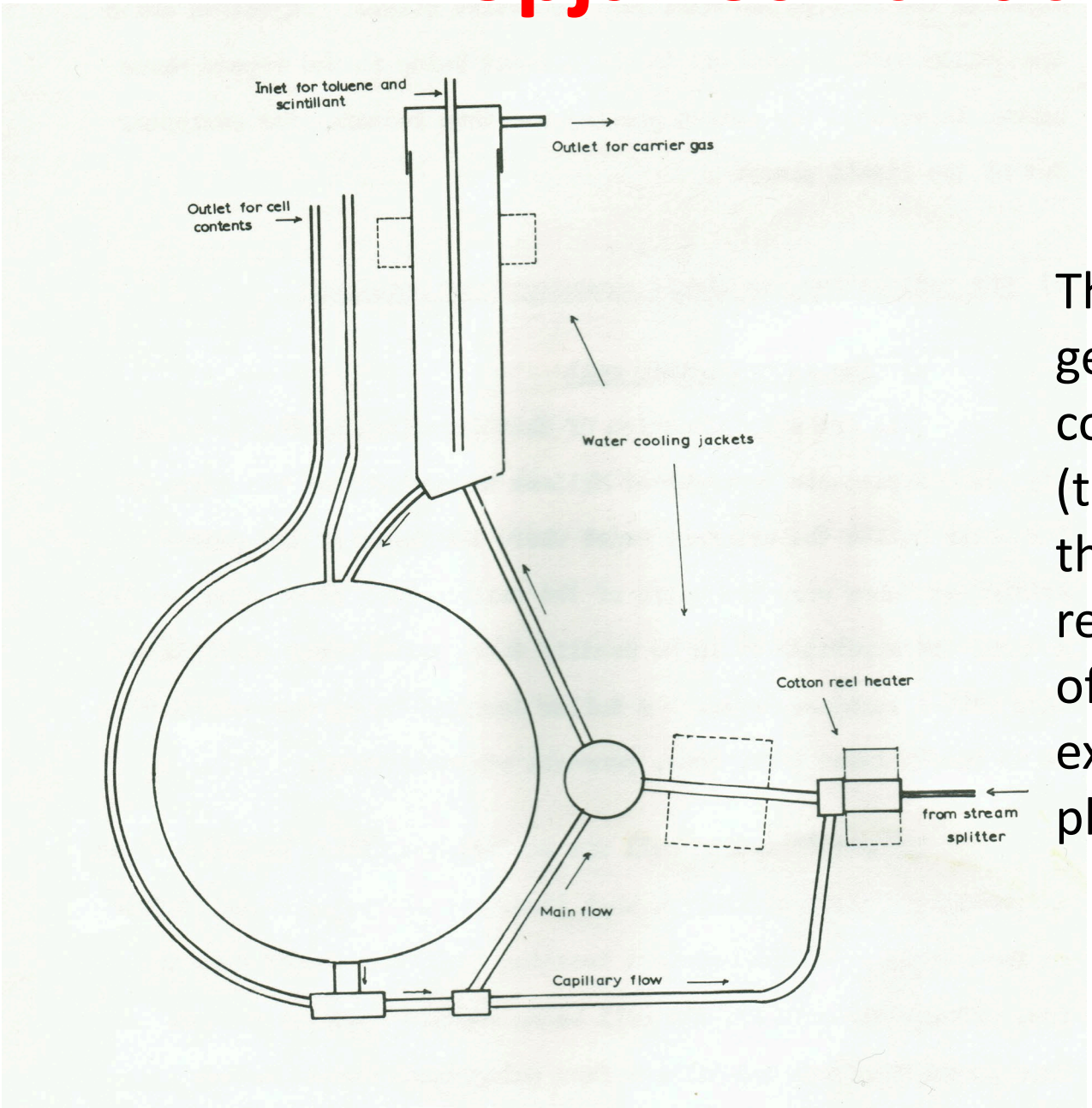
https://www.youtube.com/watch?v=Ia3kWlS_OZU

Stream splitter for radio GC



S. Barnes, PhD Thesis

Popjak scintillation cell



The key to this device was the mixing generated by the gas from the GC column causing (scintillation) fluid (toluene) to flow out of the bottom of the scintillation chamber, both to aid recirculation and to provide a source of solvent vapors that more efficiently extracted the compounds in the gas phase.

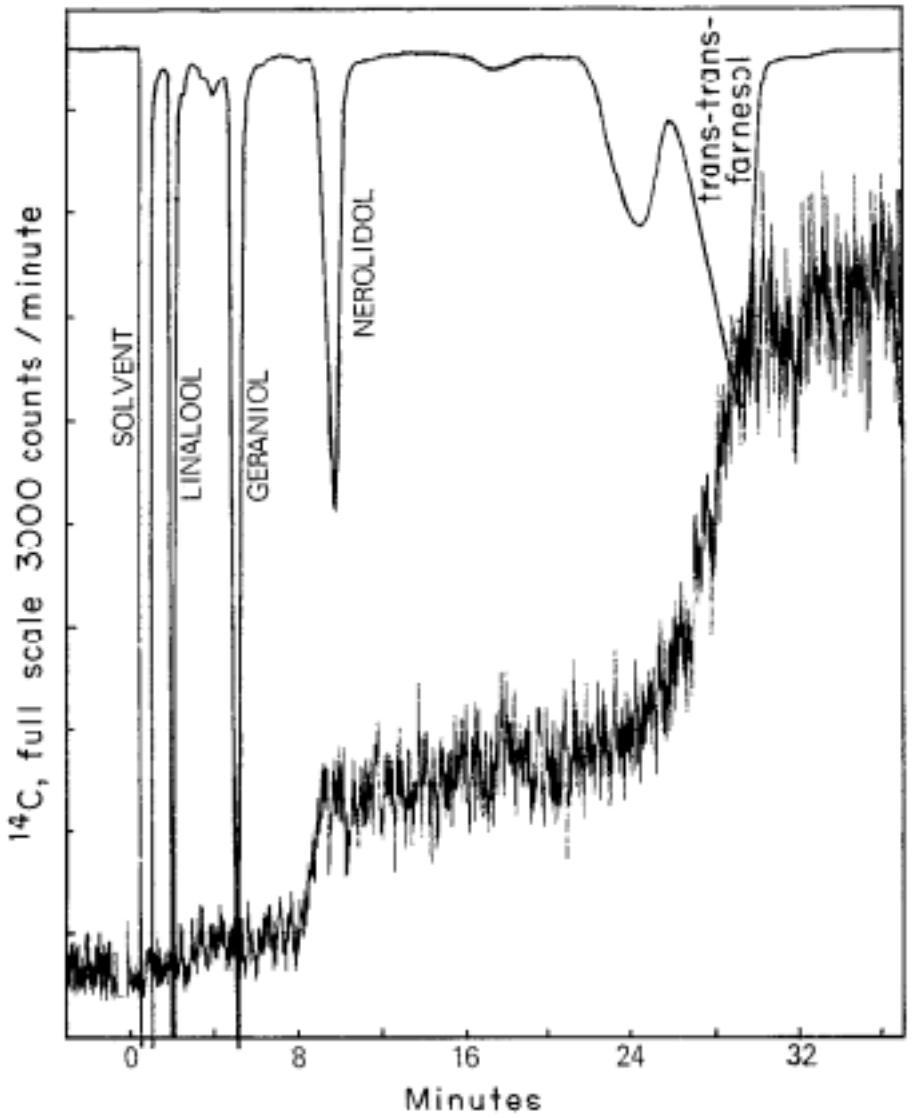
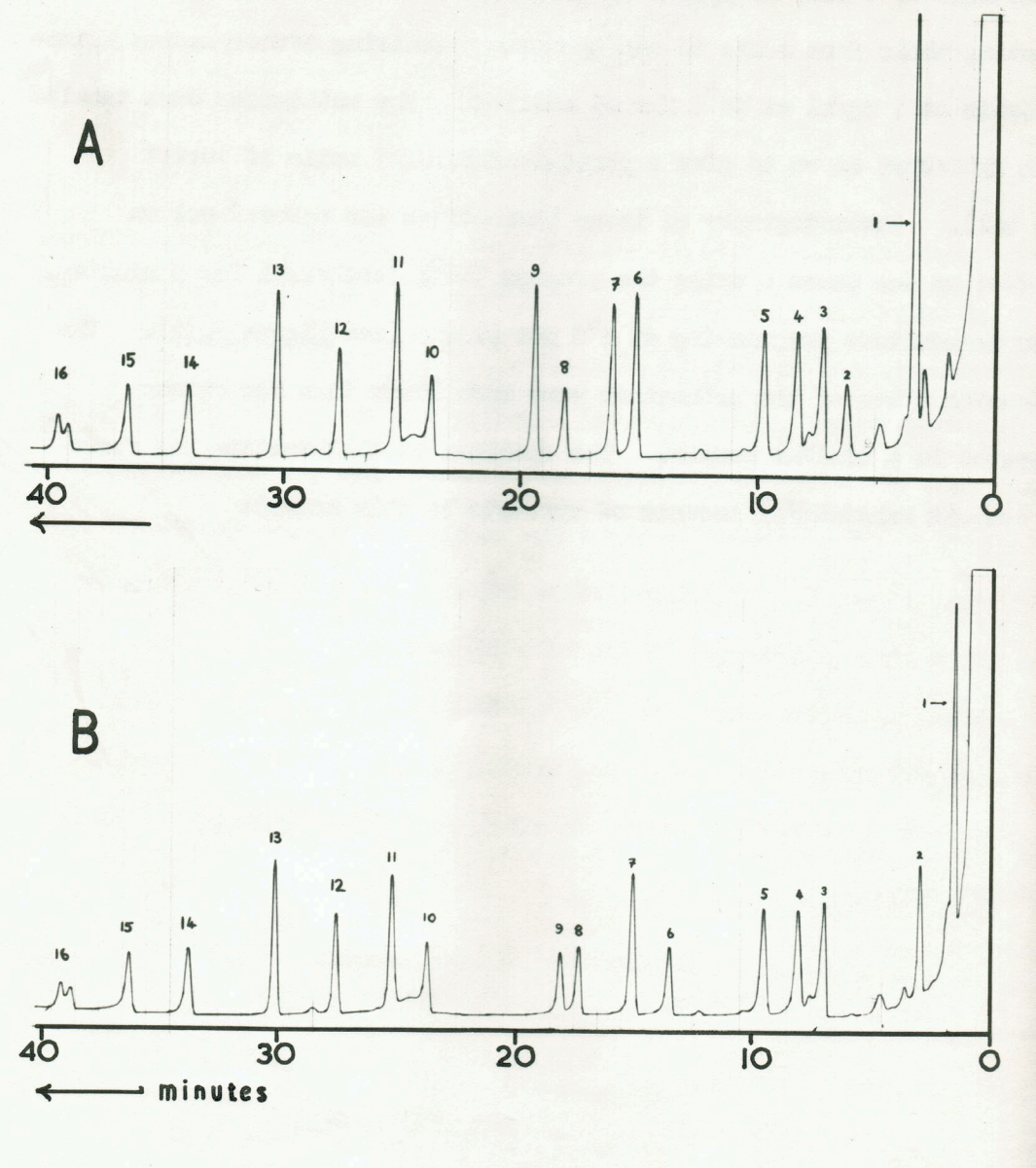


FIG. 19. Gas-liquid radiochromatogram of F_1 (*cf.* Fig. 4) from the chromatography on DEAE-cellulose of the butanol extract of an incubation of liver microsomes with farnesyl pyrophosphate. Nearly 30% of the total radioactivity in the specimen was accounted for by ^{14}C in nerolidol. Cochromatography with added markers of linolool, geraniol, nerolidol, *cis-trans-* and *trans-trans-*farnesol; simultaneous recording of mass and radioactivity detector.

Application to the discovery of a new intermediate in squalene biosynthesis

It's worth reading this 1969 article in J Biol Chem for the depth of analysis that was undertaken to prove the identity of this intermediate

GC of glycolytic and Krebs cycle intermediates

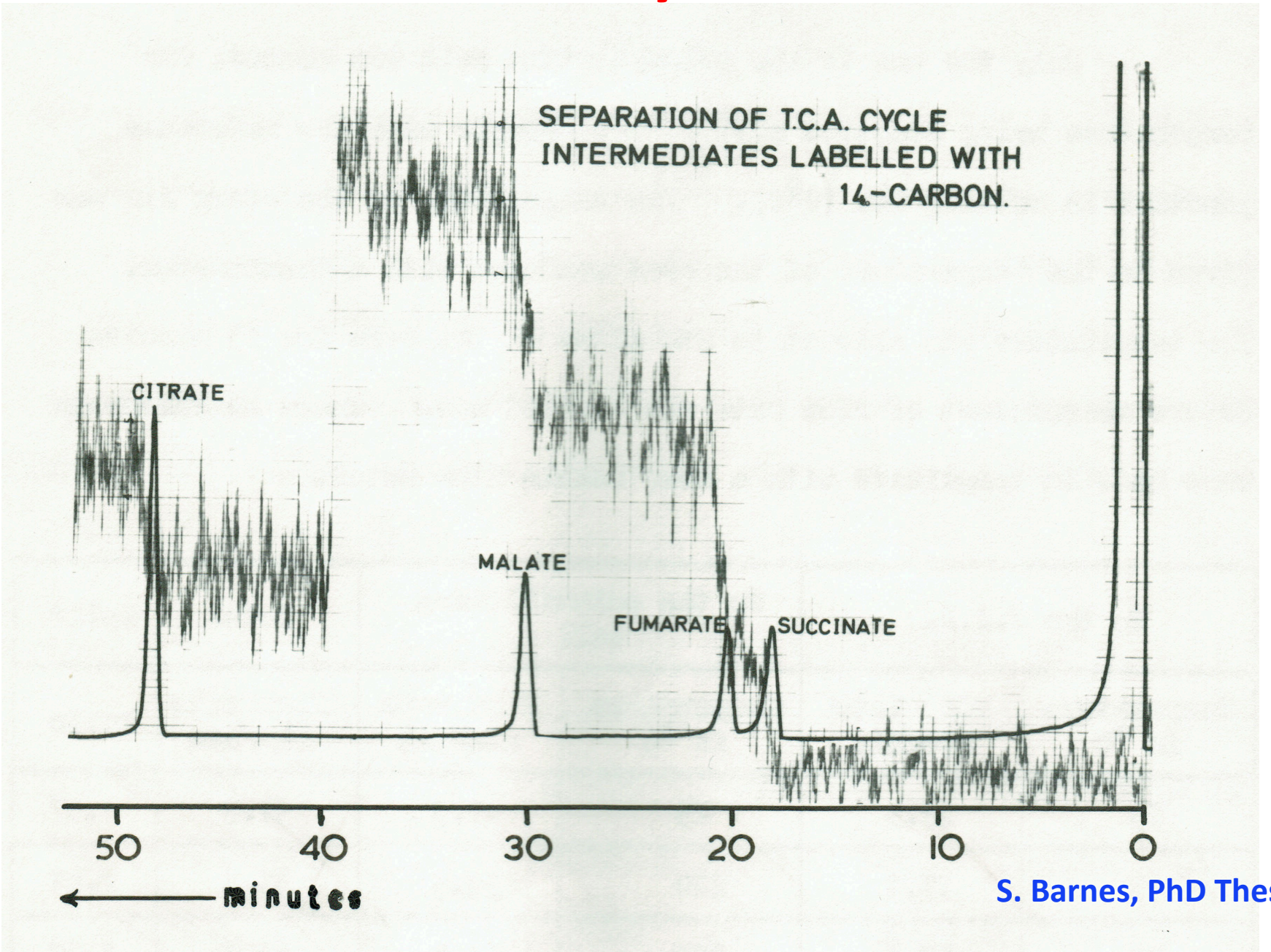


Temperature programming of TMS ester/ethers on a 5' x ¼ inch packed column of Chromosorb W coated with OV-1 liquid phase

1=pyruvate , 2=?? ,
3=phosphate , 4=succinate ,
5=fumarate, 6=oxaloacetate,
7=malate, 8=αKG,
9=hexadecane, 10=αGP,
11=citrate, 12=α–D-glucose,
13=β–D-glucose, 14=docosane,
15=F6P, 16=G6P

