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A NEW APPROACH TO DRIED BLOOD SPOT ANALYSIS FOR NEWBORN SCREENING USING HIGH RESOLUTION LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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A NEW APPROACH TO DRIED BLOOD SPOT ANALYSIS FOR NEWBORN SCREENING USING HIGH RESOLUTION LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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This work is dedicated to Sandy, Summer and
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ABBREVIATIONS

ACN	acetonitrile
CE	collision energy
C0	Carnitine
C18	Stearoylcarnitine
C_{18}	octadecyl
Da	Dalton(s)
DBS	dried blood spots
ESI^+	positive electrospray
FA	formic acid
FDA	Food and Drug Administration
GC/MS	gas chromatography mass spectrometry
HCl	hydrochloric acid
HILIC	hydrophilic interaction chromatography
HPLC	high performance liquid chromatography
HQC	high quality control
LC-MS/MS	liquid chromatography tandem mass spectrometry
LCHAD	long-chain L-hydroxyacyl-CoA dehydrogenase deficiency
LLOQ	lower limit of quantification
LQC	low quality control

MCAD	medium chain acyl co-A dehydrogenase deficiency
MeOH	methanol
mM	millimolar
MP A	mobile phase A
MP B	mobile phase B
MRM	multiple reaction monitoring
MSUD	maple syrup urine disease
NBS	newborn screening
NMR	nuclear magnetic resonance
Q1	first quadrupole
Q2	second quadrupole
Q3	third quadrupole
STD	standard (calibration)
UHPLC	ultra high pressure liquid chromatography
µM	micro molar
VLCAD	very long chain acyl-CoA dehydrogenase deficiency

ABSTRACT

A NEW APPROACH TO DRIED BLOOD SPOT ANALYSIS FOR NEWBORN SCREENING USING HIGH RESOLUTION LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

By John H. Miller IV, B.S.

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The primary purpose of newborn screening is to quickly identify children that are at risk of having a specific disorder in order to start treatment, prevent early death and reduce the chances of permanent physical or mental damage. The current and widely accepted approach used for identification of metabolism disorders involves a flow injection analysis with mass spectrometry detection of acylcarnitines and amino acids. Although this approach is widely accepted and has shown to be sufficient for identification of multiple metabolism disorders the method is not fully quantitative and results often have to be confirmed by second-tier tests.

The primary focus of this research was to improve the accuracy and selectivity of this screening method by employing a high resolution chromatographic separation for the combined analysis of twelve acylcarnitines and seven amino acids. This method is an improvement over the current methodology allowing for separation of key isomers that are diagnostic for different metabolism disorders, reducing the need for multiple second-tier tests to confirm results and shortening the time to diagnosis.

In order to further improve the efficiency of newborn screening we developed an in-line desorption device, which allows for direct analysis of DBS eliminating the need for punching disks from the filter paper cards. Our device was the first published paper that demonstrated the ability to directly analyze dried blood spots, without the need for any offline sample processing. Using this device, we validated a method to quantify biomarkers related to Maple Syrup Urine Disease, a disorder that requires a second-tier test for confirmation.

To further improve the accuracy of dried blood spot analysis we evaluated a technique to correct the sample volume in low and high hematocrit samples. The level of hematocrit in blood spotted on filter paper cards affects the volume of sample analyzed, leading to errors in accuracy. Diffuse reflectance was used to relate differences in sample hematocrit on dried blood spots. We validated our technique with eighteen donor samples at various levels of hematocrit. Correcting sample volume for hematocrit showed improved precision and accuracy over the standard approach, ultimately reducing the potential to misidentify samples.

CHAPTER 1

BACKGROUND TO NEWBORN SCREENING DRIED BLOOD SPOT ANALYSIS

1.1 Introduction

1.1.1 Background to newborn screening

Since the late 1960's it has been a requirement that all state laboratories screen newborns for inherited metabolism disorders (Sahai & Marsden, 2009). Initially, the state laboratories screened for just one disorder, phenylketonuria (PKU), but gradually additional tests were added to the screening panel as they were identified. The primary purpose of implementing newborn screening testing was to quickly identify children that were at risk of having a specific disorder in order to start treatment, prevent early death and reduce the chances of permanent physical or mental damage. The cost of screening every newborn was justified by the ability to save lives by early diagnosis and treatment versus the expenses associated with long term health effects of untreated babies (Millington, 2005). Current screening methods are able to identify as many as 40 different disorders from a single sample (Chace, Kalas, & Naylor, 2003; Sweetman, 2001). Blood is collected from each newborn within the first week of life using the same technique developed for the analysis of PKU by Robert Guthrie (Guthrie & Susi, 1963). The newborn's heel is pricked and the blood is spotted on filter paper cards, allowed to dry and sent to state laboratories for testing. Several different tests are

performed on each sample; some tests require separate analysis for identification of specific disorders. Acylcarnitines and amino acids, markers for a variety of metabolism disorders, are typically screened in a single analysis employing flow injection with mass spectrometry detection.

Acylcarnitines are diagnostic markers for both fatty acid oxidation defects and organic acid disorders. Carnitine (C0) plays an essential role in the transport of fatty acids to the mitochondrial for the production of energy (Sahai & Marsden, 2009). The acylcarnitine nomenclature is based on the chain length of the fatty acid attached to C0, for example a C6 is the base carnitine attached to a six carbon fatty acid. Structures with unsaturated fatty acid are identified as C6:1 where the 1 indicates a single unsaturated bond. Dicarboxylic fatty acids and hydroxylated fatty acid species are represented by "-DC" and "-OH" respectively. The formation of an acylcarnitine is show in Figure 1.1. where "R" represents the long chain fatty acid that binds with C0.

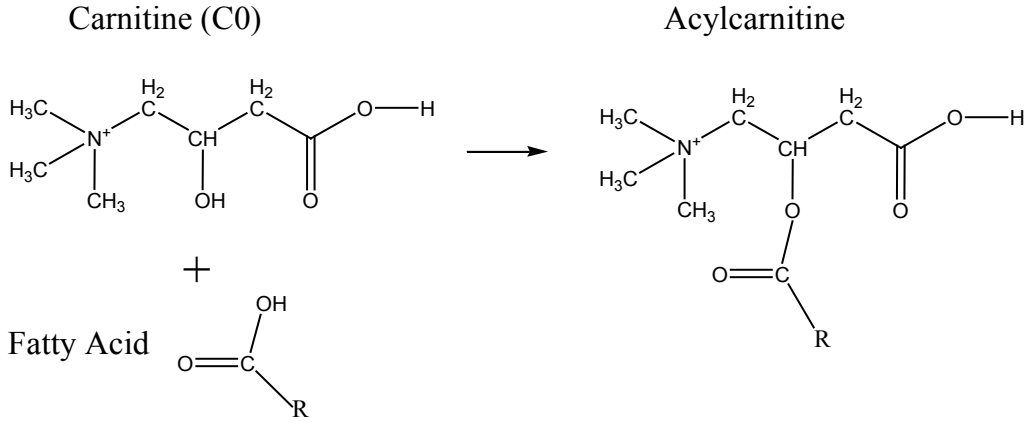


Figure 1.1: Reaction of free carnitine with a fatty acid to produce an acylcarnitine

When there is a deficiency in an enzyme specific to the metabolism of fatty acids, it results in an increase in blood levels of acylcarnitines (Sahai & Marsden, 2009). Since the enzymes are selective based on the chain length, the enzyme responsible for a disorder can be identified based on elevation of specific acylcarnitines. Elevated levels of C6, C8 and C10 are an indication of medium chain acyl CoA dehydrogenase deficiency (MCAD) (Sahai & Marsden, 2009). Elevated C16-OH, C16 and C18:1-OH are an indication long-chain L-hydroxyacyl-CoA dehydrogenase deficiency (LCHAD), and elevated C14, C14:1 and C16 are indications of very long chain acyl-CoA dehydrogenase deficiency (VLCAD) (Sahai & Marsden, 2009).

