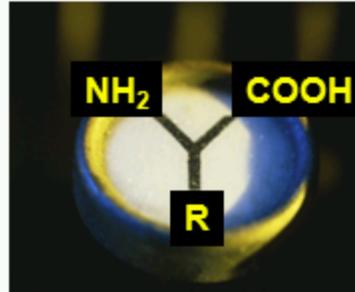


To butylate or Not to butylate - Should that even be a question?



The Historical and Present Day Basis for
Derivatization in the MS/MS Analysis of DBS in
Newborn Screening

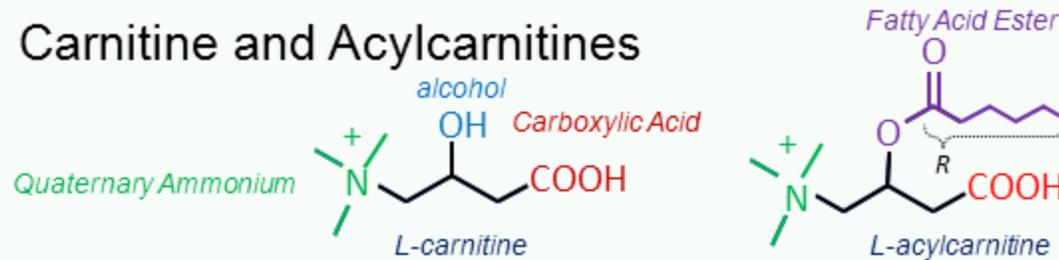
Donald H. Chace, PhD MSFS FACB

*Director-Pediatrix Analytical - Center for Research, Education and Quality - Pediatrix Medical Group
Guest Researcher - Newborn Screening and Molecular Biology Branch - NCEH, CDC*

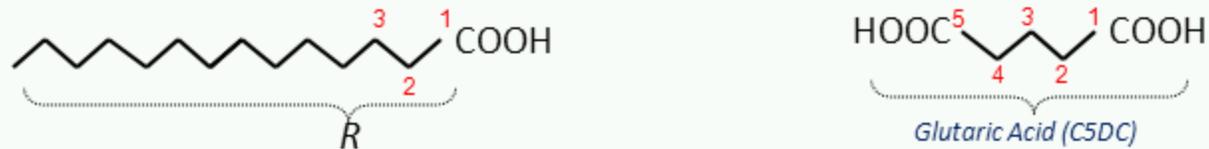
The Historical and Present Day Basis for Derivatization in the MS/MS Analysis of DBS in Newborn
Screening

The Chemical Players starring in "To B.E. or not to B.E."

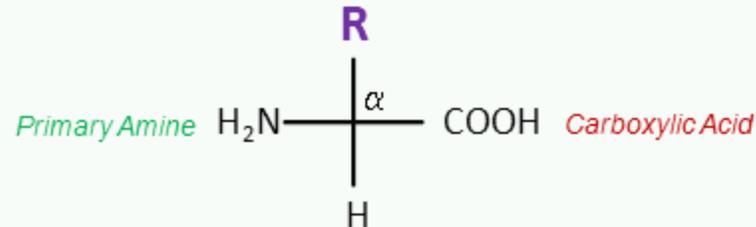
▶ Carnitine and Acylcarnitines



▶ Fatty Acids



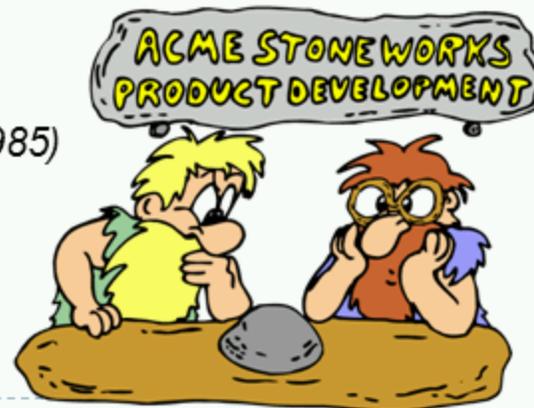
▶ Amino Acids



The Chemical Players starring in "To B.E. or not to B.E."