S. Barnes, PhD Thesis

Radio-GC of Krebs Cycle intermediates

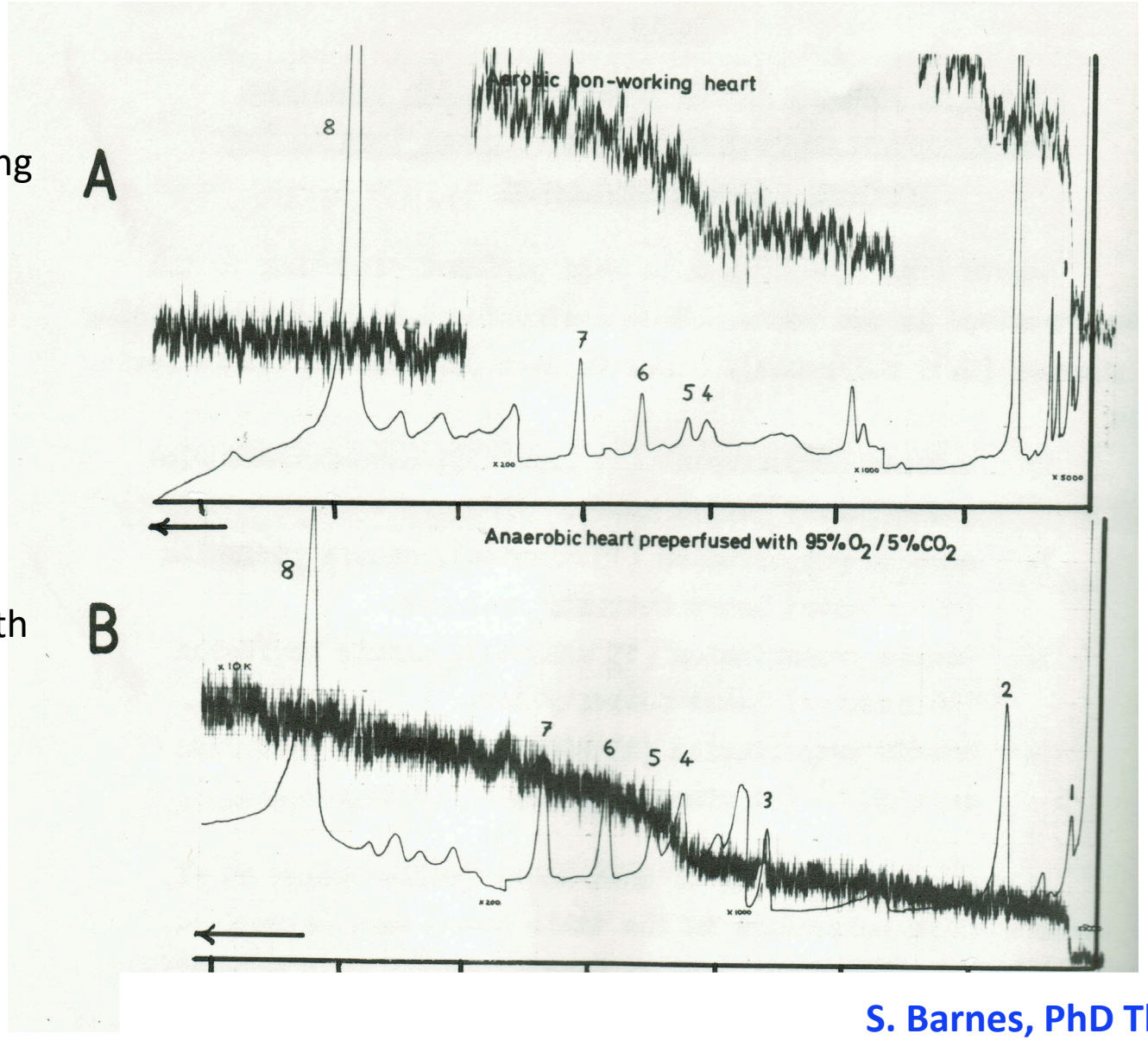


S. Barnes, PhD Thesis

Radio GC analysis of beating heart

Aerobic non-working heart

Anaerobic beating heart perfused with 95% O_2/5% CO_2



S. Barnes, PhD Thesis

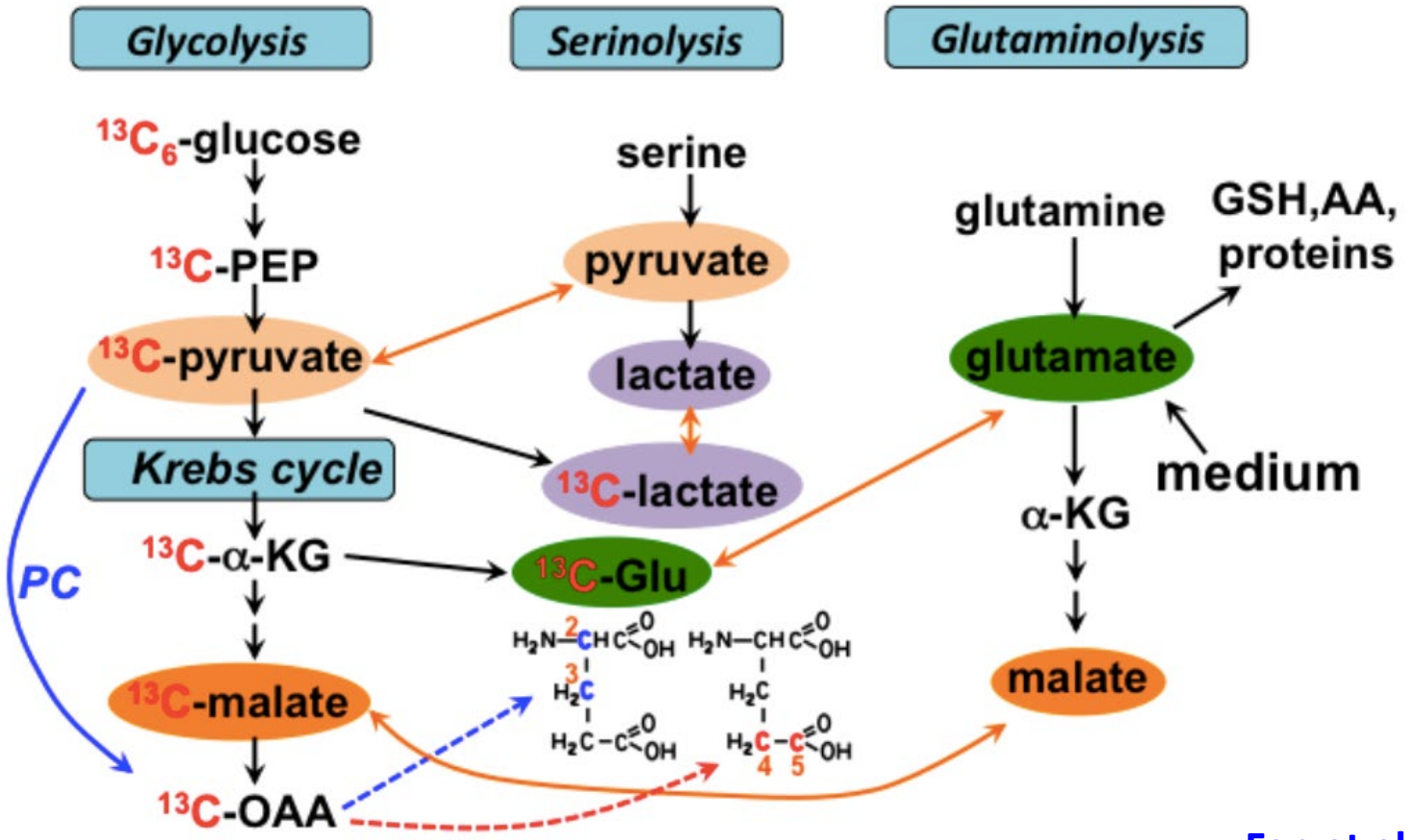
Fluxomics

See talk by Teresa Fan

https://www.uab.edu/proteomics/metabolomics/workshop/2018/videos/fan_day3.html

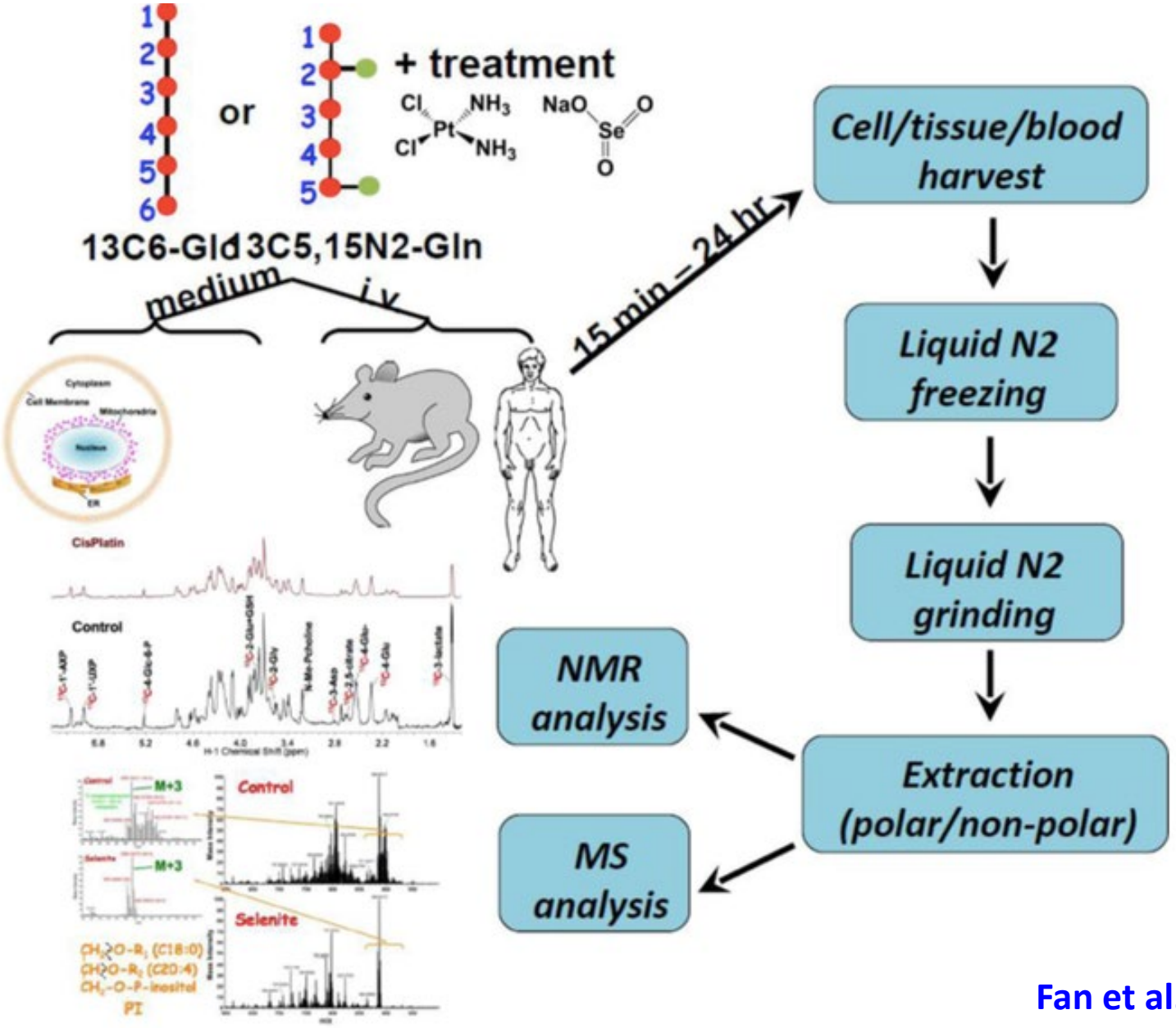
Fluxomics with stable isotopes

- A feature of many metabolites is that they have multiple origins

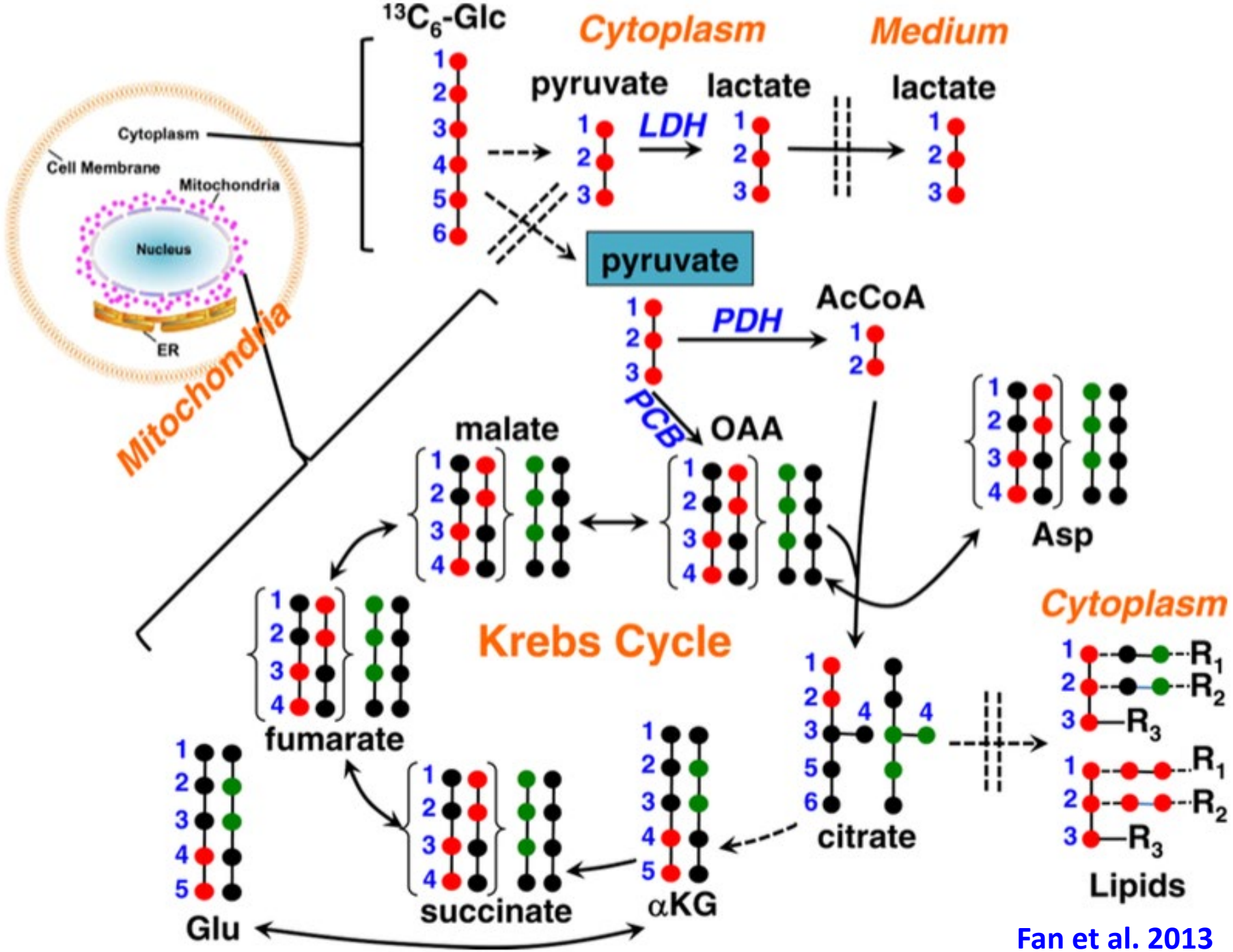


Fan et al. 2013