Organic acid disorders arise from impairment of enzymes responsible for the breakdown of amino acids prior to the citric acid cycle (Sahai & Marsden, 2009). As a consequence excess levels of short chain organic acids are formed and conjugate with carnitine. Several organic acid disorders involving vitamin B12 result in elevated levels of Propionylcarnitine (C3) (Sahai & Marsden, 2009). Other enzyme deficiencies in the metabolism of amino acids will result in elevated levels of specific amino acids. Phenylketonuria (PKU) is a classical amino acid disorder in which there is a deficiency in the enzyme responsible for the metabolism of phenylalanine (Sahai & Marsden, 2009). This results in abnormally high levels of phenylalanine, which can cause mental retardation if untreated.

Newborn screening methods typically screen for six to eleven amino acids and nine to twenty acylcarnitines. Positive samples are flagged for clinical diagnosis, in which an experienced clinician analyzes the mass spectral data and starts an intervention process with the primary care physician and parents. Diagnosis for a specific disorder requires testing of

additional blood and urine samples using a quantitative technique and chromatographically separates the analytes by either gas chromatography or high performance liquid chromatography. Additional markers are analyzed, such as urinary organic acids, for clinical diagnosis of certain disorders. Clinical diagnoses tests are typically conducted by independent laboratories which results in additional time and expense to determine the health of the child. Once the child is identified as having a specific disorder, the exact nature of the deficiency is evaluated by testing the level of enzyme activity or gene sequencing. A typical test for enzyme activity requires profiling the metabolism of a specific analyte in the presence of an enzyme (Wolf, Grier, Allen, Goodman, & Kien, 1983). Although there are some colorimetric tests that can be employed at newborn screening centers they are not fully quantitative and often have to be confirmed by a second laboratory. Gene sequencing is more complex and requires a second sample to be taken from the newborn (Tiranti et al., 2004). The samples are usually analyzed using a Western Blot approach along with gel electrophoresis to identify specific differences in the genome.

1.1.2 Current methodology

There are two widely used approaches in newborn screening for acylcarnitines and amino acids. The original and widely accepted method requires the analytes to be derivatized prior to analysis and a more recent approach does not require derivatization. Both techniques allow for identification of several different metabolism disorders in a single analysis. The original technique used by most state laboratories was developed at Duke University and involves the derivatization of the carboxylic acid functional group with n-butanol and

hydrochloric acid to form butyl esters (Millington, Kodo, Norwood, & Roe, 1990). The final extracts are analyzed by flow injection analysis (Figure 1.2). Flow injection implies that the sample is injected without chromatographic separation; instead, the sample is injected into the flow path of the mobile phase, which carries the plug of sample to the mass spectrometer. The flow rate is reduced prior to the sample reaching the mass spectrometer which improves the sensitivity of the method by broadening the elution plug so that the signal can be averaged over a longer time period. Flow injection analysis can be done rapidly in 2-5 minutes, allowing for hundreds of samples to be analyzed in a single day (Sweetman, 2001).

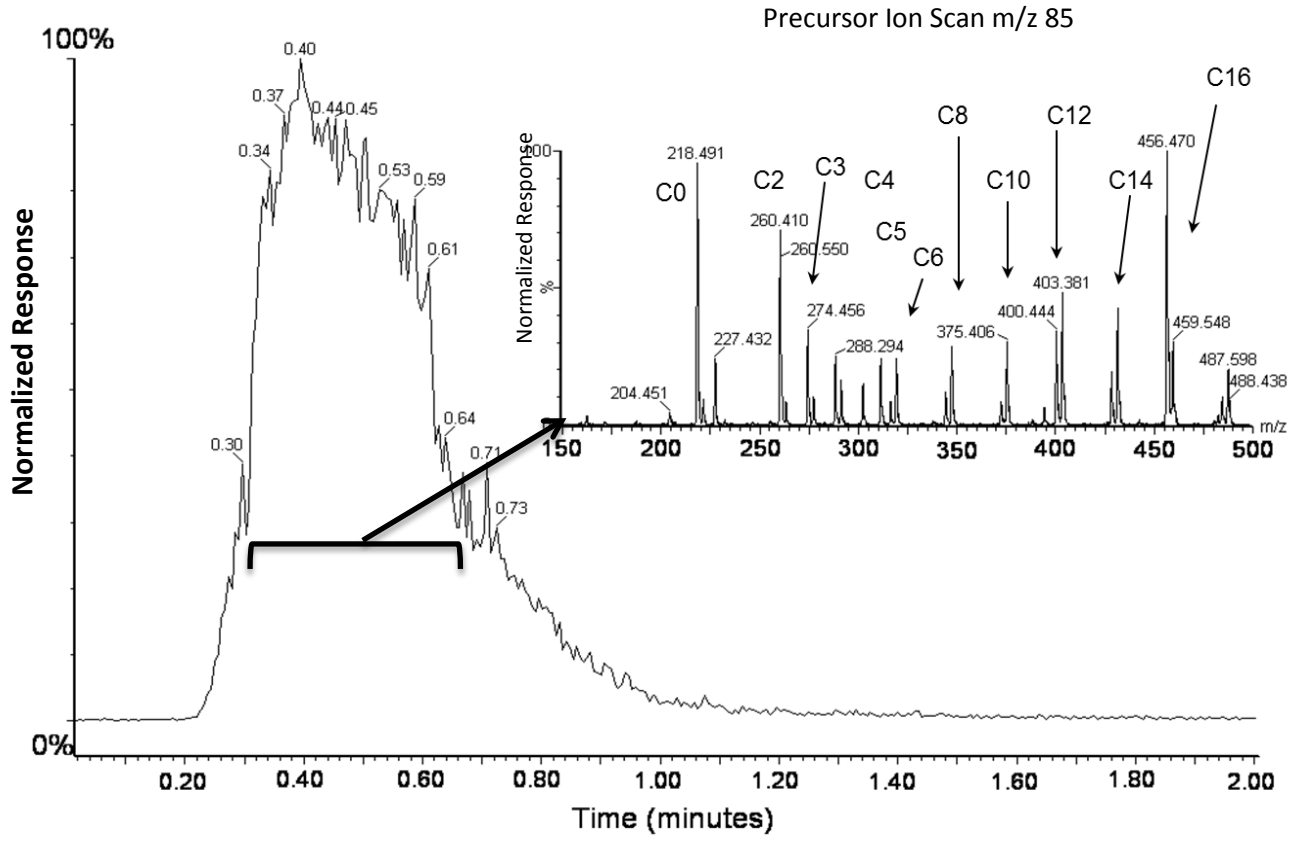
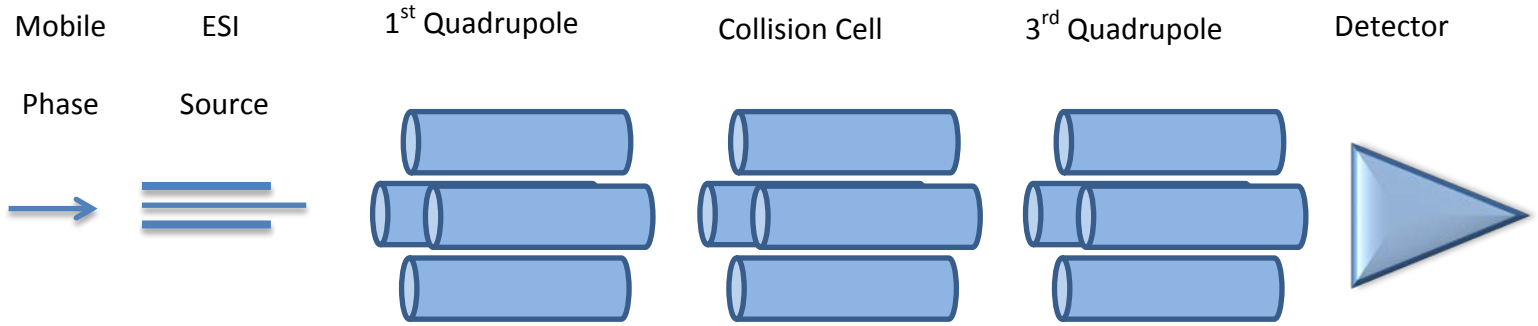


