

Expanded Newborn Screening by Tandem Mass Spectrometry: Considerations on its Simplification and Potential Further Expansion.

Authors: Lisa Sapp, Alex Cherkassiy, Yijun Li, Rebecca Pitts, Alla Ostrinskiya, Annie Wang, and Blas Cerda



1 Introduction

The most widely used tandem mass spectrometry-based method for detecting inborn errors of metabolism relies on the butylation (derivatization) of amino acids, acylcarnitines and free carnitine. This assay is typically referred to as the derivatized assay. The method allows for the measurement of 30-40 markers that are used for the screening of more than 30 genetic metabolic disorders simultaneously. As impressive as the current method is, questions still remain: Can the assay be simplified? Can it accept new analytes?

Here we report our study in simplifying the assay and expanding it by including new analytes. This poster shares our findings regarding the challenges and caveats when attempting to expand the analyte menu including potential interferences and biases and how they can be avoided or minimized.

4 Linearity: The Non-Derivatized Method

Table 1: Linearity analysis of amino acids and SA

	ALA	ARG	GLU	GLY	LEU	LEU	MET
r	0.995	0.996	0.995	0.996	0.996	0.996	0.996
SE	0.007	0.007	0.007	0.007	0.007	0.007	0.007
	ASP	GLN	PRO	TYR	VAL		
r	0.996	0.996	0.996	0.996	0.996	0.996	0.996
SE	0.007	0.007	0.007	0.007	0.007	0.007	0.007

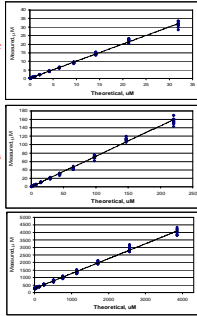
Table 2: Linearity analysis of acylcarnitines

	CS	CA	CD	GA	GA	CS	CDG	GA
r	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996
SE	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007
	CS	CA	CD	GA	GA	CS	CDG	GA
r	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996
SE	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007

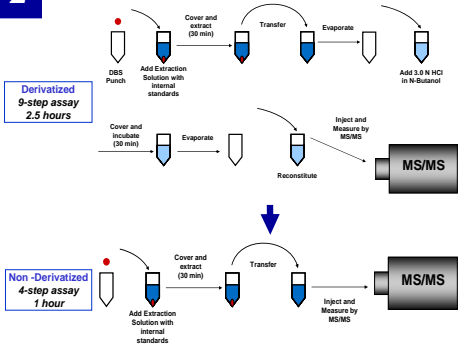
Each analyte exhibits a linear response over a wide range of concentrations.

The blood spots used for this study were prepared using whole blood obtained from a commercial source. The blood was processed by adjusting the hematocrit concentration to 17 mg/dL and adding the blood with several amino acids, carnitine, acylcarnitines and succinylacetone at known concentrations that cover the clinically significant levels. The processed blood was dispensed on a filter paper card to form blood spots on the filter paper matrix. The blood spots were allowed to dry overnight and then processed according to the non-derivatized protocol outlined in panel 2.

Figure 1: Representative Linearity Plots



2 Simplification of the Method



5 Recovery and Precision

Table 3: Percent recoveries at different blood enrichment (spike) levels

Spiked	ALA	ARG	CA	CD	GLU	GLY	LEU	LEU	MET	PRO	TYR	VAL	CS	CA	CD	GA	GA	CS	CDG	GA		
Recovery	91	79	91	83	45	21	4	18	42	63	54	14	14	17	13	52	85	52	85	85	85	85

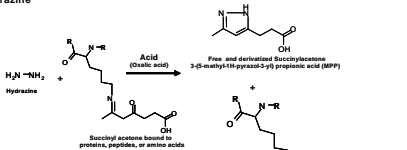
% Recovery	ALA	ARG	CA	CD	GLU	GLY	LEU	LEU	MET	PRO	TYR	VAL	CS	CA	CD	GA	GA	CS	CDG	GA		
Recovery	91	79	91	83	45	21	4	18	42	63	54	14	14	17	13	52	85	52	85	85	85	85

Total NCV includes: within run, between run within day, and between day imprecision. The same blood spots used in the Linearity study were processed according to the non-derivatized method in panel 2 and used to determine Recoveries and Total Imprecision.

3 Incorporation of New Analytes

Two new target analytes have been successfully incorporated into the non-derivatized method.

- Proline** is a marker for Hyperprolinemia Type I and II. Quantitative analysis of proline in newborn DBS can be achieved by extracting as any other analyte and using D₂O-proline as internal standard.
- Succinylacetone (SA)** is a marker for Tyrosinemia Type I. Extraction of SA is simultaneous with the extraction of amino acids and acylcarnitines by reacting SA with hydrazine.



6 Tyrosinemia Type I: Test on a True Positive

Tyrosine catabolism is a metabolic pathway clearly not a direct relationship and the presence of Tyrosine and succinylacetone are more appropriate marker for the detection of this disorder.

Figure 2: Tyrosine Catabolism

Figure 3a: Tyrosine Spectra

Figure 3b: Tyrosine Concentrations

Figure 4a: Tyrosine Spectra

Figure 4b: Tyrosine Concentrations

Figure 5a: SA Spectra

Figure 5b: SA Concentrations

The increased specificity of succinylacetone as a marker, is demonstrated when an affected patient sample is compared to known normal samples for corresponding tyrosine and succinylacetone (SA) concentrations. The samples were processed according to the non-derivatized method described here. Figures 3 and 4 show that there is a striking difference between the tyrosine and SA levels in this patient. While the affected patient displays normal tyrosine levels at 25 hrs of age, SA concentration is significantly elevated. It is not until the patient is 14 days old that the tyrosine levels are elevated.