Pre-DBS History of MS/MS and NBS

- ▶ Carnitine and Acylcarnitines Analysis before 1990
 - ▶ GC/MS (~1985)
 - ▶ Pre-formed cation of carnitine made it involatile. Required removal of "quat", extensive prep.
 - ▶ HPLC only (no MS)
 - ▶ Free Carnitine and short chain acylcarnitines
 - ▶ Immunoassays and Radio-immunoassays
 - ▶ Free Carnitine measured before and after hydrolysis with a strong base (NaOH)
 - FC: Free Carnitine (FC before hydrolysis)
 - TC: Total Carnitine (FC after hydrolysis)
 - AC: Total Acylcarnitine (TC – FC)
- ▶ *LC-MS of Carnitine and Acylcarnitines (>1985)*
 - ▶ *Fast Atom Bombardment Ionization (FAB)*
 - ▶ *Manual Analysis, Drop of glycerol on probe*
 - ▶ *Precursors of 99 (Methyl Esters) -*
 - ▶ *Derivatization using MeOH + 6 N HCl*



ACME = Acyl Carnitine Methyl Esters

Pre-DBS History of MS/MS and NBS

Post 1990 - DBS and MS/MS Development

- ▶ Duke – NC MCAD frequency study.
 - ▶ MCAD deficiency close to that for PKU
 - 1:10,000 -1:20,000
 - ▶ MCAD treatable condition
 - ▶ Develop a Screening Test for MCAD
 - ▶ Only MS/MS can be used.
 - ▶ Need a DBS method
- ▶ Step 1: Compare to an Existing NBS Method
 - ▶ For acylcarnitines – does not exist
- ▶ Step 2: Similar compounds, same DBS platform
 - ▶ Phenylalanine and the DBS
 - ▶ Develop MS/MS for Phe then compare to Flurometry DBS



Post 1990 - DBS and MS/MS Development

Chance Favors the Prepared Mind... *or the lucky!*

- ▶ **Use existing MS/MS method for acylcarnitines**
 - ▶ FAB MS/MS and Pre 99 scans
 - ▶ Methyl Ester analysis for AA did not work (Interference for Phe)
 - ▶ Alternative Derivative: Butyl Ester
 - ▶ interference disappeared

- ▶ **Validation Phase**
 - ▶ In development of analysis of Phe – discovered many other compounds – amino acids in a full scan profile.

- ▶ **Eureka Moment**
 - ▶ ... Leu, Tyr and Met – Oh My!

- ▶ **AC's adapted from ME to BE for NBS**
 - ▶ FIB and improved sensitivity
 - ▶ Better long chain AC sensitivity
 - ▶ Better separation of C5DC from C8
 - ▶ Clinical Method = ME, NBS = BE

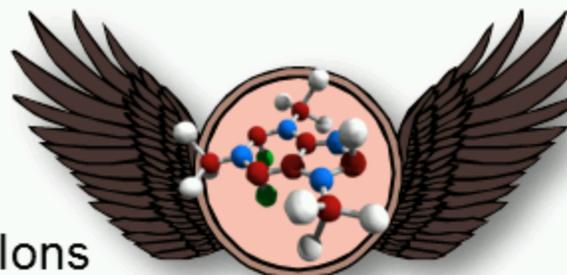


Chance Favors the Prepared Mind... or the lucky!

Basis for Derivatization – Improved Ionization

▶ What is **ionization**?

- ▶ The process in which a molecule becomes an ion.
- ▶ Ion = charged molecule.
- ▶ Two charges: Positive (+) and Negative (-)
- ▶ Neutral equals no net charge.



▶ Mass Spectrometers require Ions

- ▶ Ions give molecules **wings** and fly through a vacuum.
- ▶ Mass spec works by repelling, attracting with the same and opposite charges to measure mass.

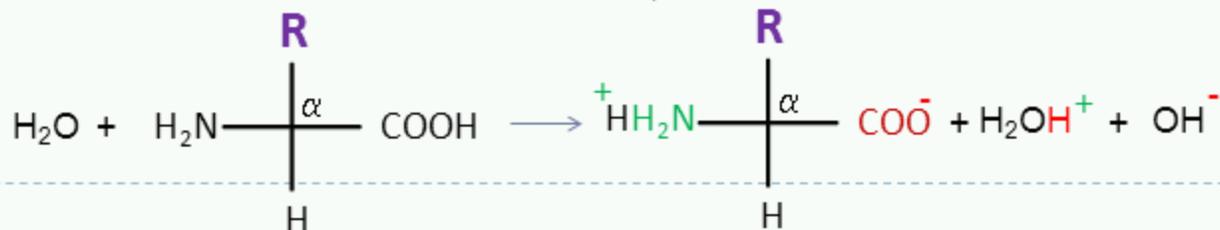


Basis for Derivatization – Improved Ionization

Ionization Efficiency

- ▶ The relative amount of + or - charge of a specific type relative to the total quantity of molecules.
 - ▶ Detection efficiency = total number of charge molecules detected = analytical sensitivity.
 - ▶ Includes ionization efficiency as part of detection efficiency.