Figure 1.2: Flow injection analysis for an extracted dried blood spot sample derivatized with n-butanol with insert showing the mass spectra for acylcarnitines analyzed by precursor ion scan for the production of 85 m/z

The mass spectral analysis can be very complex; however, most laboratories use specialized software to help identify and quantify the analytes of interest. Also, there is no separation between the analytes and matrix components which could affect the quantitation of some analytes, especially for compounds that do not have labeled internal standards. For analysis of butylated acylcarnitines, the mass spectral data is collected for positive precursor ions of the 85 m/z production. Under these conditions the mass spectrometer allows all ions to pass through the first quadrupole and into the collision cell. The ions are fragmented and only ions with 85 m/z pass through the third quadrupole and associated precursor ion from the first quadrupole is identified. The derivatized amino acids are analyzed in a similar fashion, typically monitoring the neutral loss of 102 m/z. A neutral loss scan function only allows specific ions that lose a designated mass to pass through to the third quadrupole. In scan mode the instrument has to ramp the voltage applied to the quadrupole in a specified time to filter ions from low to high mass to charge (m/z). Although this allows for multiple compounds to be detected in a single scan it results in lower sensitivity since the time that the mass spectrometer is detecting an individual mass is a fraction of the scan time. An alternative approach is used to improve the sensitivity for difficult to detect compounds is called multiple reaction monitoring (MRM). In this technique the mass spectrometer monitors a specific molecular mass and a specific fragment ion in a specified dwell time. Multiple reaction monitoring provides the best sensitivity for an analyte since the mass spectrometer does not need to scan across a series of masses. Figure 1.3 shows a schematic of mass spectrometer and the different scanning modes used.



Scan Type	Q1	Q2	Q3	Analytes Detected
Parent Ion Scan	Scan all masses	Fragmentation	Only mass with 85 m/z	Acylcarnitines
Neutral Loss	Scan all masses	Fragmentation	Only masses that lost 102 m/z	Amino Acids
MRM	Selected molecular ion mass	Fragmentation	Selected fragment mass	Selected Amino Acids

Figure 1.3: Schematic of a triple quadrupole mass spectrometer with descriptions of different scan modes used by newborn screening for analysis of amino acids and acylcarnitines

1.1.3 Quantification of acylcarnitines and amino acids

Quantification of amino acids and acylcarnitines is based on the response factors determined using isotopically labeled internal standards. Dried blood spots are extracted in a solution containing isotopically labeled internal standards at known concentrations. In the derivatization based screening method the internal standards are also derivatized during the sample preparation to form their butyl esters. The response of the internal standard is used to determine the concentration of the analyte based on Equation 1.1 where the response factor is either previously determined or assumed to be equal to one. It is recommended that the response factors be determined prior to analysis using Equation 1.2 in order to more accurately quantify each analyte. However, most laboratories set cut-off values independently based on response of normal samples and set a cut-off above this range to identify abnormal samples. Although, this has been proven to be acceptable for screening there are potential problems with using a single point response factor for quantitating samples. This technique assumes that the response of the internal standard is equivalent to the response of the analyte and that the analyte and internal standard are equally affected by matrix suppression or enhancement, however this is not always true (Trufelli et al., 2011).

Equation 1.1: Conventional calculation for concentration for analytes extracted from dried blood spots

$$Analyte\ Conc. = \frac{Analyte\ Response}{I.S.\ Response} \times Conc.\ I.S. \times Response\ Factor \times \frac{Extraction\ Volume}{Sample\ Volume}$$

Equation 1.2: Response factor determination for external solutions

$$Response\ Factor\ = \frac{Internal\ Standard\ Response}{Analyte\ Response}$$

1.1.4 Issues associated with newborn screening

Although there are several advantages to using this method for screening, there are also several inherent problems. The method primarily used but most state laboratories require multiple extraction steps including derivatization and multiple evaporation steps. Derivatization has been employed to improve sensitivity of some compounds, but there are issues as to the efficiency of the derivatization for others (Sapp et al., 2007). Hydrochloric acid used in the derivatization step is caustic, hazardous and destroys laboratory equipment with prolonged exposure. Even though the flow injection analysis allows for hundreds of samples to be analyzed in a single day, it lacks the selectivity to differentiate between disorders associated with isobaric analytes. The screening method is not fully quantitative and relies on individual laboratory cut-off values to determine test positive samples. Another concern is the volume of sample analyzed has been shown to be affected by differences in hematocrit (Mei et al., 2001). Hematocrit affects the diffusion properties of blood on the filter paper. Samples with high hematocrit will not diffuse as much as samples with low hematocrit. Since newborn screening uses a predefined punch size to analyze samples (either 3.0 mm or 6.0 mm) the actual volume will vary depending on hematocrit. It has been previously shown that samples with abnormally low or high hematocrit have dramatic differences in sample volume which could result in the misidentification of disorders (Mei et al., 2001).

A major drawback to flow injection analysis is the lack of separation of isobars; compounds having the same mass but are structurally different. In some cases the different isobars are diagnostic for separate disorders, and clinical diagnosis is required for proper identification of a disorder (Bennett, 2009; Sweetman, 2001). Leucine, isoleucine, and hydroxyproline all have the same molecular ion 131 m/z and appear as a single entity in newborn screening tests. Although leucine and isoleucine are both used as indicators for maple syrup urine disease (MSUD) their response factors are different and elevated levels of one isomer can influence the screening result (Chace et al., 1995). In addition, a variation in hydroxyproline levels can lead to inaccurate diagnosis of MSUD (Matern, Tortorelli, Oglesbee, Gavrilov, & Rinaldo, 2007). There are also several acylcarntines that have the same mass, C4-isomers butyrylcarnitine and isobutrylcarnitine are symptomatic of two different disorders; short-chain acyl-CoA dehydrogenase (SCAD) and isobutryrl-CoA dehydrogenase deficiency respectively (Chace et al., 2003). C5-isomers valerylcarnitine and isovalerylcarnitine appear at 245 m/z and other species such as 3-hydroxyisovaleryl carnitine, 2-methyl-3-hydroxybutyryl carnitine and butyrylcarnitine dicarboxylic acid (C4DC) appear at 262 m/z. These species are indicators of specific disorders, but are not separated using flow injection analysis and require additional testing for confirmation of the disorder. All final diagnostic tests for analysis of amino acids and acylcarnitines involve some form of chromatographic separation.