Stable isotope resolved metabolomics

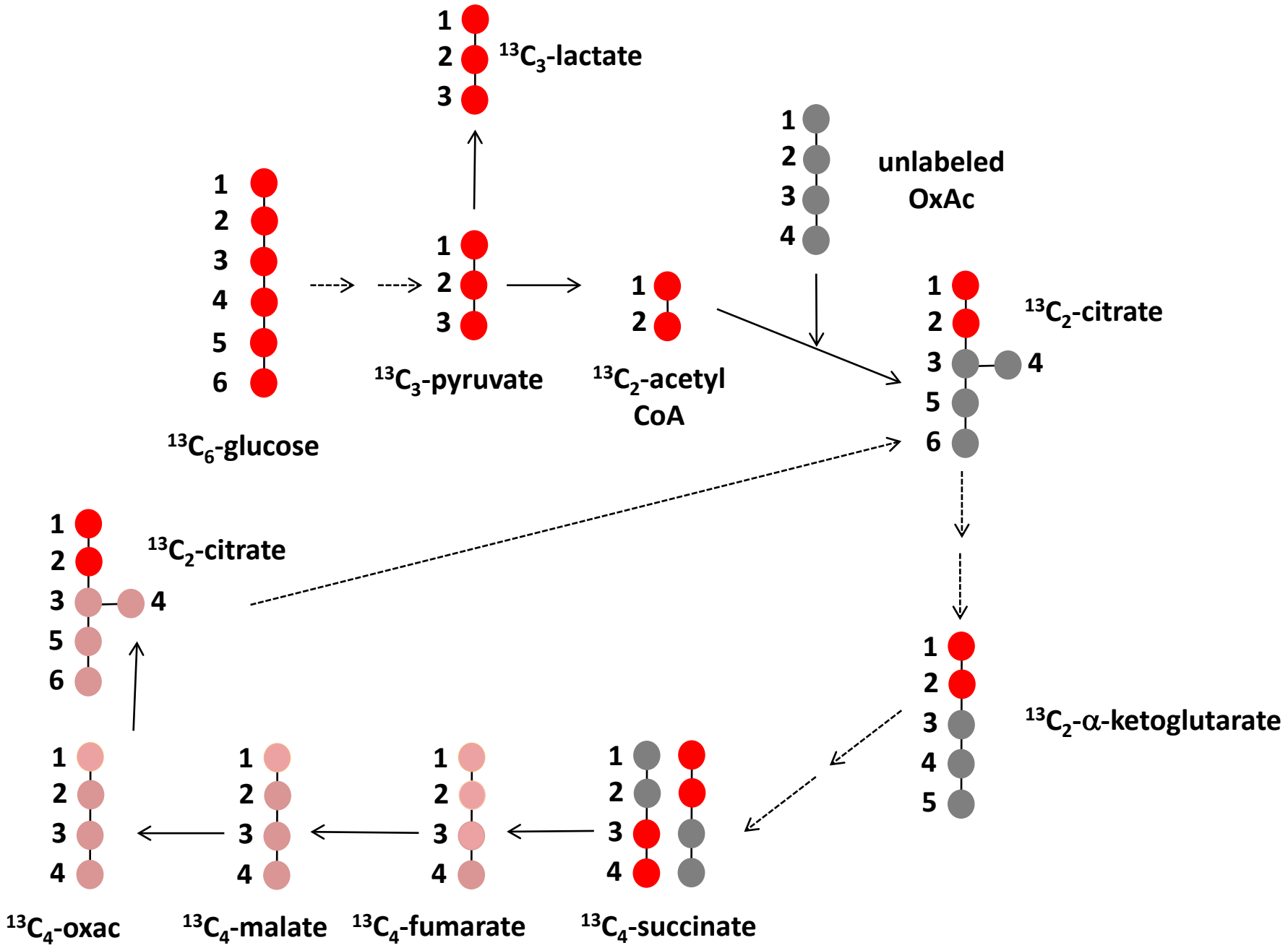


Fan et al. 2013

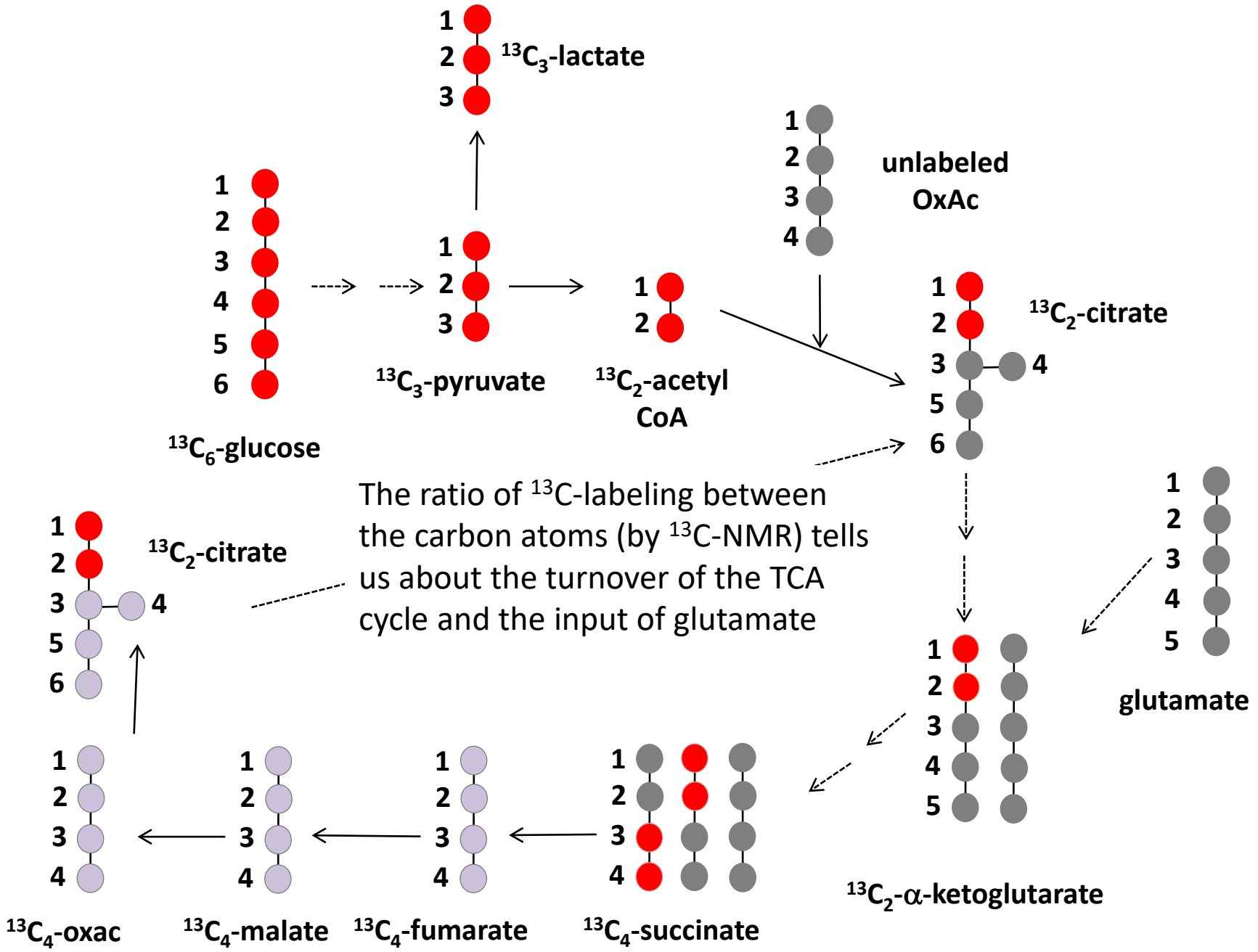


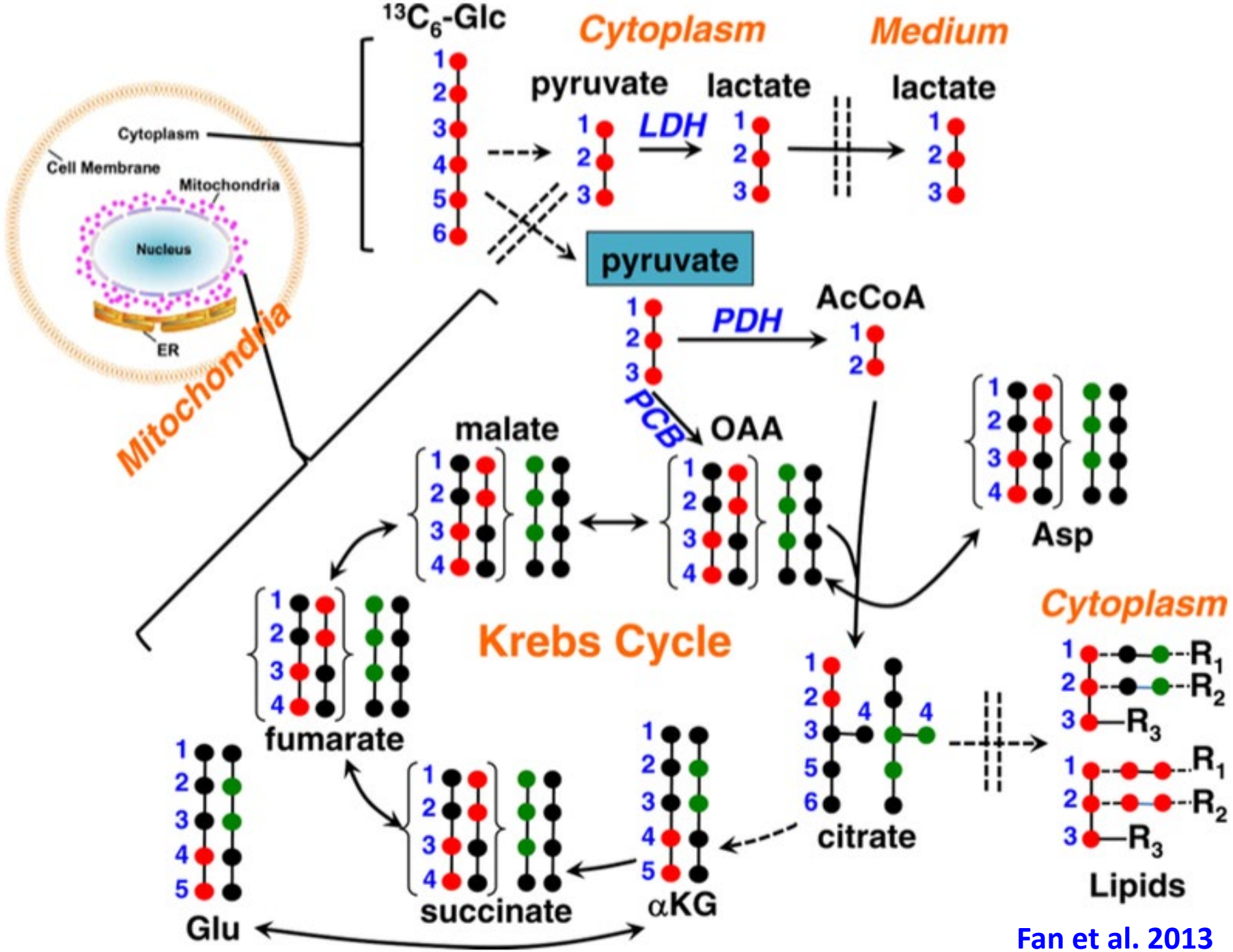
Fan et al. 2013

Ideal metabolism of glucose



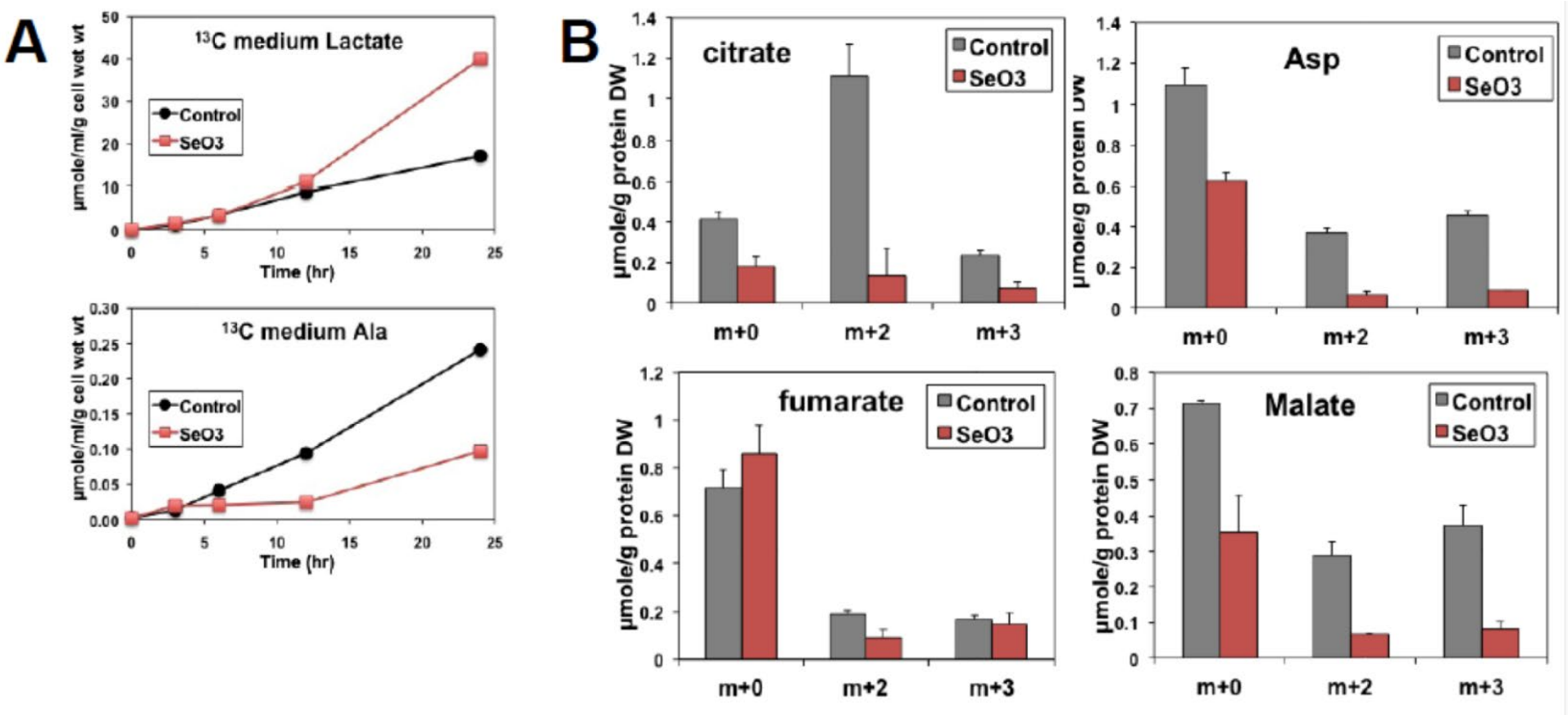
Effect of glutamate turnover





Fan et al. 2013

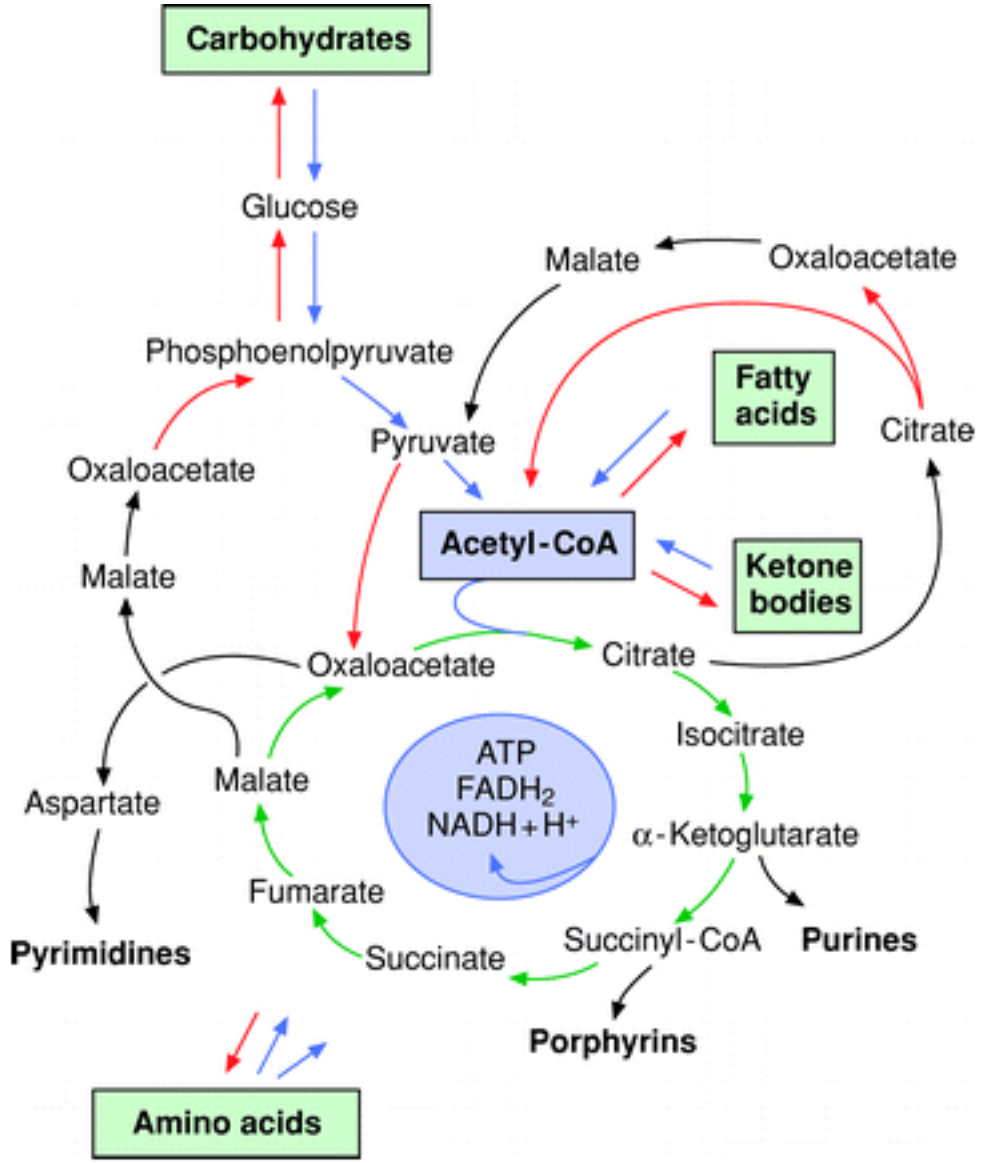
Effect of selenite on pools of intermediates



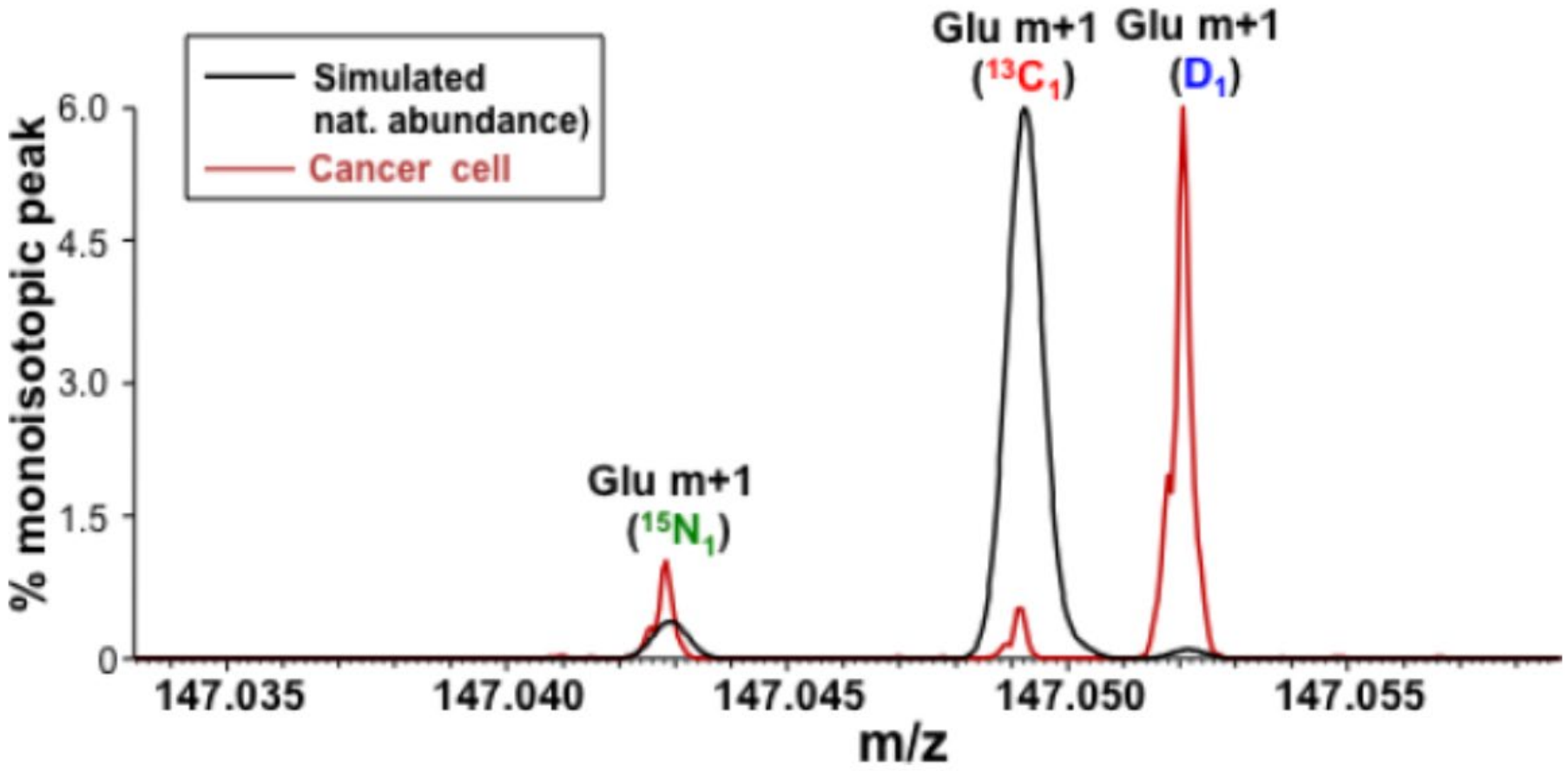
Pyruvate carboxylase converts pyruvate to oxaloacetate and by-passes the early steps in the Krebs cycle. Treatment of the cells with selenite blocks this step and the ^{13}C-content of citrate sharply decreases