7 Potential Interferences: Ornithine and Proline

The ESI-MS/MS measurement of Proline and Ornithine from the same mixture presents an interesting challenge. If one is not careful with the ESI settings, Ornithine ions can dissociate in the source to produce a fragment ion that is isobaric with the Proline protonated molecular ion. Further, the source-produced Ornithine daughter ion fragments in the collision cell in the same way as Proline. Therefore, if Ornithine is present in a mixture, this process can interfere with the measurement of Proline. Figure 5a shows the fragmentation of Proline in the collision cell. Figure 5b depicts the decomposition of Ornithine in the source to generate a fragment that may interfere with the measurement of Proline.

Figure 5a: Pro fragmentation.

Figure 5b: Ornithine in-source decomposition.

Figure 6: Effect of source settings on Ornithine interference.

Experimental proof of the potential interference: Figure 6 shows that this potential interference can be controlled by carefully selecting the ESI settings for the measurement of Ornithine. In this experiment, dried blood spots with increasing Ornithine concentration and constant Proline concentration were used to monitor the Proline concentration as a function of Ornithine spike. From the results it is obvious that carefully optimized settings will minimize the potential interference.

8 Formiminoglutamic acid (FIGLU)

The failure of folate to break down histidine results in accumulation of an intermediary metabolite, formiminoglutamic acid (FIGLU). The main peak of butylated FIGLU produces a fragmentation at m/z 85 and thus it shows in the acylcarnitine spectra overlapping with a C-acylcarnitine. The accumulation of butylated FIGLU thus has the potential to interfere with the measurement of C-acylcarnitines.

Figure 7: Differential Butylation in Dried Blood Spots.

Overall, these results indicate that when using the derivatized assay, there is a high potential for discriminating against Ile and Allo-Ile. The clinical significance of this trend continues to be evaluated in our laboratories.

10 Butylation Efficiencies of Leu, Ile and Allo-Ile

In order to elucidate whether or not there is differential esterification of Leu isomers, individual solutions of Leu, Ile, and Allo-Ile were each prepared at 50 μM concentrations. These solutions were dried down and derivatized with n-butanol. Excess reagents were evaporated and the samples were reconstituted with a solution that contained ³H-Leu. The samples were injected into a tandem mass spectrometer and analyzed by a neutral loss scan of 46. These experiments quantitate the amount of non-derivatized Leu isomers after esterification. The independent quantification of non-derivatized Leu, Ile and Allo-Ile is used to determine the degree of butylation on each species. The lower the concentration of non-derivatized (non-derivatized) amino acid the higher the butylation efficiency.

Isomer	Non-derivatized (μM)	Esterified (μM)	Butylation efficiency
Leu	0.7	4.3	87%
Ile	4.4	0.6	12%
Allo-Ile	3.3	1.7	34%

From Table 6 it can clearly be seen that there is a high degree of differential butylation among the Leu isomers with Leu being the most prone to butylation and Ile the most resistant to esterification. These results are quite striking and reveal a trend not reported before. These results suggest that when measuring dried blood spots for Leu/Ile/Allo-Ile, the main element being analyzed is Leu and not the sum of all isomers as originally thought.

11 Butylation Efficiencies of Leu, Ile and Allo-Ile

Having determined that butylation does not occur at the same rate for all Leu isomers, we evaluated the clinical impact of this differentiation by measuring newborn dried blood spots with the derivatized assay. For these experiments, 3 identical sets of 200 samples each were processed as follows:

Figure 7: Differential Butylation in Dried Blood Spots.

Overall, these results indicate that when using the derivatized assay, there is a high potential for discriminating against Ile and Allo-Ile. The clinical significance of this trend continues to be evaluated in our laboratories.

9 Leucine and Isoleucine

A very intriguing observation during the development of the non-derivatized assay was a significant bias between the derivatized and the non-derivatized assays for the measurement of Leucine and Isoleucine.

Leucine and isoleucine are isomers and both are detected by tandem mass spectrometry at m/z 188 when they are butylated (N: 102 scan) or m/z 132 as the protonated free acids (N: 46 scan). Therefore, when a mixture of Leucine and isoleucine is measured, the peak at m/z 188 (derivatized) or 132 (non-derivatized), is expected to represent the sum of the signals generated by these two amino acids. However, when measuring this standard mixture with the derivatized and non-derivatized assays we observed that this expectation may not always be true.

Table 5: Leu/Ile quantitation-Derivatized vs. Non-Derivatized

	NIST	Non-Derivatized	Derivatized
Leu + Ile	4.55	4.1	2.8
Accuracy		90%	62%

Evidently, there is a clear bias between the two assays. The fact that it is the derivatized assay that shows the lower recoveries suggests that there is some discrimination against one of the two amino acids during butylation.

12 Summary

- Based on these studies, we have shown that it is possible to measure succinylacetone (the primary marker for the detection of Tyrosinemia Type I) and that it is possible to do it simultaneously with amino acids and acylcarnitines using a non-derivatized assay procedure.
- Both the derivatized and non-derivatized methods are quite complex assays. However, it must be recognized that these assays are complex and are associated with a number of potential processes that can interfere or bias the results.
- One of the most significant findings of this study is the fact that the esterification step in the derivatized assay discriminates against Ile and Allo-Ile. The clinical significance of this process is under investigation in our laboratories.