Another issue not previously evaluated in newborn screening is the potential for the matrix to affect the quantification of analytes. Although labeled internal standards are used for quantification, the response of the internal standard could be affected differently relative to the analyte by other compounds in the matrix (Trufelli et al., 2011). In addition there is the

potential for matrix related components, such as phospholipids, to dramatically reduce the ionization of some analytes to an undetectable level. Since there is no chromatographic separation employed in the screening method, all the analytes appear at the same time as the other matrix components. Matrix effects can dramatically affect quantification of analytes even if labeled internal standards are used and should be evaluated in newborn screening methods (Lindegardh, Annerberg, White, & Day, 2008). The FDA requires bioanalytical methods to be evaluated for matrix effects to ensure that quantification is accurate and precise (US FDA. Guidance for Industry: Bioanalytical Method Validation, 2001).

1.1.5 Misdiagnosis of patients

Established ranges have been determined for each analyte in order to differentiate between normal healthy levels and diseased levels. Due to overlap in these ranges, cut-off values have to be set. However, it has been reported that as many as 20% of babies initially identified as having a disorder are later determined to be healthy (Sahai & Marsden, 2009). Although, adjusting the cut-off values higher would reduce the number of false positives it would also increase the occurrence of false negatives (Sweetman, 2001).

False positive cases occur when newborns are identified as having a disorder, but after additional testing the child is determined to be healthy. This has less of an impact to the child, but it has shown to result in psychological and emotional issues for the parents (la Marca et al., 2007; Schulze et al., 2003; Sweetman, 2001). It has been reported that less than 27% of newborns identified by current screening methods as having methylmalonic acidaemias are diagnosed with this disorder after employing a more selective second-tier test (Matern et al., 2007). Propionylcarnitine (C3) is the primary marker for methylmalonic acidaemias, however

methylmalonic acid has been identified as a more selective marker. This example demonstrates the poor specificity of current markers for some diseases that could be addressed by adding more specific markers to the screening method. Proper diagnosis of MSUD using newborn screening methods also has a high false positive rate due to lack of chromatographic separation between leucine, isoleucine and hydroxyl proline, as discussed earlier. Most laboratories have implemented second-tier testing for confirmation of disorders that have a high false positive rate. These second-tier tests often involve a second analysis for more specific markers and require a chromatographic separation.

1.1.6 Requirement for second-tier testing

As discussed above, one of the major issues with the current newborn screening method is it is not completely diagnostic, and markers are not always specific to a single disorder (Matern et al., 2007; Sweetman, 2001). Newborn screening laboratories have implemented more selective second-tier tests for some disorders to confirm test positive samples prior to physician notification (Matern et al., 2007). Although these second-tier tests do provide additional specificity, additional instrumentation and personnel are needed for these analysis. Some laboratories screen for more specific markers such as succinylacetone for diagnosis of Tyrosinemia Type 1 (Al-Dirbashi et al., 2008; Matern et al., 2007; Turgeon et al., 2008). Several second-tier tests have been published for the diagnosis of elevated C3 which is a marker for four potential disorders: methylmalonic academia, cobalamin deficiency, homocystinuria and propionic academia (la Marca et al., 2007; Magera, Helgeson, Matern, & Rinaldo, 2000; Matern et al., 2007; Weisfeld-Adams et al., 2009). The current approach to second-tier testing is that one second-tier test is employed for each disorder which results in

multiple second-tier tests. Having a more comprehensive screening method would eliminate the need for multiple second-tier tests, reducing costs and ensuring faster and more accurate identification of certain disorders. To date there is not a comprehensive method that can chromatographically separate both acylcarnitines and amino acids in a single analysis that would be suitable for screening or as a second-tier test.

1.2 Chromatographic separation of acylcarnitines and amino acids

1.2.1 Current methods for acylcarnitines and amino acids

Analysis of acylcarnitines and amino acids can be very complex due to the nature of these analytes. Amino acids are very polar compounds and are not well retained on conventional reverse phase columns such as an octadecyl (C_{18}) phase. This is due to the fact that amino acids contain both an acidic and basic functional group and they are ionized under most of the typical chromatographic conditions. This makes amino acids more water soluble and therefore they stay in the mobile phase and do not interact with a hydrophobic stationary phase. Acylcarnitines have extremely diverse polarity from carnitine being the most polar to stearoylcarnitine (C18) which is very non-polar. The polarity differences between these compounds are affected by the hydrophobic carbon chain attached to a portion of the molecule. The length of this chain will affect the hydrophobicity of the molecule, resulting in longer retention on a reverse phase column. Another issue with analysis of both amino acids and acylcarnitines is that most of these analytes do not contain a chromophore necessary to absorb UV light. Therefore, these analytes cannot be analyzed by UV or fluorescence techniques without some type of derivatization to enhance their detection. Although there have been several published methods for the analysis of amino acids and acylcarnitines, very

few methods have included both in the same analysis. In addition, a rapid screening method should be capable of chromatographically resolving all these analytes and isobaric species with run time less than 5 minutes

Published methods for analysis of acylcarnitines employ a variety of chromatographic conditions and modes of detection. As stated previously, analysis of acylcarnitines for UV absorbance or fluorescence detection required some form of derivatization. Pentafluorophenacyl trifluoromethanesulfonate (Minkler, Ingalls, & Hoppel, 2005) and 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate (Minkler, Kerner, North, & Hoppel, 2005) have been used for derivatization of acylcarnitines for UV and fluorescence detection respectively. Although these methods are suitable for quantitative analysis of acylcarnitines the analysis times are extremely long and they would not be practical for a rapid screening method or for a second-tier test. An alternative approach would be to use electrospray ionization with mass spectrometry detection. However, the current published methods are still not suitable for newborn screening due to analysis times that are longer than 10 minutes (Ghoshal, Guo, Soukhova, & Soldin, 2005; Maeda et al., 2007), the requirement for alternative instrumentation (Chalcraft & Britz-McKibbin, 2009) or that they utilize ion pair reagents (Vernez, Wenk, & Krahenbuhl, 2004). Comprehensive methods for analysis of acylcarnitines, such as dicarboxylic acid and hydroxylated species have been reported, however quantification of all these species adds additional complexity to the analysis and are not necessary for screening (Maeda et al., 2008). Others have reported the separation of key isobars such as C4 and C5 which does allow for differential diagnosis for short chain acyl-coenzyme A dehydrogenase deficiency (SCAD) and isobutyryl-CoA dehydrogenase deficiency (IBCD) or isovaleric academia (IVA) (Forni, Fu, Palmer,

& Sweetman, 2010). Recently there was a report for the analysis of 48 acylcarnitines from dried blood spots using UHPLC-MS/MS on a C_{18} column with a 15 minute analysis time (Gucciardi, Pirillo, Di Gangi, Naturale, & Giordano, 2012). This method does provide chromatographic separation for a comprehensive list of acylcarnitines including several isobars, but the analysis time is still not practical for a rapid test. Table 1.1 provides a comparison of all the published methods for acylcarnitine analysis.