Fan et al. 2013

Anaplerotic reactions

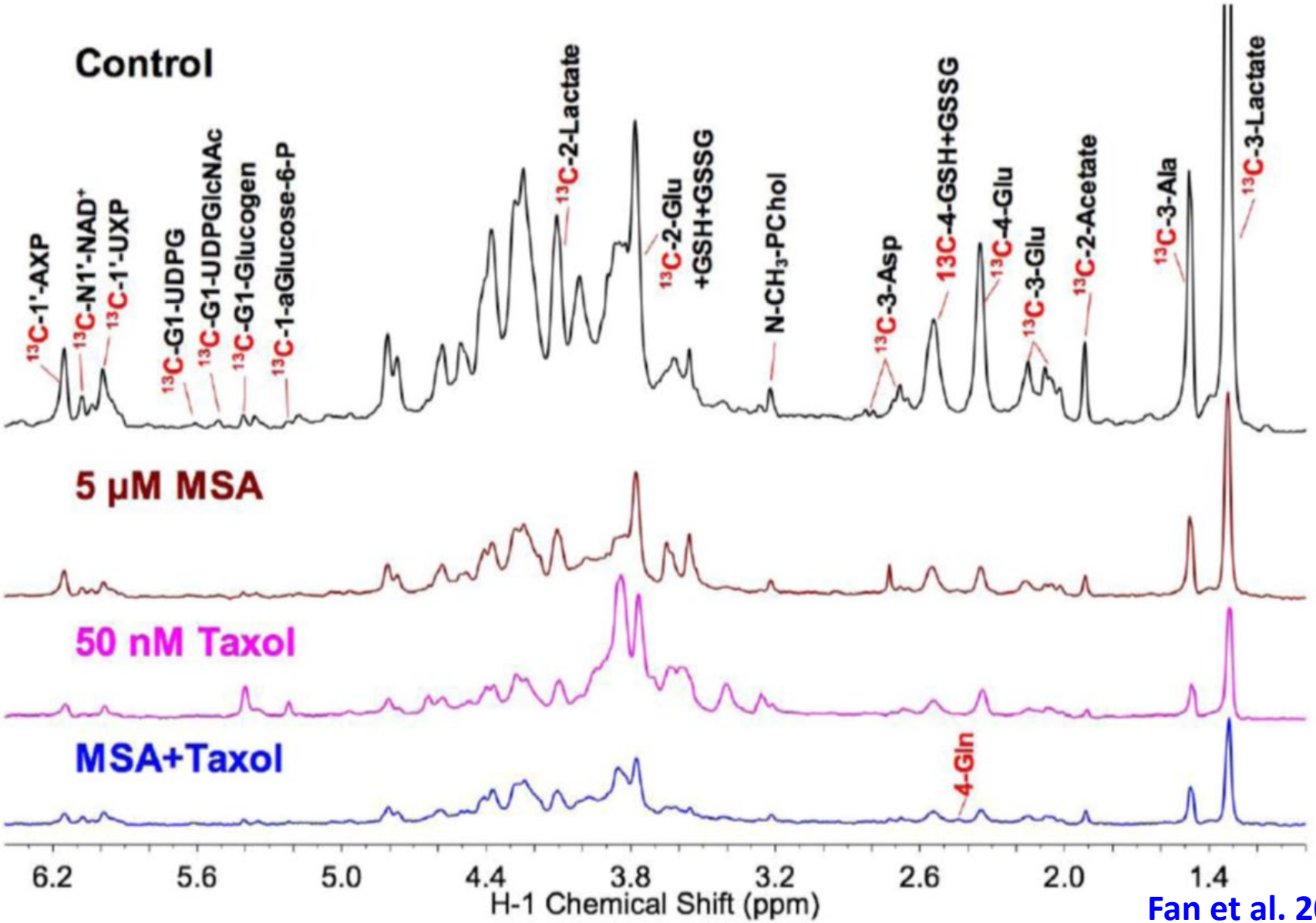


High resolution FT-ICR-MS



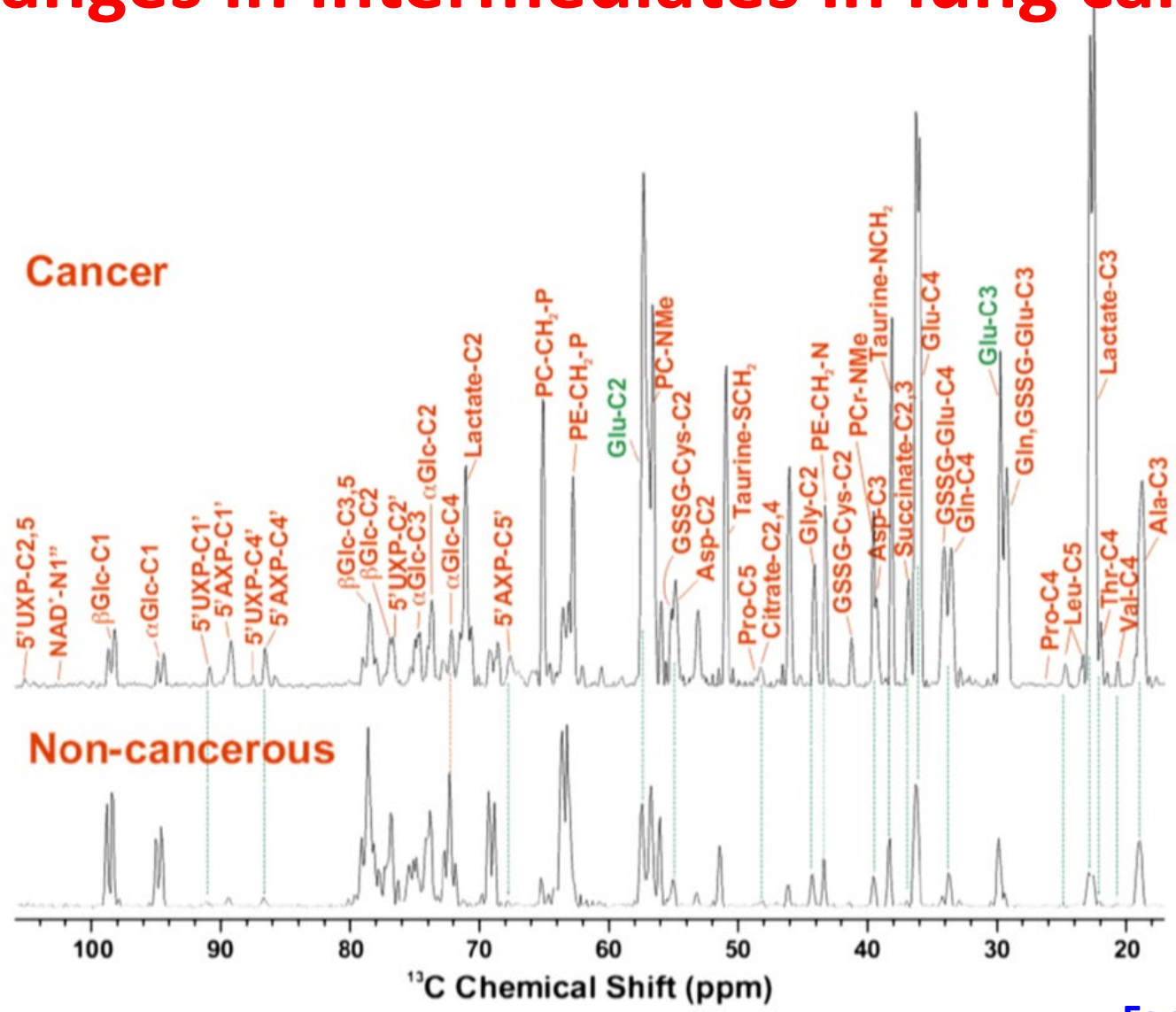
Fan et al. 2013

Use of ^{1}H-^{13}C-NMR



Fan et al. 2013

Changes in intermediates in lung cancer

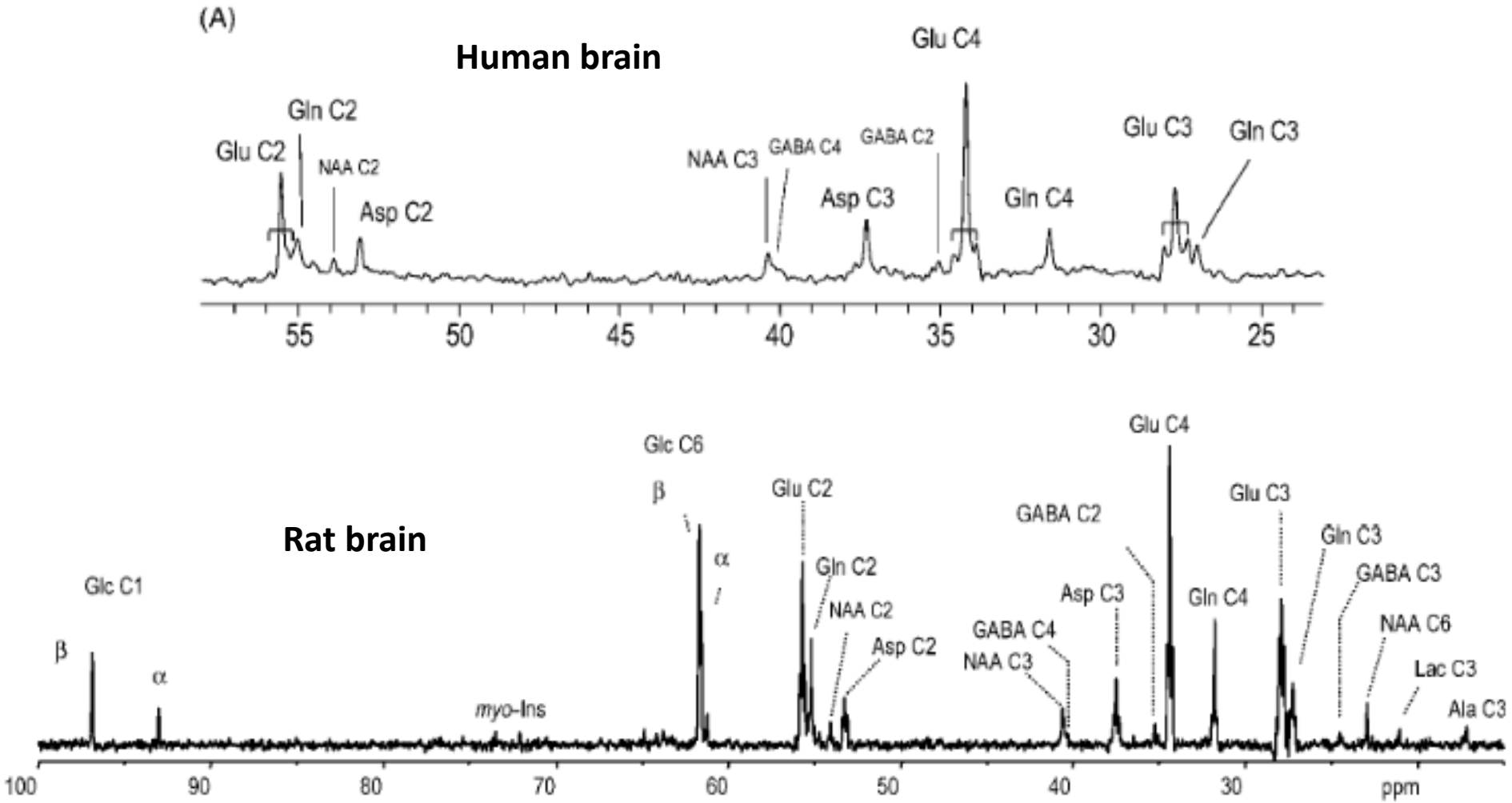


Fan et al. 2013

Biological NMR

- If ^{13}C-labeled precursors are used, there is a very much enhanced set of ^{13}C NMR resonances
- You have a choice between analysis of a biological extract (have all the time you need)
- And direct analysis in tissue:
  - Surface coil technology in the living animal
  - Magic Angle Spinning on a piece of tissue

NMR analysis of metabolites from ^{13}C-labeled precursors using pulse sequences



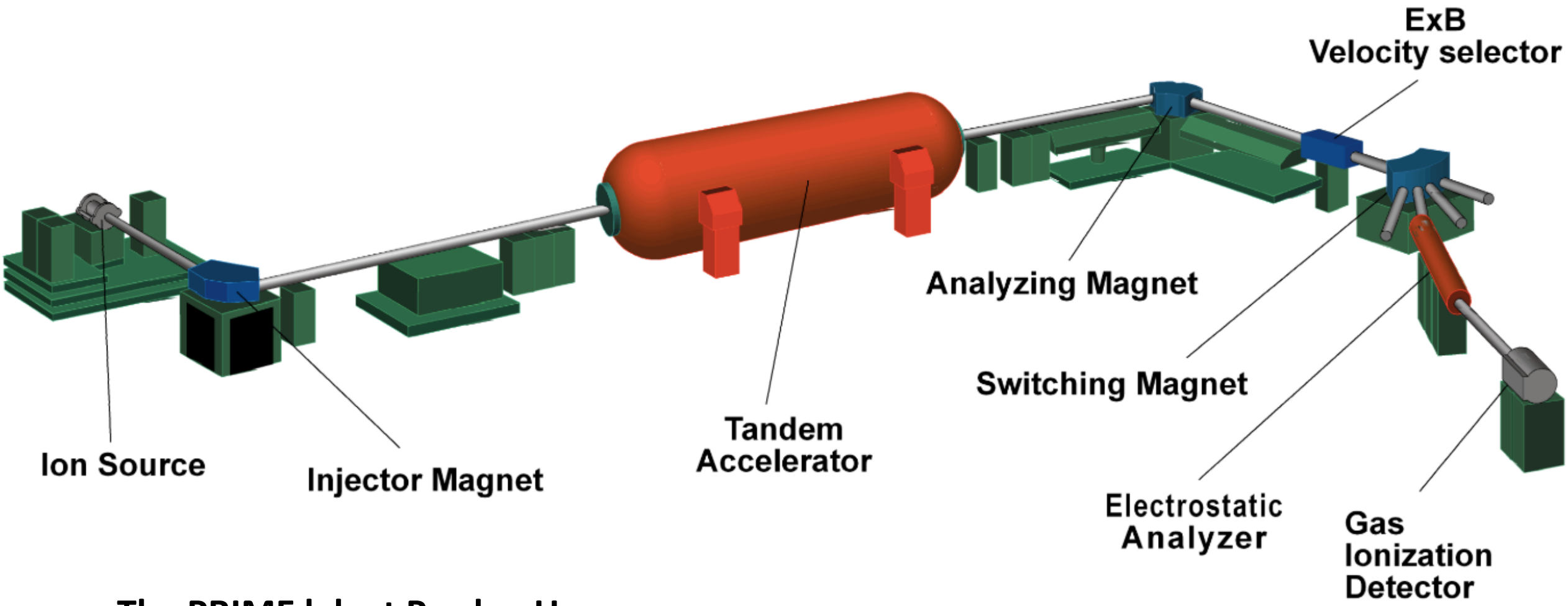
Probing the depths of metabolite penetration into tissues

Ultimate sensitivity by sacrificing metabolite identity at physiologic sites by ^{14}C-labeling the precursor of interest