- ▶ Molecules must be polar and/or ionizable to be detected by MS/MS.
 - ▶ Must have groups that can be charged (NH₃, COOH, OH, SH) in a polar solution (water plus polar organic solvents)

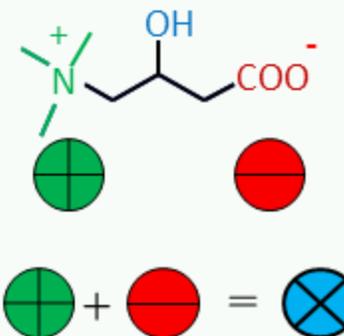


Ionization Efficiency

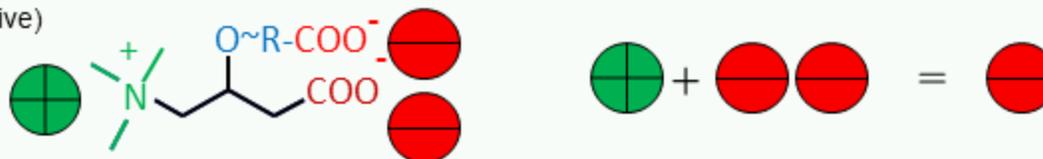
Why We Derivatized Then and Why Now! Part 1

- ▶ **Positive Ion Mode MS/MS**
 - ▶ Requires a NET positive charge. (For AC's and AA's THINK positive)

- ▶ **Amino Acids and Acylcarnitines have a –COOH group.**
 - ▶ COOH likes to be Negative in aqueous solutions at neutral pH.
 - ▶ Neutralize that Negativity and Stay Positive!
 - ▶ Zwitter ions at the pKa are net neutral.
 - ▶ Some compounds are really negative (Twice the negativity)
 - ▶ AA's have R-groups that are acids (Glutamate, Aspartate)
 - ▶ AC's that have a dicarboxylic fatty acid (C5DC, C4DC, C3DC)



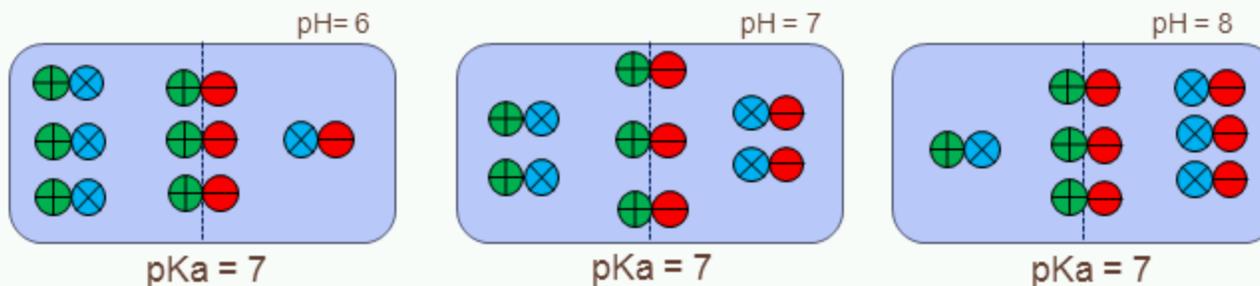
- ▶ **Even a permanent positive charge is no match for a double negative!**
 - ▶ Although carnitine has a permanent positive charge – two negatives don't make positive. (net negative)



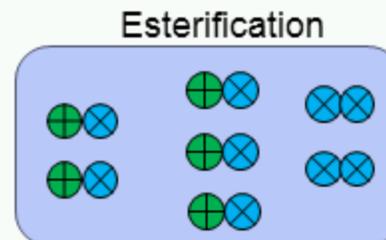
Why We Derivatized Then and Why Now! Part 1

Why We Derivatized Then and Why Now! Part 2

- ▶ So why does a free acid analysis (non-derivatization) of an acylcarnitine or amino acid work in positive ion MS/MS?
 - ▶ In aqueous solution (ACN/Water) – both positive and negative ions are present.
 - ▶ *(that's life – you take the negative with the positive!)*



- ▶ How can you shift the analysis in your favor?
 - ▶ Shift the ions in favor of positive by lowering mobile phase pH
 - ▶ Permanently neutralize the acid group by making an ester (a covalent bond, permanent neutral)



Why We Derivatized Then and Why Now! Part 2

Acidic Alcohol Esterification - more than just neutralization of acid groups



▶ Ionization takes place in a border zone!