Almost all the reported methods for amino acid analysis involve some form of derivatization either pre-column or post-column. Derivatization is used to enhance the retention of these analytes in reverse phase chromatography and to improve the detection by UV absorbance or fluorescence. There are several commercial kits available for this analysis including Agilent's Aminoquant method and Water's AccQ•Tag™ Ultra derivatization method (Armenta et al., 2010). Although chromatographic separation for amino acids using these methods can be achieved in approximately 10 minutes, both of these kits involve additional derivatization reagents and specialty columns for the analysis. Kasper recently published a review of current methods for the analysis of amino acids in biological samples by HPLC with UV or fluorescence detection, ion-pair chromatography, capillary electrophoresis, GC/MS, UPLC/MS and 2-dimensional NMR (Kaspar, Dettmer, Gronwald, & Oefner, 2009). A comprehensive list of published methods for the chromatographic separation of amino acids is included in Table 1.1. To date there are no published papers involving the separation of both amino acids and acylcarnitines with a run time that would be comparable to the analysis time to the newborn screening flow injection technique.

Table 1.1: Comprehensive list of methods for used for analysis of acylcarnitine and amino acid

Compound Class	Analytes	Analysis time	Instrumentation	Comments	Reference
Acylcarnitines	15 acylcarnitines	70 min	HPLC-UV	Derivatization with pentafluorophenacyl trifluoromethanesulfonate	(Minkler et al., 2005)
	7 long chain acylcarnitines	13 min	HPLC-FLD	Derivatization with 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate	(Minkler, Kerner et al., 2005)
	17 acylcarnitines	33 min	LC-MS/MS	Analysis of Human Serum and Urine	(Maeda et al., 2007)
	17 acylcarnitines	33 min	LC-MS/MS	Analysis of Human Serum and Urine	(Maeda et al., 2008)
	7 acylcarnitines and 13 amino acids	16 min	CE-MS/MS	Requires alternative instrumentation and only analyzes for select acylcarnitines	(Chalcraft & Britz-McKibbin, 2009)
	10 acylcarnitines	17 min	LC-MS/MS	Poor chromatography for several of the acylcarnitines, no derivatization required	(Ghoshal et al., 2005)
	10 acylcarnitines	10 min	LC-MS/MS	Previous method modified from 17 min to 10 minutes, no derivatization required	(Ghoshal, Balay, & Soldin, 2006)
	48 acylcarnitines	15 min	UPLC-MS/MS	Most comprehensive method, but it does not include quantitative analysis of amino acids	(Gucciardi et al., 2012)

Compound Class	Analytes	Analysis time	Instrumentation	Comments	Reference
Acylcarnitines	6 long chain acylcarnitines	12 min	LC-MS/MS	Method not comprehensive and used for the analysis of PC-12 cells	(Jauregui et al., 2007)
Amino Acids	25 amino acids	10 min	UHPLC-FLD or LC/MS/MS Waters AccQ-Tag derivatization.	Requires specialized reagents and columns. Derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate	(Armenta et al., 2010)
	15 amino acids	60 min	HPLC Electrochemical Detection Derivatization with o-phthalaldehyde–2-mercaptoethanol or ophthalaldehyde	Requires Derivatization, specialized detection and long analysis time	(Tcherkas, Kartsova, & Krasnova, 2001)
	14 amino Acids	60 min	HPLC FLD Derivatization with o-phtalaldehyde, 2-mercaptoethanol as catalyst	Not validated for biological samples, requires derivatization and long analysis time (glues)	(Peris-Vicente, Gimeno Adelantado, Carbó, Castro, & Reig, 2006)
	21 amino acids	30 min	HPLC-FLD Derivatization with o-phtalaldehyde, 2-mercaptoethanol as catalyst	Not validated for biological samples, requires derivatization (wine)	(Soufleros, Bouloumpasi, Tsarchopoulos, & Biliaderis, 2003)

Compound Class	Analytes	Analysis time	Instrumentation	Comments	Reference
Amino Acids	23 amino acids	17 min	HPLC FLD OPA derivatization	Requires derivatization	(Fekkes, 1996b)
	38 amino acids	115 min	Ion Exchange LC-UV Derivatization with ninhydrin reagent	Requires derivatization and extremely long analysis time	(Le Boucher, Charret, Coudray-Lucas, Giboudeau, & Cynober, 1997)
	16 amino acids	15 min	Ion Pair LC/MS/MS Ion pair reagent: nonafluoropentanoic acid	Ion pair chromatography	(de Person, Chaimbault, & Elfakir, 2008)
	26 amino acids in plasma and 22 amino acids in urine	30 min	Ion Pair UHPLC Ionpair reagents: Tridecafluoroheptanoic acid	Ion pair chromatography	(Waterval, Scheijen, Ortmans-Ploemen, Habets-van der Poel, & Bierau, 2009)
	16 amino acids	60 min	HILIC LC/MS/MS	Allows for separation of isomers but long analysis time	(Langrock, Czihal, & Hoffmann, 2006)
	21 amino acids	10 min	Monolithic Silica –FLD Derivatization with with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F)	Fast method monolithic column but requires derivatization	(Song, Funatsu, & Tsunoda, 2012)

Compound Class	Analytes	Analysis time	Instrumentation	Comments	Reference
Amino Acids	32 amino acids	25 min	CE-MS	No derivatization required but long analysis time	(Soga, Kakazu, Robert, Tomita, & Nishioka, 2004)
	20 amino acids	5.5 min	UHPLC-UV&CD Derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F)	Fast analysis but not validated for biological samples (food samples)	(Eto, Yamaguchi, Bounoshita, Mizukoshi, & Miyano, 2011)
	30 amino acids	30 min	GC/MS Derivatization with propyl chloroformate	Requires alternative instrumentation and derivatization	(Kaspar, Dettmer, Gronwald, & Oefner, 2008)

1.2.2 Implementation of a high resolution chromatographic separation

There is a growing need to incorporate chromatographic separation into the current newborn screening method for metabolism disorders (Bennett, 2009). The current newborn screening method is acceptable for fast identification of certain metabolism disorders; however there is not sufficient selectivity to accurately identify many of these disorders. Also, some of the critical diagnostic markers are isobars which are not separated by flow injection analysis. In addition, more specific markers are known for some of these metabolism disorders and have been implemented in second-tier tests, but are not included in the newborn screening. By adding a chromatographic separation to the current newborn screening method, separation of isomers could be achieved and additional analytes could be added to the method. A chromatographic separation would allow for differential diagnosis of several key metabolism disorders. Butryrlcarnitine (C4) and isobutryrlcarnitine (ISO C4) are examples of unresolved isomers which are diagnostic for separate disorders and can only be differentiated chromatographically. Also not previously evaluated in newborn screening is the possibility that the matrix suppresses the ionization of analytes, potentially affecting the quantification. In flow injection analysis the analytes are eluted as a plug, such that there is no separation of matrix components from the analytes. Since there is no separation, there is the potential for analytes at higher concentrations to reduce the ionization of analytes at lower concentrations. This competing ionization can result in poor sensitivity and could potential increase errors in identification of a disorder. Employing a chromatographic separation could resolve matrix components from the analytes, improving the sensitivity and improving the quantification of the analytes.