Technology the same as the one used for radiocarbon dating

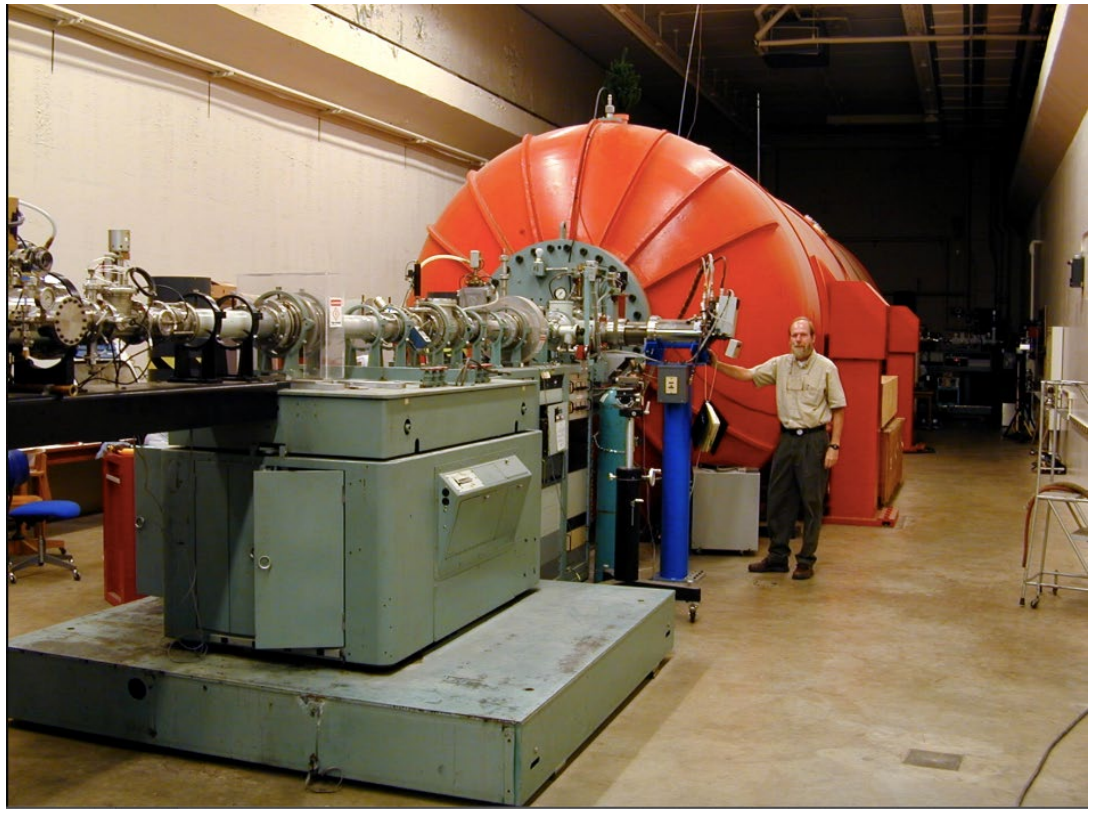
Accelerator mass spectrometry (AMS)

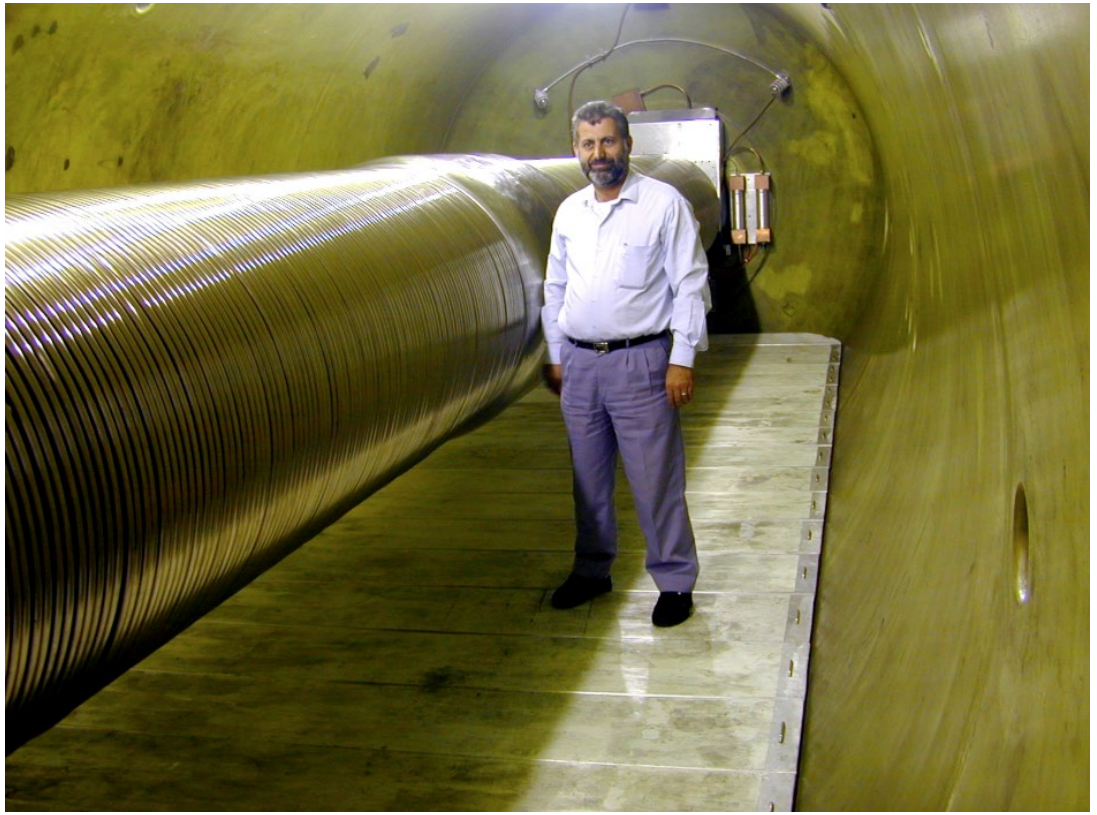
The ultimate mass spectrometer

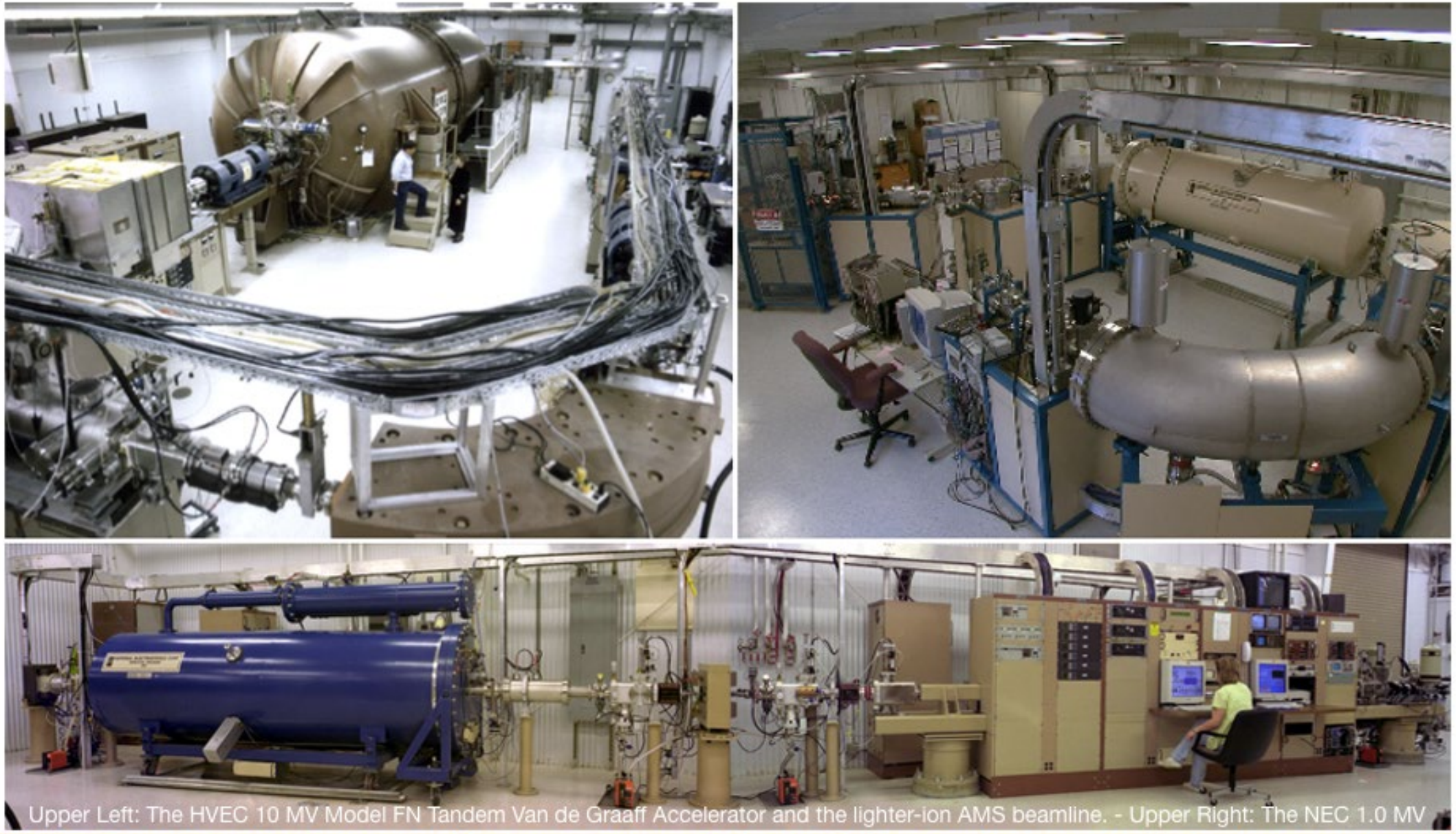


The PRIME lab at Purdue U

The Van der Graaf accelerator – PRIME lab



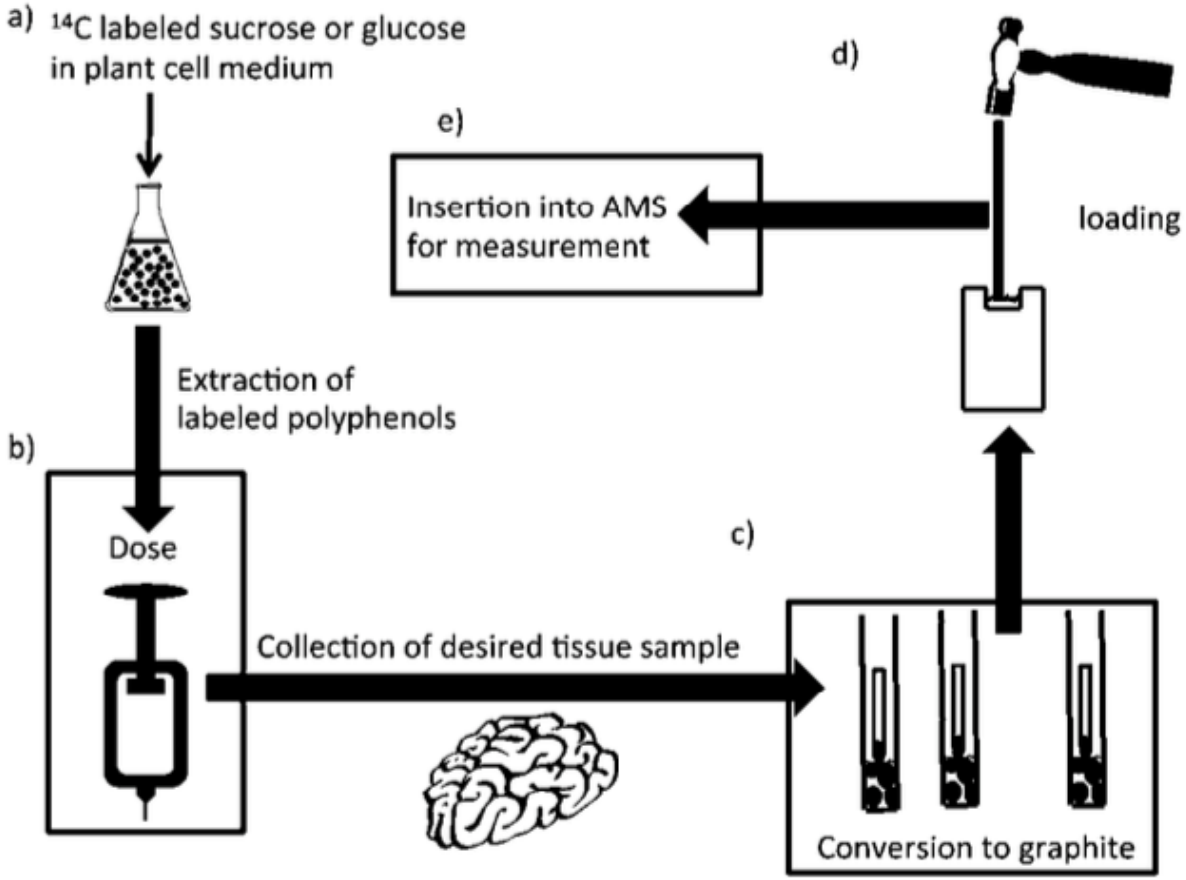


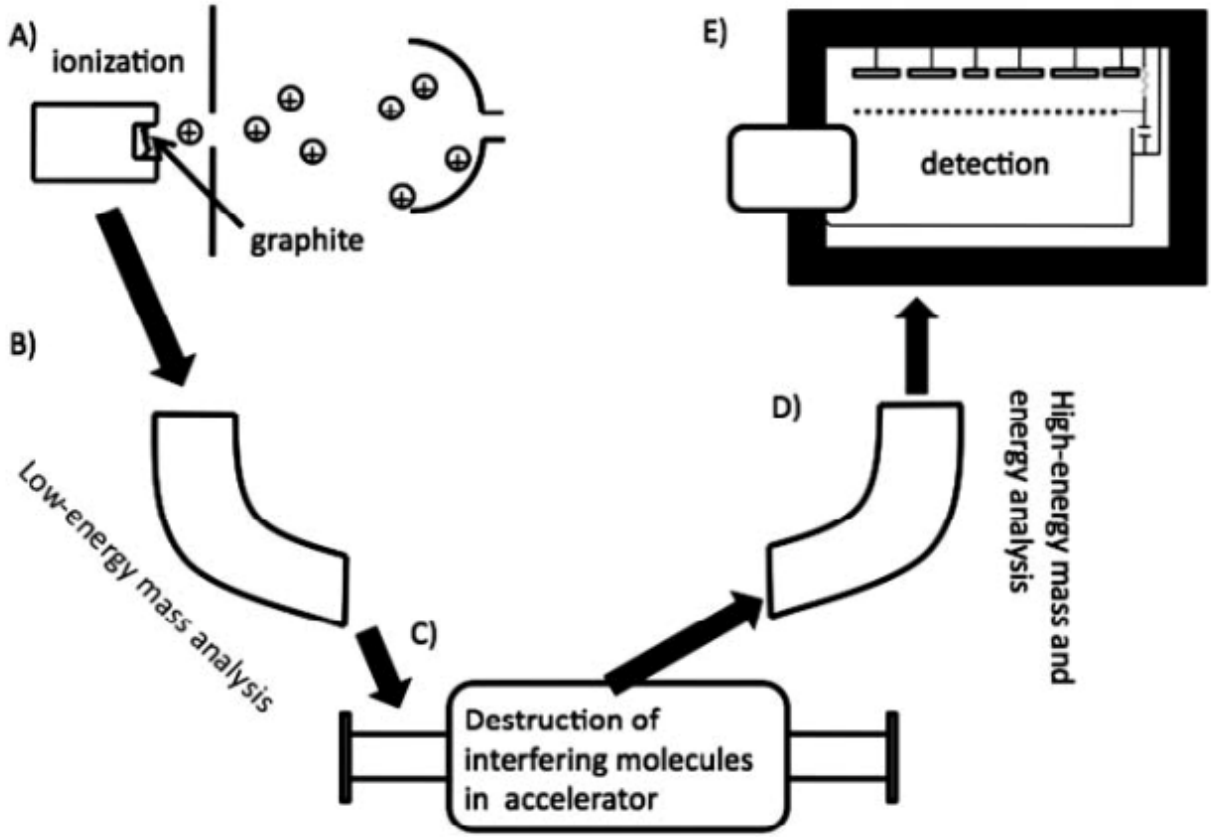


Upper Left: The HVEC 10 MV Model FN Tandem Van de Graaff Accelerator and the lighter-ion AMS beamline. - Upper Right: The NEC 1.0 MV Model 3SDH-1 Tandem Accelerator for BioAMS. - Lower: The NEC 1.7 MV Model 5SDH-2 Tandem Accelerator for Ion Beam Analysis and neutron production.