- ▶ One side has a lot of friends who like to H-bond.
 - ▶ The aqueous, polar phase
- ▶ Other side is the cold deep dark unfriendly environment of nothing – the Vacuum.
 - ▶ The non-polar, not interacting nothing.
 - ▶ Other than the MS/MS “tractor beam”



▶ AC's and AA's functional groups like amines, acids, alcohols like water and polar solvents. They don't want to leave into a vacuum.

- ▶ Their non-polar aliphatic groups don't like water and enjoy sitting on the border with the vacuum.
- ▶ FAB – ion beam strikes liquid like splashing ducks in a bathtub
 - They go flying, get excited (charged) and pulled into mass spec by negative and positive plates and poles.



▶ Electrospray

- Zapped by 10,000 volts, they get charged and their environment (water) reduced by air drying.
- They run for the hills (ionize into a vacuum) when they get too close to each other (droplet explodes) and are attracted into the mass spectrometer.



Acidic Alcohol Esterification - more than just neutralization of acid groups

Bottom Line – Indisputable Evidence

- ▶ MS/MS analysis of Amino Acids and Acylcarnitines is in positive ion mode.
- ▶ Only AC's and AA's with a net + charge will be detected.
- ▶ The more positive ions present in solution, the more ions that can enter the MS/MS to be detected and reflected and thus higher ion counts (*relatively speaking*)
- ▶ Derivatization generates more positive ions and thus more ion counts.
- ▶ Ions Counts = Sensitivity
- ▶ Derivatization = Better Sensitivity

The Bottom Line



Bottom Line – Indisputable Evidence

Butyl Esters are not Picture Perfect ...

- ▶ Derivatization does convert AC to FC.
 - ▶ Quantification of FC tends to be higher.
 - ▶ Can be corrected – we are working on it!
 - ▶ Glutamate = 260, C2 = 260
 - ▶ Lots of Glutamate! – contributes to C2!
 - ▶ Can be corrected – we are working on that too!
- ▶ Derivatization ruins the lab dryers over time.
 - ▶ Need all teflon dryers (*or all fiber glass Corvettes*).
- ▶ Derivatization is an MS cleaner!
 - ▶ Because of acidic environment
 - ▶ Fewer LC clogs
 - ▶ Cleaner source
 - ▶ Paper fibers dissolved.
 - ▶ (note – base is use to hydrolyze AC)



Butyl Esters are not Picture Perfect ...

We Report - You Decide... *(read the fine print)*

- ▶ Derivatization shifts the m/z of dicarb ACs away from key hydroxy AC compounds. *(Victor is up next!)*
- ▶ Not Derivatizing (FA's) result in inability to distinguish important AC's
 - ▶ Loose selectivity for C3DC, C4OH, C5OH, C4DC
- ▶ Free Acids and Free Carnitine and Succinylacetone
 - ▶ Two for one method may be problematic for underivatized FC.
 - ▶ we are working on this too!
- ▶ Free Acid Analysis is Simpler?
 - ▶ One or two less step
 - ▶ Direct extraction and analysis uses different solvents
 - ▶ Methods are therefore different by more than derivatization.



We Report - You Decide... (read the fine print)

My Quandary Summarized and More Questions

- ▶ **My quandary – Is FA a better method?**
 - ▶ Does it improve the analytical sensitivity and selectivity of acylcarnitines and amino acids?
 - ▶ Does it improve the clinical sensitivity and selectivity (False Pos, False Neg)?
- ▶ **Free Acids made possible by Electrospray Ionization**
 - ▶ FreeAcid analysis made possible by Electrospray (can't be done with FAB/FIB).
- ▶ **Free Acid is faster per sample?**
 - ▶ 30 minute less preparation time for one sample but when analyzing by batch it is an unimportant savings – simply an offset of time.
- ▶ **Free Acid is better for FC quantification?**
 - ▶ Not proven at least not yet – some evidence suggest the opposite is true.
- ▶ **We now have at least 4 different MS/MS methods being used in the US.**
 - ▶ BE with or without SA and FA with or without SA then add kit based and non-kit based which may have different extraction solvents and you have 8 different MS/MS methods!



My Quandary Summarized and More Questions