1.2.3 Theory and approach for chromatographic separation

Conventional high performance liquid chromatography has been unacceptable for newborn screening due to long analysis times and the inability to separate all of these compounds in a single analysis. Innovations in ultra-high pressure liquid chromatography utilizing smaller particles and higher linear velocities, as shown by MacNair, Jorgenson and coworkers has been able to obtain high efficiency separations in short analysis times (MacNair, Lewis, & Jorgenson, 1997). The theoretical plate height, which is a measure of a column's efficiency, can be defined by the van Deemter Equation (Equation 1.3) (Miller, 2005). Minimizing the theoretical plate height (H) ultimately improves the columns efficiency. Three kinetic interactions play a role in the column efficiency; eddy diffusion (A), molecular diffusion (B) and mass transfer (C). The B and C terms are both influenced by the linear velocity (u) of the mobile phase. The optimal linear velocity (u_{opt}) is dependent on particle size and diffusion of the anaylte in the mobile phase (D_m) (Equation 1.4) (MacNair et al., 1997). Eddy diffusion (A) is directly related to the particle size (d_p) and packing efficiency (λ) of the column (Equation 1.5) (Miller, 2005). Therefore, smaller particles reduce eddy diffusion and improve column efficiency.

$$H = A + \frac{B}{u} + Cu \qquad \text{(Equation 1.3)}$$

$$u_{opt} \approx \frac{3D_m}{d_p} \qquad \text{(Equation 1.4)}$$

$$A = 2\lambda d_p \qquad \text{(Equation 1.5)}$$

Using smaller particles at optimal conditions reduces the theoretical plate height, which results in an increase the number of theoretical plates obtained per unit length of column. This

allows shorter columns packed with smaller particles to achieve similar performance as longer columns packed with larger particles. Ultimately this reduces analysis times without sacrificing column efficiency.

Another critical aspect to our chromatographic separation is the stationary phase that was selected. The stationary phase is critical to providing good chromatographic peak shape and acceptable retention. There are several choices for stationary phases in liquid chromatography from very polar silica (Si-OH) phases to very non-polar C_{18} bonded stationary phases. Conventional reverse phase columns, such as C_{18} stationary phases, retain hydrophobic analytes more than hydrophilic analytes. However, several of the amino acids and acylcarnitines are extremely hydrophilic and they would not be well retained on a conventional C_{18} column unless they were derivatized first. An alternative stationary phase, called hydrophobic interaction chromatography or HILIC, employs a polar stationary phase and has been used to separate compounds with diverse polarity (Hemström & Irgum, 2006). In addition, the elution order is reversed when compared to separations on a C_{18} column, hydrophilic solutes are more retained than hydrophobic.

The basic HILIC stationary phase consists of an unmodified silica particle with Si-OH as the functional group, however there are also several bonded phases available (Hemström & Irgum, 2006). The exact mechanism for retention of polar analytes is debated, although it is thought that multiple types of interactions can occur including; adsorption, electrostatic and dipole-dipole (Hemström & Irgum, 2006). Adsorption of analytes into and out of a water solvated layer surrounding stationary phase particles appears to be a driving force for retention (Hemström & Irgum, 2006). Therefore, hydrophilic analytes have a higher affinity for the water

and will partition into this layer more readily, increasing their retention. Conversely, hydrophobic analytes will have a higher affinity for the mobile phase and are less retained. The overall result of this separation mechanism is longer retention for hydrophilic solutes than for hydrophobic solutes. Modified silica bonded stationary phases provide alternative selectivity for HILIC retention, include; diol, cyanopropyl, amide, anion exchange, cation exchange and β-cyclodextrin (Hemström & Irgum, 2006). Hemström and Irgum have published a comprehensive review of all the different HILIC bonded phases that are commercially available (Hemström & Irgum, 2006). Several of these stationary phases provide unique selectivity for acidic and basic compounds and while others are good for the separation of isomers. Although, underivatized silica provides acceptable retention for polar solutes the amide bonded phase shows improve peak shape for basic compounds and requires less time for re-equilibration after gradient separation using buffers (Hemström & Irgum, 2006). A major advantage to HILIC chromatography is the mobile phase is rich in organic solvent, which provides better desolvation, ultimately improving sensitivity for electrospray ionization (ESI). Overall, HILIC chromatography provides a unique selectivity that is ideal for separation of for both acylcarnitines and amino acids.

The use of silica-bonded cyclodextrin as a secondary separation mechanism further enhances the selectivity of this chromatographic system allowing for the separation of critical isobars. Cyclodextrins have been used to separate enantiomers, diasteriomers and structural isomers in multiple applications including CE, GC and HPLC (Ward & Baker, 2008). The cyclodextrin ring is made up of multiple glucopyranosides attached together forming different size rings; α (alpha) - six member ring, β (beta) – seven member ring and γ (gamma) an eight

member ring (Tang & Ng, 2008). The size of the ring is important to the enhancing the selectivity of this separation. If compounds are too big to fit inside the cyclodextrin ring then there will not be any differences in retention of two isobars. Also if both of the molecules easily fit into the cyclodextrin then the cyclodextrin will not provide added selectivity either. The ideal case is when one of the two isomers will fit into the cyclodextrin and the other does not fit as well, due to steric hindrance or repulsive interactions with the surface of the cyclodextrin. There are various types of interactions that can occur between the cyclodextrin and the analyte molecule, including van der Waals, hydrophobic, electro static and hydrogen bonding (Li & McGuffin, 2007). The interior of the cyclodextrin ring is hydrophobic, allowing for better retention of non-polar compounds; however the top of the cyclodextrin ring is more hydrophilic due to hydroxyl groups, allowing for better retention of compounds that can hydrogen bond with these hydroxyl groups. Therefore the retention is related to its size and structure of the molecule, and is affected by its orientation and interaction with the cyclodextrin (Singh, Bharti, Madan, & Hiremath, 2010; Li & McGuffin, 2007). This makes cyclodextrin an ideal phase for separating structural isomers, since one isomer will be generally more retained than the other.

We combined both of these separation mechanisms to develop a rapid high resolution chromatographic separation for the analysis of acylcarnitines and amino acids. The chromatographic separation can be conducted in approximately the same time as the current flow injection analysis. In addition, we were able to chromatographically separate key isobars that are diagnostic markers for different metabolism disorders, improving the selectivity of the analysis.

1.3 Automated dried blood spot analysis

1.3.1 Recent advancements in automation for dried blood spot analysis.

Dried blood spot analysis has been extremely advantageous to newborn screening allowing for samples to be easily collected, shipped and stored. However, the analysis of dried blood spots involves more complexities due to multiple processing steps that are typically done manually or with limited automation. This slows down sample processing and adds to the workload of newborn screening laboratories. Another disadvantage of dried blood spot analysis is a 3 mm punch has to be removed from the card for the analysis. Once this spot is removed there is no way to directly track the sample back to the original card from which it was taken. This lack of connection makes it difficult to ensure there are no samples that are mixed up due to processing errors. Therefore, in order to improve the efficiency of dried blood spot analysis and to minimize the potential for processing errors, we investigated the ability to directly analyze newborn screening cards without the need to punch disks from the card.