The AMS facility at the Lawrence Livermore National Laboratory

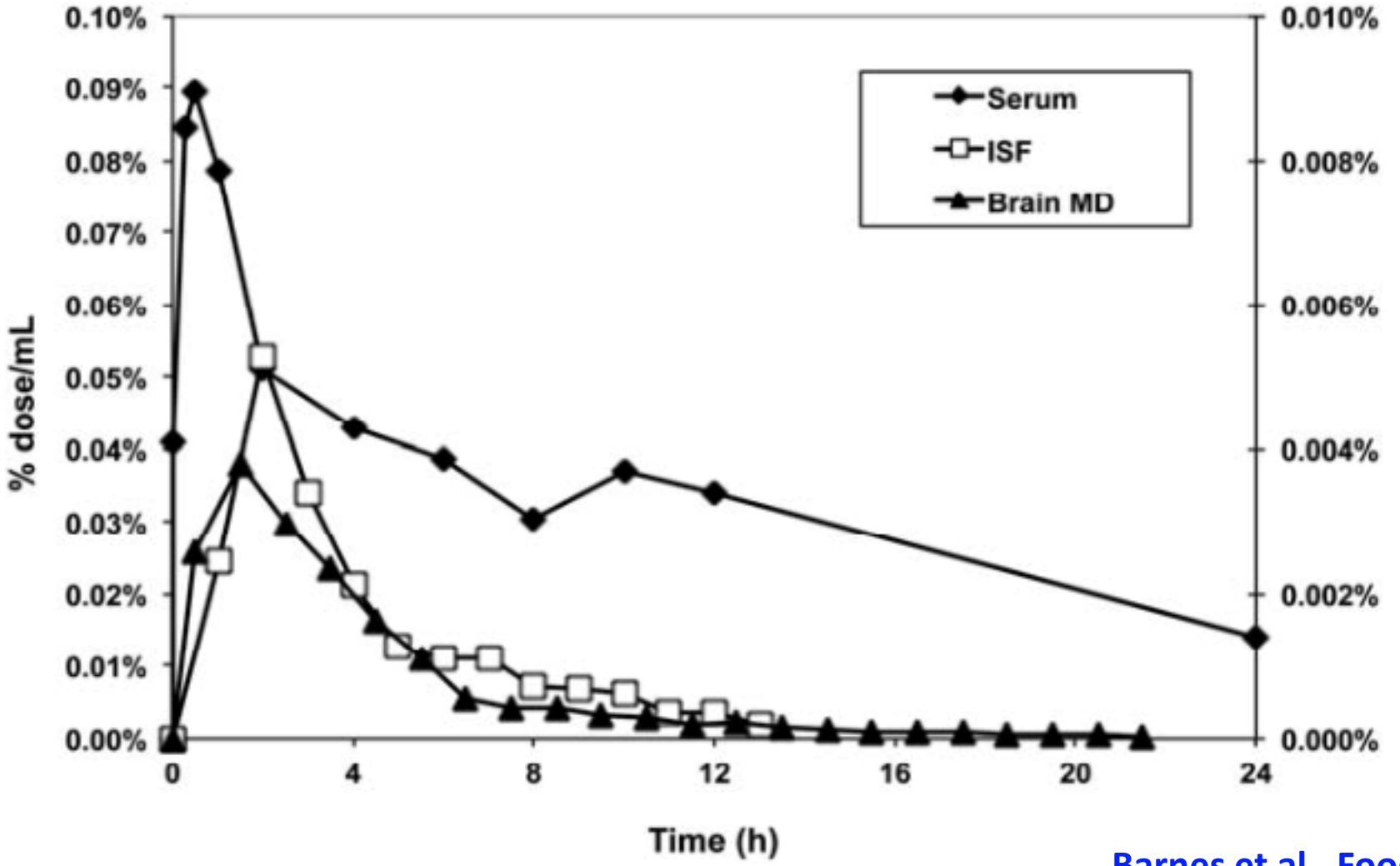
Tracing the appearance of a ^{14}C-labeled precursor





Barnes et al., Food Funct 2011

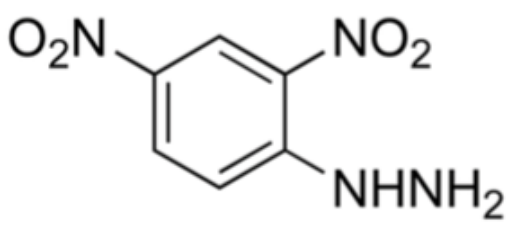
Tracing the movement of ^{14}C-intermediate in tissues