Others have also investigated the direct analysis of dried blood spots using similar approaches, however their research has been aimed at improving the efficiencies of dried blood spot analysis for use in clinical trials in pharmacokinetic studies to support new drug development (Barfield, Spooner, Lad, Parry, & Fowles, 2008; Beaudette & Bateman, 2004; Li & Tse, 2010; Spooner, Lad, & Barfield, 2009; Uyeda et al., 2011; Youhnovski et al., 2010) and for therapeutic drug monitoring (Edelbroek, Heijden, & Stolk, 2009). This work has been a driving force in developing automated techniques so that hundreds of samples can be analyzed rapidly with little to no operator intervention. Table 1.2 is a comprehensive list of articles published for the direct analysis of dried blood spots. There are two primary designs that have been used

for direct analysis of dried blood spots; flow through and direct surface sampling. The first device developed in 2009 required a disk to be punched from the dried blood spot and placed in a cartridge (Déglon, Thomas, Cataldo, Mangin, & Staub, 2009). The cartridge was inserted in-line with the mobile phase and the analytes were eluted off the DBS. Although this approach was successful it was extremely labor intensive and time consuming, since every sample had to be individually processed. This design was later automated in 2011 using multiple clamping devices to hold dried blood spots so that many samples could be analyzed, greatly improving the efficiency (Déglon et al., 2011). An alternative approach was developed which involved direct sampling of the surface of the dried blood spot using a device similar to a TLC plate analyzer (Van Berkel & Kertesz, 2009). There are several advantages to this device; samples can be processed without removing a punch, variable sample size can be analyzed and it could be incorporated with in-line sample clean-up prior to analysis. The TLC sampling device was pressed on the top of a dried blood spot and the mobile phase was used to elute the analytes. This device has been recently automated with robotic instrumentation improving sample through-put efficiency.

Other techniques that have been published for the direct analysis of dried blood spots but they are not fully quantitative. The first technique involves analysis using ionization by paper spray. A small triangle of paper is placed in a clamp inside the ion source of the mass spectrometer. Mobile phase is pumped to the paper and a voltage is applied to the clamp allowing for ionization of the analytes. The analytes are eluted off the paper and are ionized and analyzed by mass spectrometry. The second technique involves the use of direct electro spray ionization (DESI). DESI employs an electrospray source that is directed at the sample's

surface. A solvent is pumped through the source which is ionized. The charge from the solvent is transferred to the surface and analytes are ionized and pulled into the mass spectrometer. Both of these methods are direct ionization techniques in which the sample is directly analyzed just prior to the mass spectrometer. Paper spray does allow for some separation depending on the properties of the paper used, however DESI is a direct analysis without any chromatographic separation. Although both of these techniques do allow for direct sampling of dried blood spots, neither of these techniques would be a dramatic improvement over the newborn screening method since they are only semi-quantitative techniques.

Both the TLC interface and flow through device have been employed for the quantitative analysis of pharmaceutical compounds to support clinical pharmacokinetic studies (Déglon et al., 2011; Heinig et al., 2011). These devices allow for quick analysis of DBS, and have been fully automated to reduce sample handling requirements, improving sample tracking with minimum off-line sample preparation. However, there are still some concerns with direct analysis of dried blood spots including incorporation of an internal standard, dilution of out of range samples and correction for hematocrit-induced variations in sample volume.

There have been multiple techniques used to add internal standard for on-line dried blood spot analysis. The internal standard can be added to the sample prior to analysis either into the blood prior to spotting it on filter paper (Abu-Rabie & Spooner, 2009) or after the blood has dried (Déglon et al., 2009; Déglon et al., 2011). Although both of these techniques appear to be acceptable they require additional sample handling that has to be done at the time of sample collection or sufficiently before sample analysis. Adding internal standard to the blood prior to spotting requires a larger volume of sample to be drawn, measured accurately before

adding the internal standard. This approach is not practical for newborn screening which is very simplistic and would add more complexity to the sample collection. A recent technique was developed by Abu-Rabie et. al. uses a piezo electric spray device to add internal standard to the dried blood spot prior to analysis and allowing it to dry (Abu-Rabie et al., 2011). An even coating of internal standard is sprayed across the surface of the dried blood spot and allows for the internal standard to bind to the matrix and paper components similarly to the analyte. Once the samples are dried the DBS is analyzed by a flow injection technique so that the internal standard elutes with the analytes. This approach was acceptable for the analysis of acetominophen and sitamaquine using direct analysis with the TLC interface. Although adding the internal standard to the DBS prior to analysis appears to an acceptable technique, it adds additional sample handling requirements and delays time to analysis. An alternative approach would be to load a plug of internal standard into a loop and inject it through the DBS as the analytes are eluted (Heinig, Wirz, Bucheli, & Gajate-Perez, 2011). This technique appears to be the best solution since it minimizes sample processing steps and can be easily integrated into most analytical systems.

Table 1.2: Current approaches used for direct analysis of dried blood spots

Technique	Internal Standard Approach	Analysis	Comments	Reference
Flow through	Added to DBS prior to analysis	Saquinavir, Imipramine, Verapamil	First Publication of on-line desorption of dried blood spots	(Déglon et al., 2009)
	Added to DBS prior to analysis	Flurbiprofen and its metabolite 4-hydroxyflurbiprofen	Method was fully validated and used for the evaluation of a pharmacokinetic study	(Déglon et al., 2011)
TLC	Internal Standard added to blood before spotting	Acetaminophen, Sitamaquine, Propranolol	First publication of TLC interface for dried blood spot analysis. No chromatographic separation	(Van Berkel & Kertesz, 2009)
	Internal Standard added to blood before spotting	Acetaminophen, SB243213, Sitamaquine, Ibuprofen, 4-Nitrophthalic acid Proguanil, Simvastatin, Benzethonium chloride	Optimization of elution conditions	(Abu-Rabie & Spooner, 2009)
	Evaluated internal standard addition to paper before and after spotting blood and on-line using loop injection	Not given, pharmaceutical compound	Incorporated column switching and on-line SPE cleanup	(Heinig, Wirz, & Gajate-Perez, 2010)

Technique	Internal Standard Approach	Analysis	Comments	Reference
TLC	Internal Standard added to blood before spotting	Acetominophen and Sitamaquine	Evaluated the robustness for direct analysis of DBS and dried plasma samples (DPS)	(Abu-Rabie & Spooner, 2011)
	IS sprayed onto dried blood spot prior to analysis	Acetominophen and Sitamaquine	Evaluated the use of spraying IS onto DBS prior to analysis. Samples were processed manually and with TLC interface	(Abu-Rabie et al., 2011)
	Internal standard added by loop injection on-line	Oseltamivir (Tamiflu®) and Oseltamivir carboxylate	Evaluated a new device that directly desorbs sample on-line with chromatographic separation	(Heinig et al., 2011)
Paper Spray	Internal Standard added to blood before spotting	Amitriptyline and Sitamaquine	Analysis of therapeutic Drugs in DBS	(Manicke, Abu-Rabie, Spooner, Ouyang, & Cooks, 2011)
	Added to DBS just prior to analysis	Propranolol and Atenolol	Evaluated the potential to analyze drugs in DBS using paper spray	(Manicke et al., 2011)
Paper Spray	No Internal Standard	Lidocaine, Amitriptyline, Sunitinib, Verapamil, Citalopram	Analysis of therapeutic drugs in DBS using silica coated paper substrate	(Zhang, Xu, Manicke, Cooks, & Ouyang, 2012)