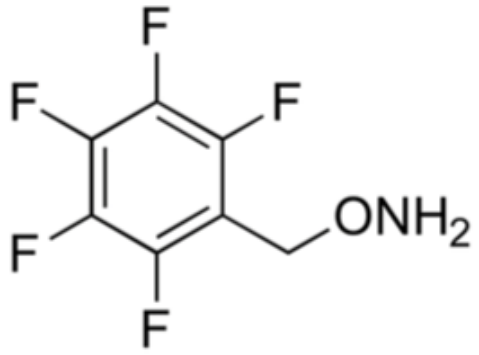
Barnes et al., Food Funct 2011

Using chemical reagents in metabolomics

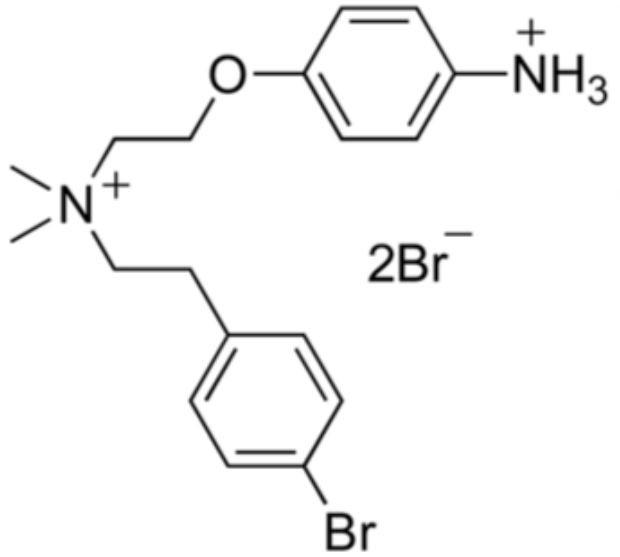
Carbonyl derivatization reagents



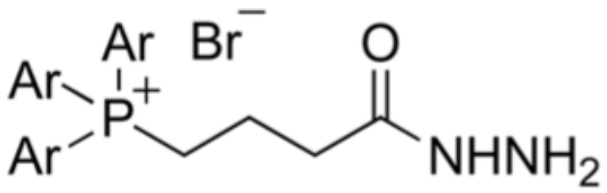
DNPH



PFBHA

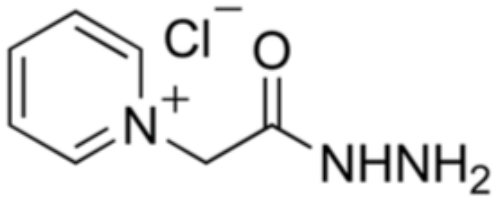


4-APEBA



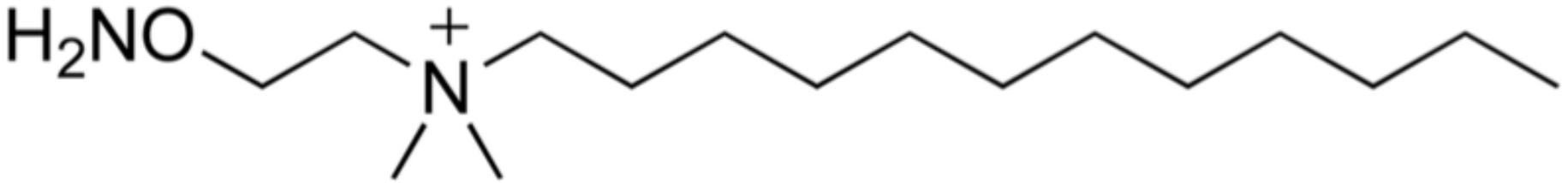
Ar = 2,4,6-trimethoxyphenyl

TMPP-PrG

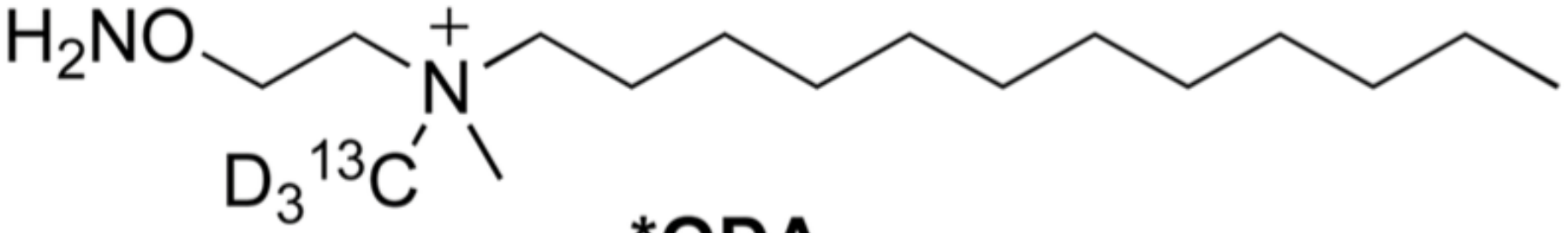


Girard-P reagent

Isotopic carbonyl reagents

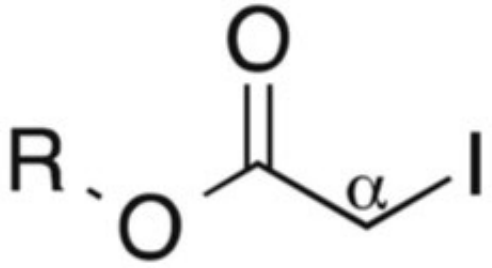


QDA

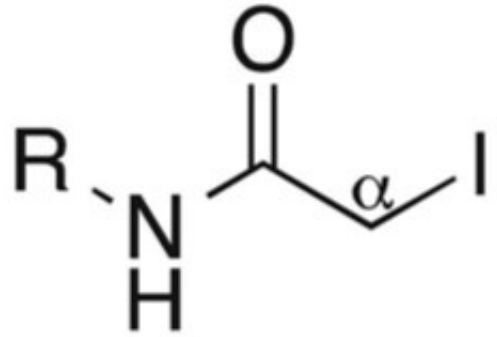


***QDA**

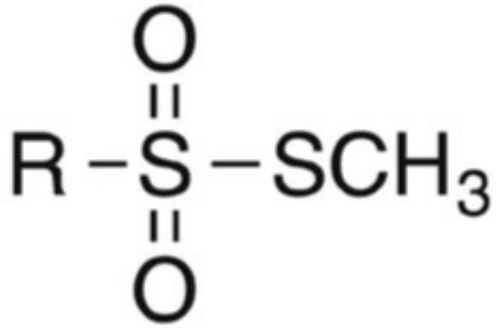
Thiol derivatization reagents



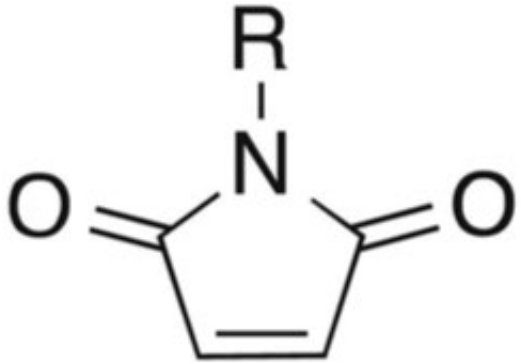
IAA



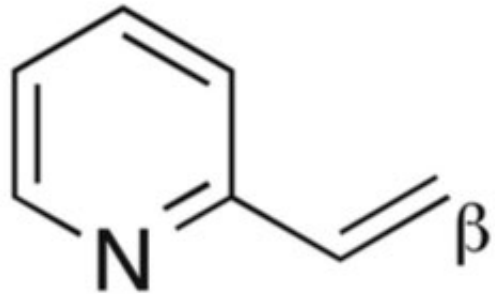
IAM



R = CH_3, **MMTS**

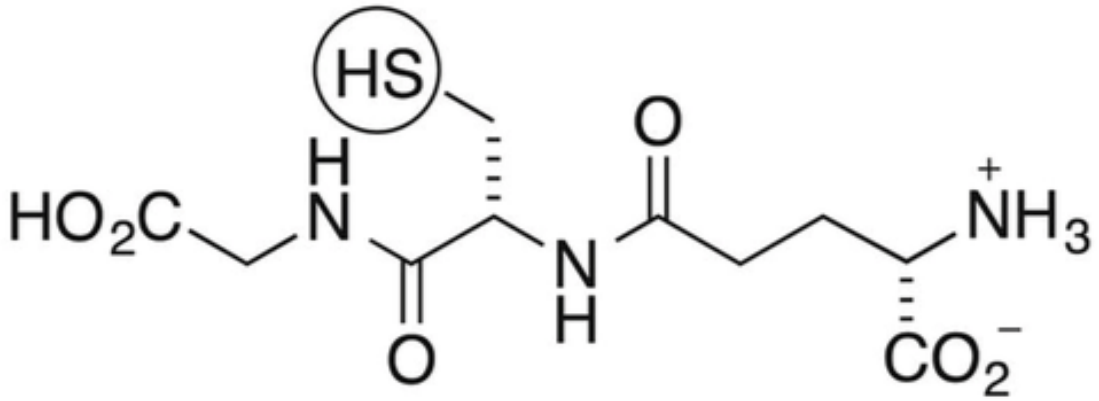


R = CH_3CH_2, **NEM**

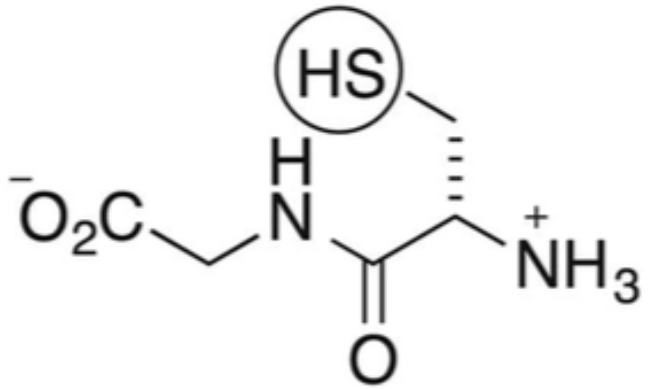


VP

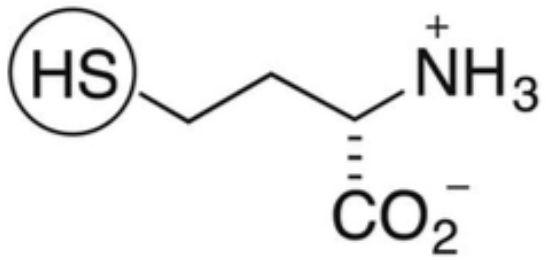
Detectable thio-metabolites



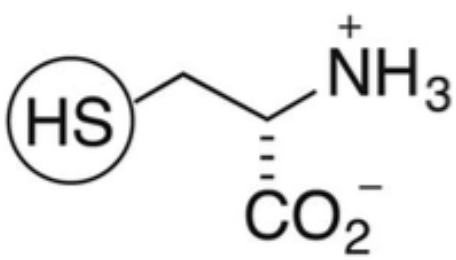
L-glutathione



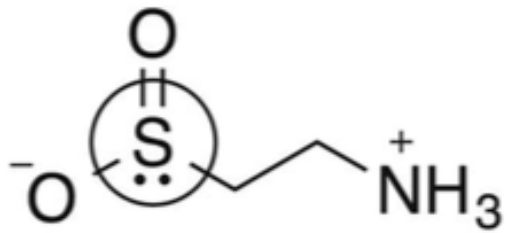
L-cysteinylglycine



L-homocysteine

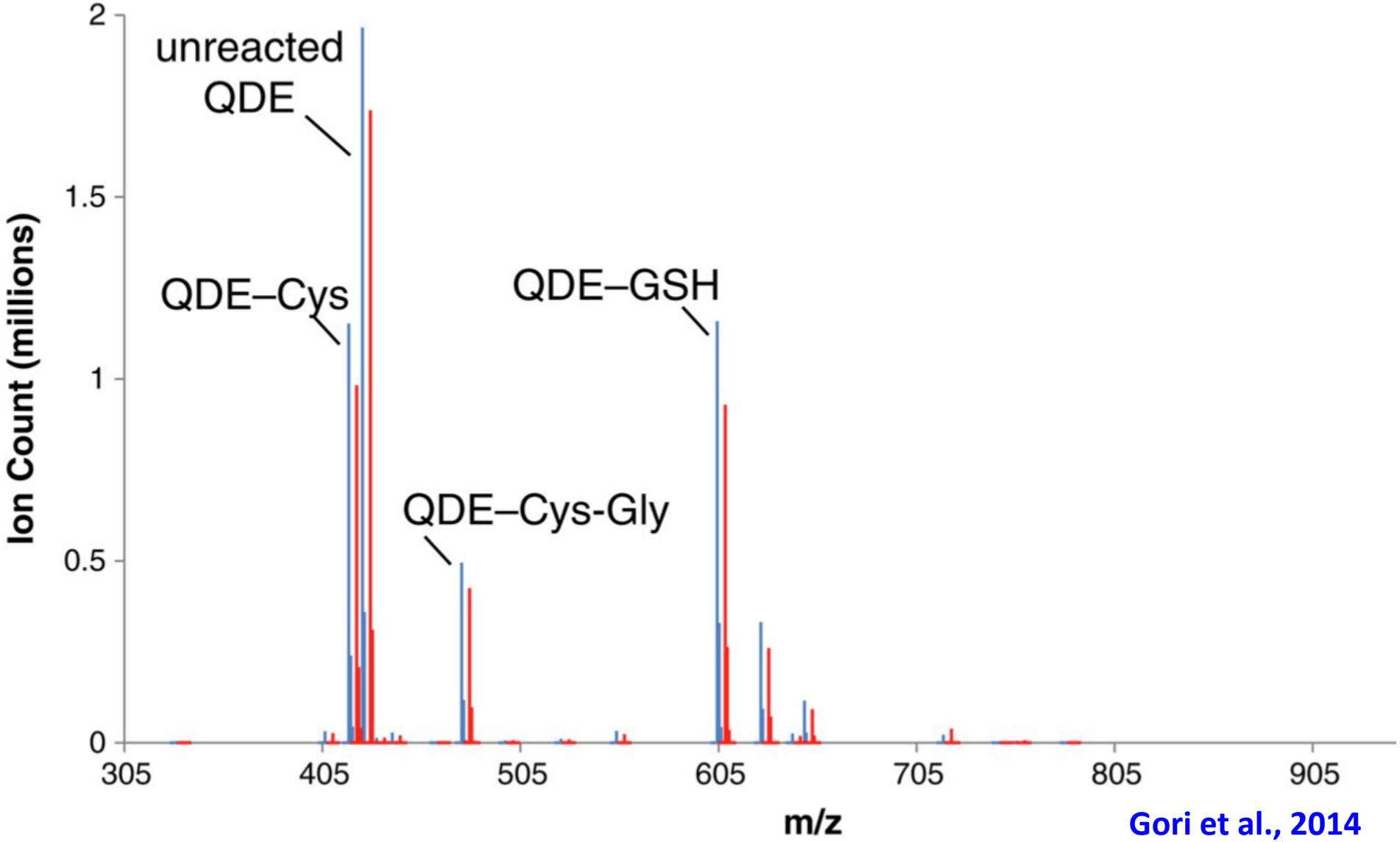


L-cysteine



hypotaurine

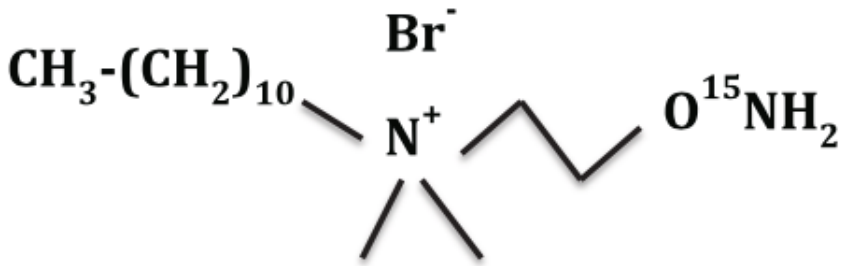
Thiol metabolites in A459 cell extract



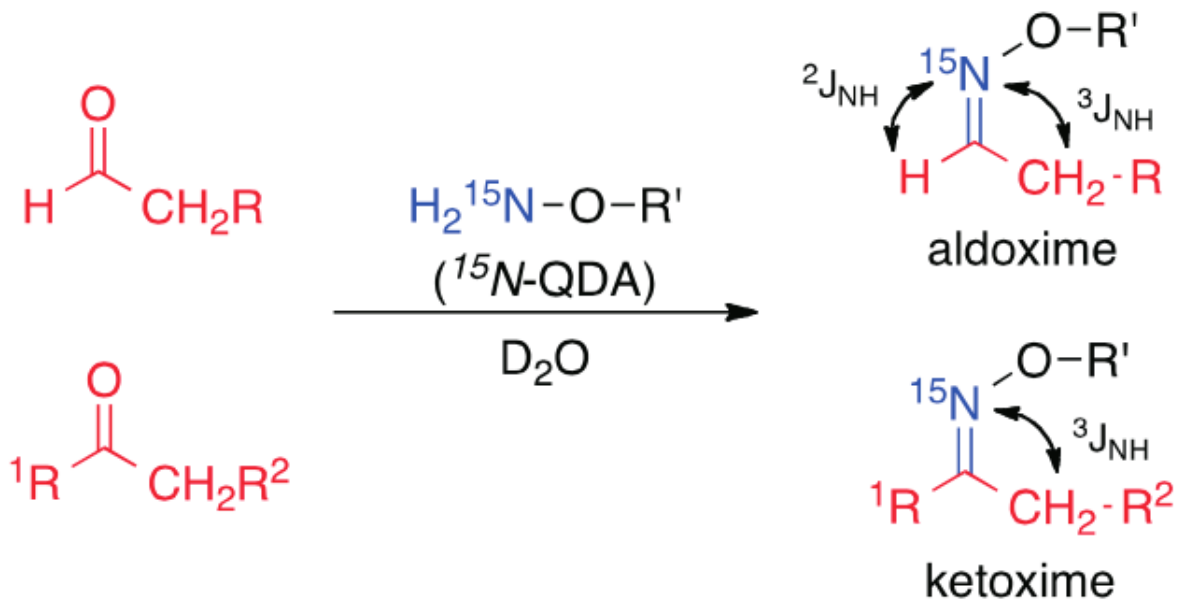
Gori et al., 2014

^{15}N-labeled derivatization reagent

A

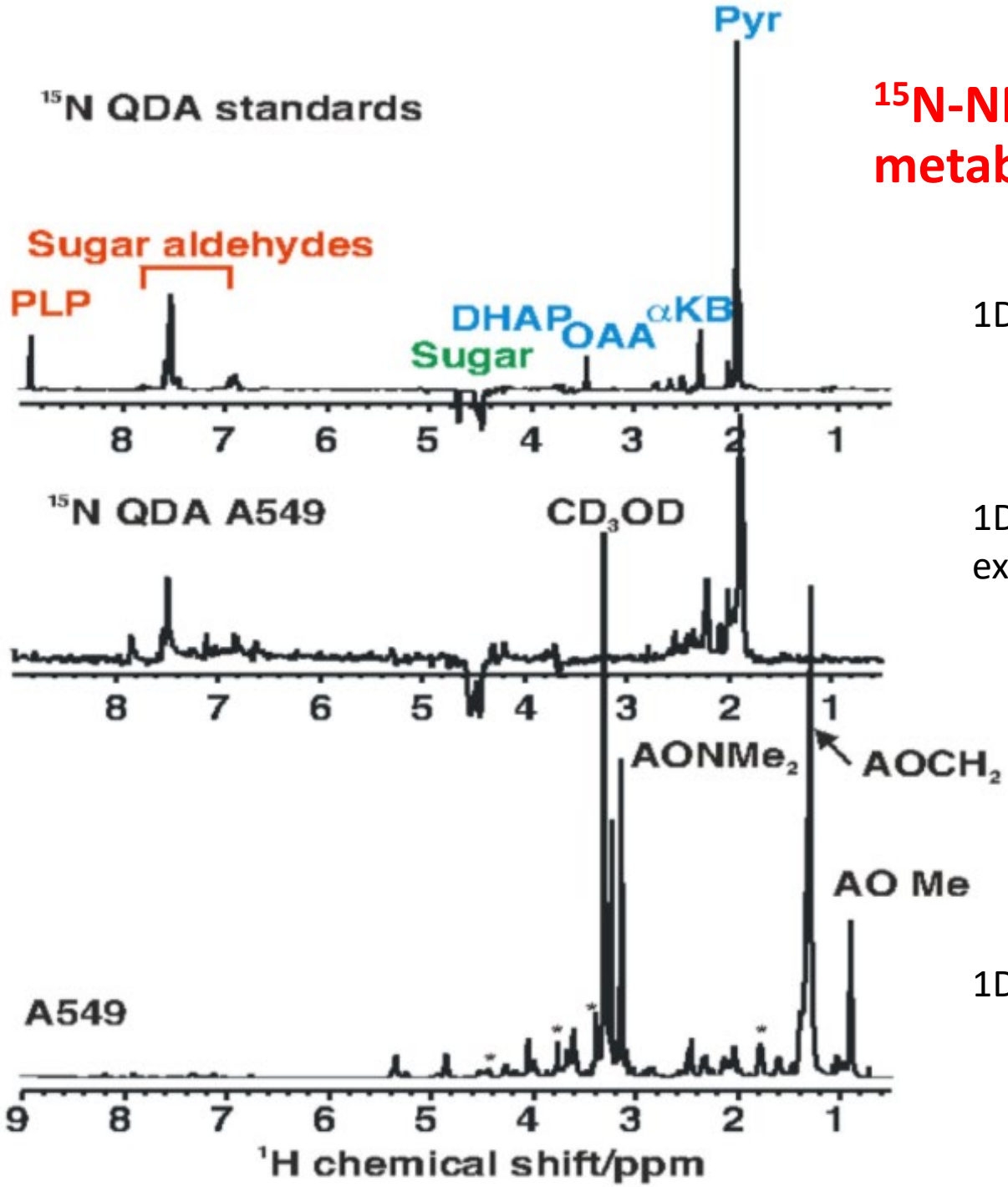


B



Lane et al., 2014

^{15}N-NMR of derivatized metabolites



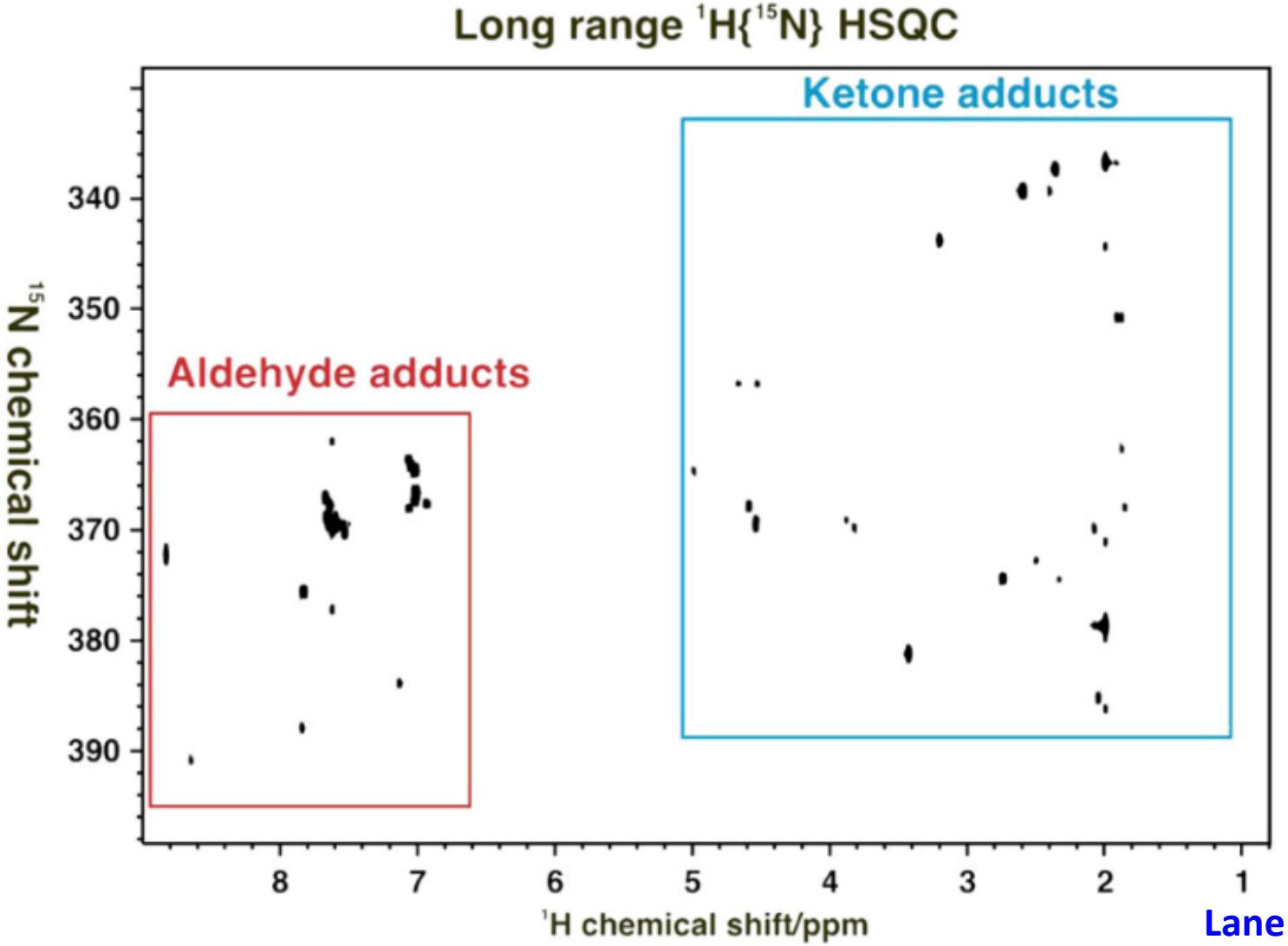
1D-HSQC spectrum of stds

1D-HSQC spectrum of polar extract of A549 cells

1D-presat spectrum of A549 cells

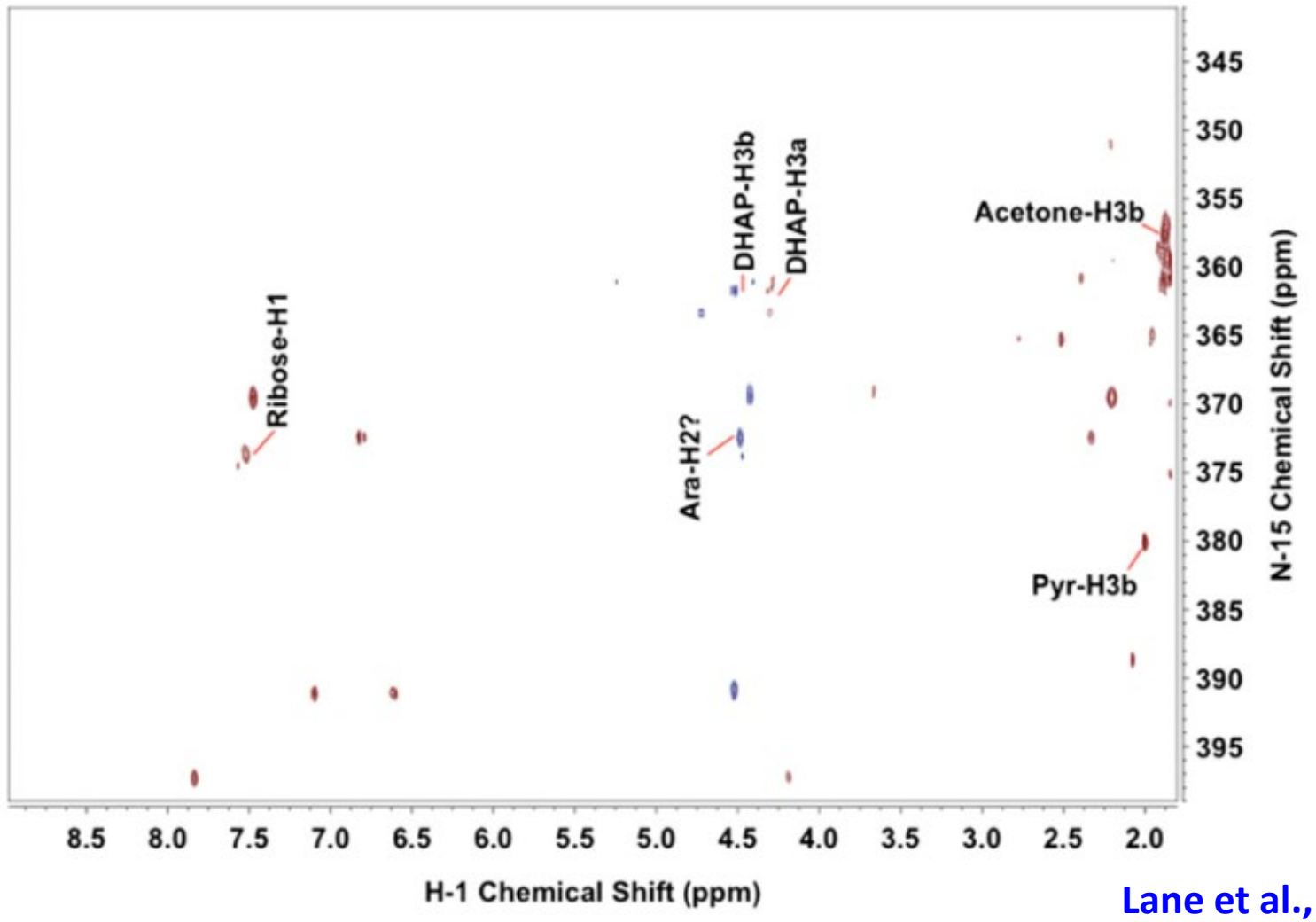
Lane et al., 2014

2D-^{1}H, ^{15}N-NMR of standards



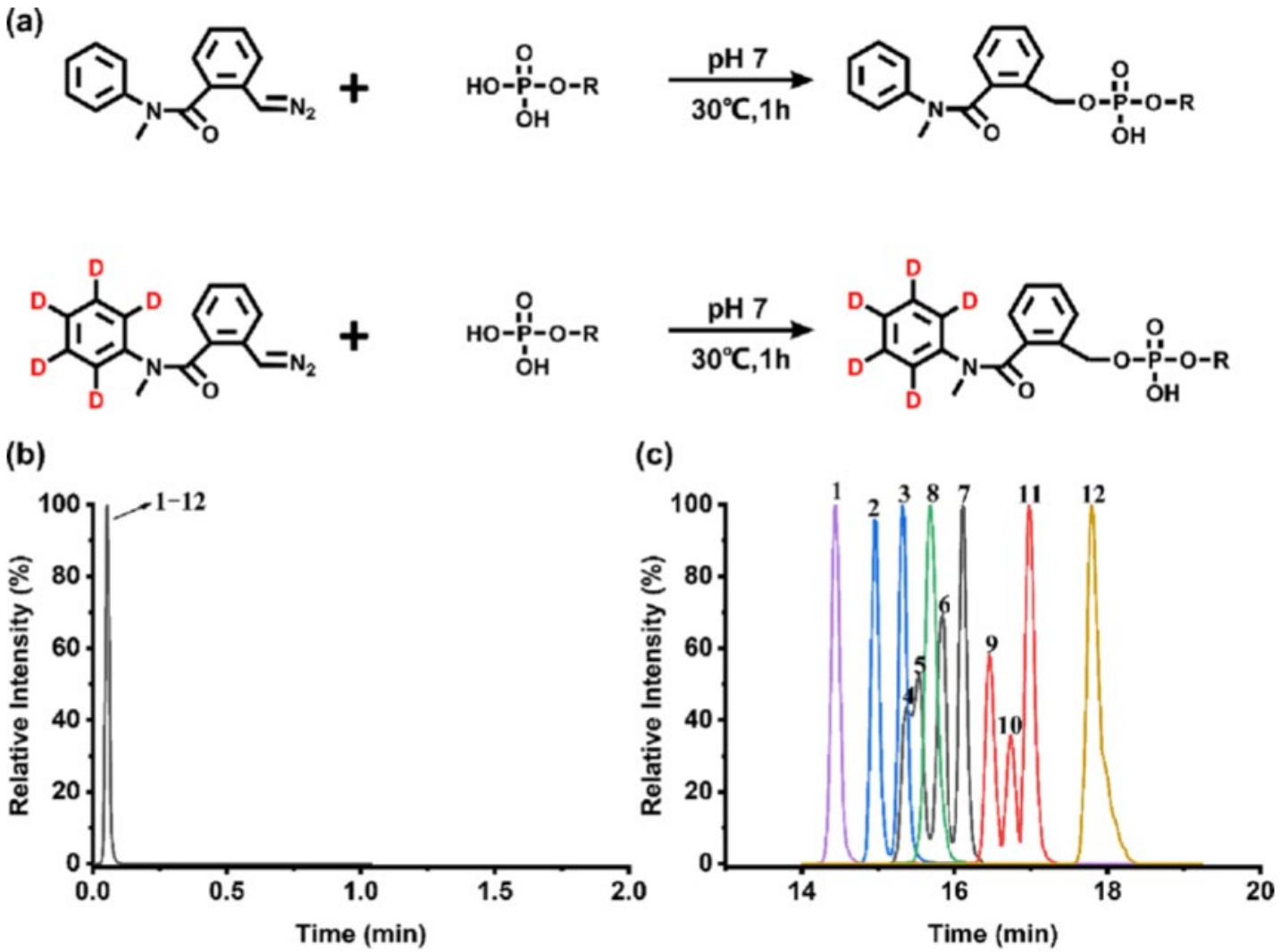
Lane et al., 2014

2D-^{1}H, ^{15}N-NMR of A459 cell extract



Lane et al., 2014

Analysis of sugar phosphates



The reagent is:
2-(diazo-methyl)-N-methyl-N-phenyl Benzamide

Has to be synthesized.

See **Ultrasensitive Determination of Sugar Phosphates in Trace Samples by Stable Isotope Chemical Labeling Combined with RPLC–MS**
Sha Li, Fei-Long Liu, Zheng Zhang, Xiao-Ming Yin, Tian-Tian Ye, Bi-Feng Yuan, and Yu-Qi Feng

Analytical Chemistry March 11, 2022

Hyperpolarized NMR

- **NMR's advantages**
  - **Non-destructive**
  - **Quantitative**
  - **Usable *in situ***
- **NMR's disadvantages**
  - **The difference in the populations of the low and high energy states is 1 part in a million, i.e., low sensitivity**
  - **If an excess in the high energy state could be achieved, NMR could become >10,000 times more sensitive, albeit that the half-life in the hyperpolarized state may be only 30 s**

JCIM JOURNAL OF CHEMICAL INFORMATION AND MODELING

Article

Cite This: J. Chem. Inf. Model. 2019, 59, 605−614

pubs.acs.org/jcim

Kinetic Analysis of Hepatic Metabolism Using Hyperpolarized Dihydroxyacetone

Alexander Kirpich,[†,‡,¶,○] Mukundan Ragavan,[§,○] James A. Bankson,[∥,⊥] Lauren M. McIntyre,[#,¶] and Matthew E. Merritt*[,§,¶]

[†]Department of Biology, University of Florida, Gainesville, Florida 32611, United States

[‡]Informatics Institute, University of Florida, Gainesville, Florida 32611, United States

[¶]Southeast Center for Integrated Metabolomics, University of Florida, Gainesville, Florida 32611, United States

[§]Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, Florida 32611, United States

[∥]Department of Imaging Physics, The University of Texas MD Anderson Cancer Center, Houston, Texas 77030, United States

[⊥]The University of Texas MD Anderson Cancer Center, UTHealth Graduate School of Biomedical Sciences, Houston, Texas 77030, United States

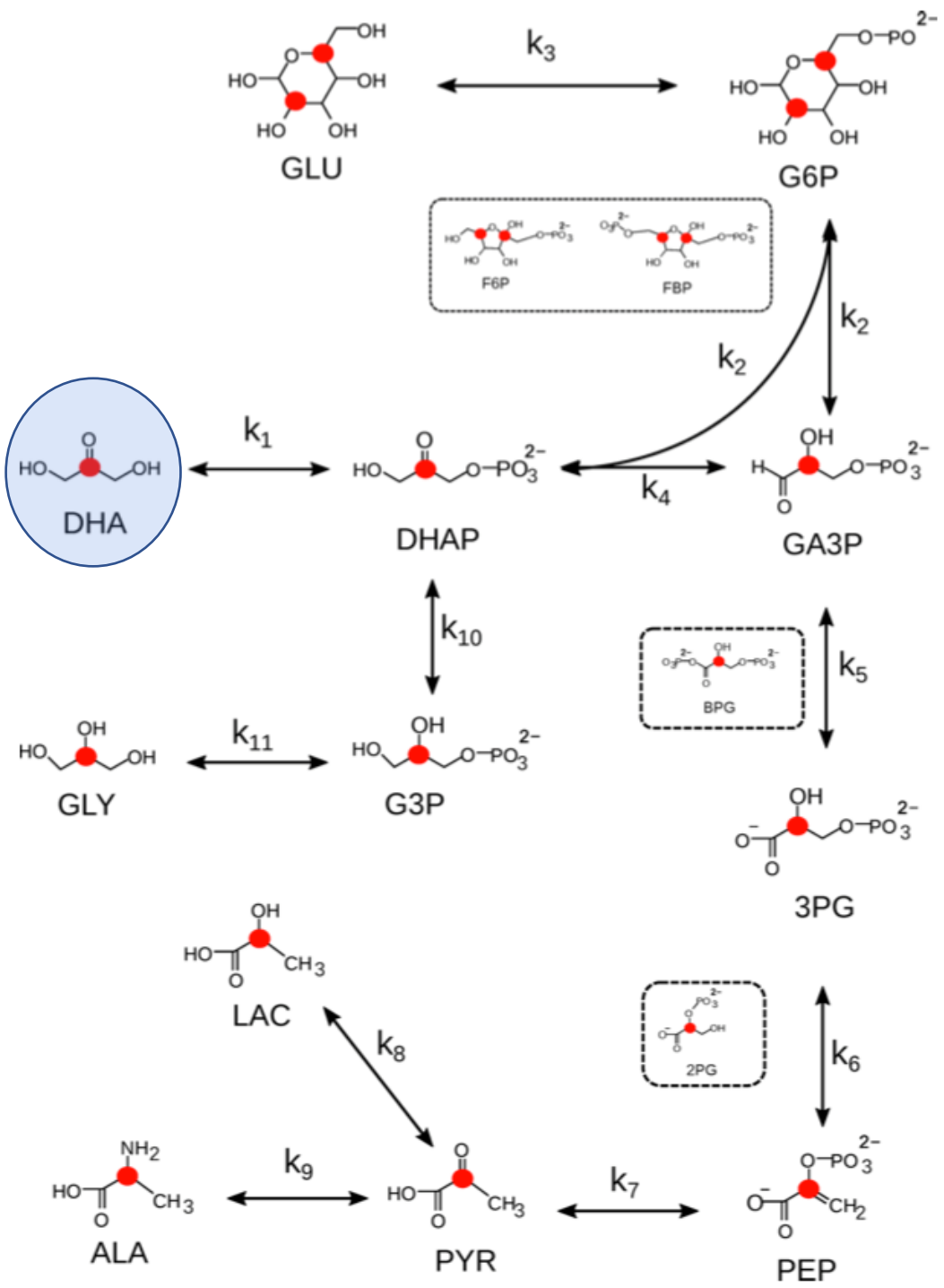
[#]Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, Florida 32611, United States

Making hyperpolarized dihydroxyacetone

- An 8.0 M solution of [2-^{13}C]DHA in a (2:1) water:dimethyl sulfoxide (DMSO) mixture was doped with 15 mM stable trityl free radical (Oxford Instruments Molecular Biotools) and 1.0 mM ProHance.
- The frozen sample was cooled to **1.05 K** in a pumped helium bath inside the magnetic field (3.35 T) of the HyperSense, and the microwave irradiation was turned on.
- Polarization took 1.5–2 h, the irradiation was turned off, and the sample was rapidly dissolved with 4 ml of hot (190 °C) PBS (10 mM, pH 7.4) and transferred to an 89-mm vertical 9.4 T NMR spectrometer for transfer into the perfusate chamber and spectral acquisition.

Animal experiment and data collection

- Isolated perfused mouse liver placed inside the wide bore of the NMR
- Liver perfused with either 0.2 mM octanoate or 0.2 mM octanoate/2 mM pyruvate
- Hyperpolarized DHA added and first free induction decay collected within 1 msec
- Subsequent FID collection was for 1 sec with a 2 sec delay before the next acquisition – 3 sec cycle time
- Total number of collections = 60, i.e., a total of 3 min
- Half-life of the ^{13}C signal was 32 sec, i.e., by 3 min the signal was 1.5625% of the starting signal



- DHA is first converted to DHA-PO_4
- DHA-PO_4 has a divergence of paths
  - To glycerol-3-PO_4 and then glycerol
  - With GA3P to FDP, G6P and glucose
  - Or by isomerizing to GA3P and then to 3PG, PEP, pyruvate, etc.
- Each step is governed by a linear rate constant, k_1, , k_2, etc.

PCCP

PAPER

View Article Online
View Journal | View Issue

Cite this: *Phys. Chem. Chem. Phys.*, 2019, 21, 22849

Production of highly concentrated and hyperpolarized metabolites within seconds in high and low magnetic fields†

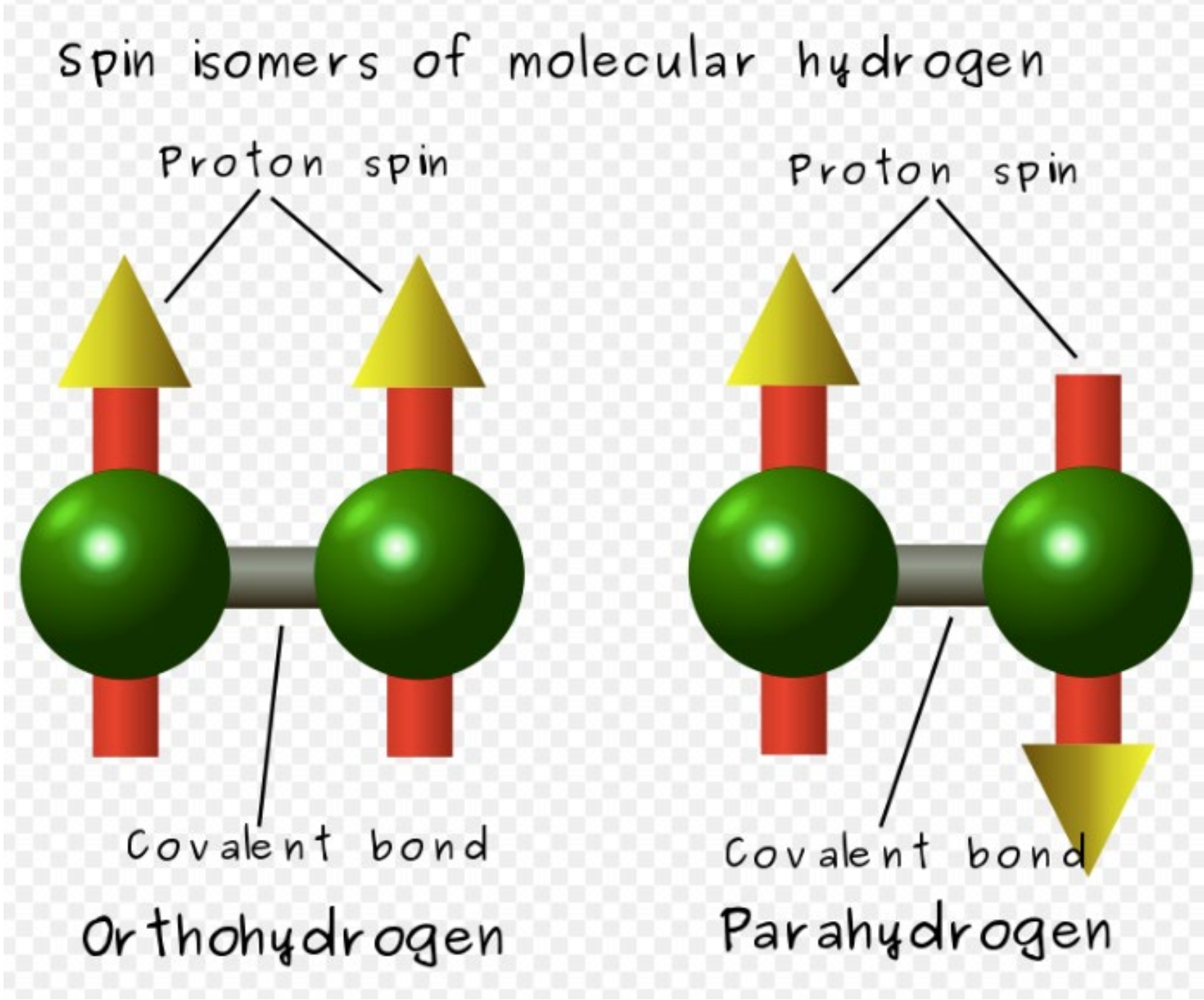
Sergey Korchak,[ab] Meike Emondts,[cd] Salvatore Mamone,[ab] Bernhard Blümich[d] and Stefan Glöggler*[ab]

Received 23rd September 2019,
Accepted 1st October 2019

DOI: 10.1039/c9cp05227e

rsc.li/pccp

Hyperpolarized metabolites are very attractive contrast agents for *in vivo* magnetic resonance imaging studies enabling early diagnosis of cancer, for example. Real-time production of concentrated solutions of metabolites is a desired goal that will enable new applications such as the continuous investigation of metabolic changes. To this end, we are introducing two NMR experiments that allow us to deliver high levels of polarization at high concentrations (50 mM) of an acetate precursor (55% ^{13}C polarization) and acetate (17% ^{13}C polarization) utilizing 83% *para*-state enriched hydrogen within seconds at high magnetic field (7 T). Furthermore, we have translated these experiments to a portable low-field spectrometer with a permanent magnet operating at 1 T. The presented developments pave the way for a rapid and affordable production of hyperpolarized metabolites that can be implemented in *e.g.* metabolomics labs and for medical diagnosis.



At room temperature, H_2 is 75% ortho and 25% para. Para is the lower energy state

At 1°K, H_2 is predominantly para.

Para H_2 aligns better with a magnetic field and efficiently transfers the polarization to another molecule

Hyperpolarized NMR Metabolomics at Natural ^{13}C Abundance

Arnab Dey, Benoît Charrier, Estelle Martineau, Catherine Deborde, Elodie Gandriau, Annick Moing, Daniel Jacob, Dmitry Eshchenko, Marc Schnell, Roberto Melzi, Dennis Kurzbach, Morgan Ceillier, Quentin Chappuis, Samuel F. Cousin, James G. Kempf, Sami Jannin, Jean-Nicolas Dumez, and Patrick Giraudeau*

Cite This: *Anal. Chem.* 2020, 92, 14867−14871

References

- Popják G, Edmond J, Clifford K, Williams V. **Biosynthesis and structure of a new intermediate between farnesyl pyrophosphate and squalene.** J Biol Chem. 1969 Apr 10;244(7):1897-918.
- Lane AN, Arumugam S, Lorkiewicz PK, Higashi RM, Laulhé S, Nantz MH, Moseley HN, Fan TW**. Chemoselective detection and discrimination of carbonyl-containing compounds in metabolite mixtures by ^{1}H-detected ^{15}N nuclear magnetic resonance.** Magn Reson Chem. 2015 Jan 23. doi: 10.1002/mrc.4199**.**
- Fan TW, Lorkiewicz PK, Sellers K, Moseley HN, Higashi RM, Lane AN**. Stable isotope-resolved metabolomics and applications for drug development.** Pharmacol Ther. 2012 Mar;133(3):366-91.
- Kirpich A, Ragavan M, Bankson JA, McIntyre LM, Merritt ME. **Kinetic Analysis of Hepatic Metabolism Using Hyperpolarized Dihydroxyacetone.** J Chem Inf Model. 2019 Jan 28;59(1):605-614.
- Korchak S, Emondts M, Mamone S, Blümich B, Glöggler S. **Production of highly concentrated and hyperpolarized metabolites within seconds in high and low magnetic fields**. Phys Chem Chem Phys. 2019 Oct 24;21(41):22849-22856.
- Dey A, Charrier B, Martineau E, Deborde C, Gandriau E, Moing A, Jacob D, Eshchenko D, Schnell M, Melzi R, Kurzbach D, Ceillier M, Chappuis Q, Cousin SF, Kempf JG, Jannin S, Dumez JN, Giraudeau P. **Hyperpolarized NMR Metabolomics at Natural (13)C Abundance**. Anal Chem. 2020 Nov 17;92(22):14867-14871.
- Hsieh CY, Sung CH, Shen YE, Lai YC, Lu KY, Lin G. **Developing a Method to Estimate the Downstream Metabolite Signals from Hyperpolarized [1-^{13}C]Pyruvate**. Sensors (Basel). 2022 Jul 22;22(15):5480.