Technique	Internal Standard Approach	Analysis	Comments	Reference
	Internal Standard added to blood before spotting	Analysis of Acylcarnitines	Similar to flow injection analysis, no chromatography shown	(Yang et al., 2012)
DESI	Internal Standard was added to the blood prior to spotting	Prazosin, Sitamaquine, Terfenadine and Verapamil	DBS were directly analyzed using desorption electrospray ionization No Chromatographic separation, analysis is only semi quantitative	(Wiseman, Evans, Bowen, & Kennedy, 2010)
Micro Extraction	Internal Standard added to blood before spotting	Verapamil, Sitamaquine, propranolol sulforaphane, sulforaphane N-acetyl cysteine (NAC) conjugate, sulforaphane glutathione (G) conjugate	Liquid micro extraction of DBS using Advion NanoMate chip-based infusion Directly extracts analytes off the surface of the sample and infuses them into the mass spectrometer	(Kertesz & Van Berkel, 2010)

1.3.2 Advantages of direct DBS analysis for newborn screening

Newborn screening is a labor intensive process requiring samples to be processed by multiple methods. In addition, the typical work load for a newborn screening laboratory ranges from 350 to 1500 samples per day (Chace, 2009). Multiple technicians and experienced chemists are required to process and analyze all these samples and results need to be reported as soon as possible so that treatment can be started for affected children. Overall, this is an ideal environment to employ automated techniques, to improve laboratory efficiency and reduce operating costs (Hamilton, 2007). Implementation of direct analysis of dried blood spots could provide a dramatic benefit to newborn screening. Also sample tracking and data analysis could be directly linked using automation devices such as bar code readers, reducing sample processing errors.

1.3.3 Development of an in-line desorption device for dried blood spot analysis

In order to address the needs of newborn screening, we have developed an in-line desorption device for direct measurement of the dried blood spot without the need punching disks from the filter paper cards eliminating off-line sample processing. This device will minimize human intervention and reduce misidentification of samples due to processing errors. Our approach involves clamping a dried blood spot between two in-line filters which provides a leak free seal. We tested the sealing properties for our device under various conditions to ensure the integrity of the sample was not compromised. The elution parameters were optimized to determine the most effective solvent composition and flow rate to quickly and efficiently elute the analytes. Finally the device was incorporated with chromatographic

separation using an Acquity UPLC HILIC Amide column to separate leucine, isoleucine and hydroxyproline, which are commonly analyzed in second-tier testing for MSUD.

1.4 Factors affecting sample volume in dried blood spot analysis

1.4.1 Evaluation of hematocrit in dried blood spots

Another critical issue in dried blood spot analysis is defining the sample volume from an excised spot. It is well known that sample volume for dried blood spots is dependent on the patient's hematocrit (Mei, Alexander, Adam, & Hannon, 2001). Hematocrit is defined as the density of red blood cell per unit volume of blood. Samples with a high hematocrit will have more red blood cells than samples with a low hematocrit. This is important because the hematocrit affects the viscosity of the blood which in turn will affect the diffusion properties of blood on the filter paper. Newborn screening cards are tested to ensure that blood with a specified hematocrit will diffuse consistently to reduce lot to lot variability (Mei et al., 2001). However, samples with a high hematocrit do not diffuse as far as samples with a low hematocrit. It has been shown that the plasma volume can vary by as much as 47% from a sample with low hematocrit to a sample with high hematocrit (Mei et al., 2001). Table 1.3 is a comprehensive list of publications related to the effect of hematocrit on dried blood spot analysis.

In order to correct for differences in hematocrit Li et. al. have suggested spotting a known volume of blood on perforated filter paper and analyzing the entire spot (Li, Zulkoski, Fast, & Michael, 2011). Although this has been shown to work for analysis of pharmaceutical compounds in blood for pharmacokinetic studies this technique is not ideal for newborn screening. Newborn screening relies on a simplistic sample collection approach where the

newborns heal is pricked with a lance and the blood is spotted on the filter paper by dabbing the paper on the newborn's heal. Spotting an exact amount of blood would require health care providers to draw blood from the child, collect the sample in a tube and then pipet a specified volume on the card. Although this would alleviate the concern related to sample volume it would introduce additional complexities to the current sample collection procedure and could cause errors related to inaccuracies in pipetting.

If the hematocrit of the blood could be determined at the time of sample collection then it could be used to estimate sample volume at the time of analysis. Although this could be done by the health care providers at the time of sample collection it would increase the work load and requires additional instrumentation for the analysis. An alternative approach would be to determine the hematocrit of the dried blood spot at the time of sample analysis. This has been previously attempted using the newborn screening test for hemoglobinopathies (Orsini, Yeman, Bodamer, Mühl, & Caggana, 2010). This test is a semi-qualitative method for the analysis of hemoglobinvariants F, A, S, D, C, and E. Samples are analyzed by HPLC UV at 415nm and the peak areas of the different hemoglobin forms are summed together to get total hemoglobin. The data was then used to relate hemoglobin content to hematocrit (Orsini et al., 2010). Although there was a good correlation between hemoglobin and hematocrit the acceptability of the method was questionable. They reported an overall percent accuracy of 108% with a range of 73.5–142%. Although there does appear to be some issues with the accuracy, this method shows feasibility to determine hematocrit in dried blood spot samples. Once the hematocrit level is determined it can be used to estimate sample volume from a

predetermined punch size, eliminating the need to pipette specified volumes on the filter paper.

Table 1.3: Reported issues related to hematocrit effect on sample volume in dried blood spot analysis

Analytes	Investigation	Comments	Reference
Acetaminophen and sitamaquine	Evaluate the effect of hematocrit on assay bias when using DBS samples for the quantitative bioanalysis of drugs	Showed little bias in sample volume within normal range, however only 15 µL of blood was spotted on cards. Only two analytes investigated	(Denniff & Spooner, 2010)
^{125}I-L-Thyroxine	Evaluated the influence of hematocrit and sample volume added to filter paper	Showed that analyte concentrations were significantly different between 75 µL and 100 µL blood was spotted on cards	(O'Broin, 1993)
^{125}I-L-Thyroxine	Evaluated hematocrit effects on sample volume and potential chromatographic effects.	Approximately a 50% difference in sample volume between 30% and 70% hematocrit. Only a 13% difference in sample volume was observed for peripheral punch and center punch	(Mei et al., 2001)
Phenylalanine	Evaluated the combined effects of hematocrit, loading blood volume and filter paper source.	Combined effect of approximately 30% was observed for phenylalanine concentrations	(Adam et al., 2000)

Analytes	Investigation	Comments	Reference
Amino acids and acylcarnitines	Evaluate the effect of hematocrit and punch location for DBS analysis	Dramatic differences in analyte concentrations were observed for low and high hematocrit samples. Some analyte concentrations in peripheral punches were significantly different than for center punches	(Holub et al., 2006)
Cyclosporin A	Evaluated hematocrit effects in DBS samples	No significant differences from 30% to 56% hematocrit were observed for a 50 µL spot and a 8 mm punch	(Wilhelm, den Burger, Vos, Chahbouni, & Sinjewel, 2009)
Compounds with diverse lipidpholicity	Evaluated the effects of hematocrit and punch location on assay bias with a variety of filter papers.	Significant differences were observed for different hematocrit levels and for center versus peripheral punch locations	(O'Mara, Hudson-Curtis, Olson,Yueh,Dunn,Spooner, 2011)
Unknown compound	Evaluated the effect of sample volume on blood spot diameter on DMPK-B and DMPK-C filter paper cards.	The diffusion properties of the two papers were dramatically different. The DMPK-C paper diffused much more evenly. Analyte concentration increased up to about 50 µL of blood then leveled off.	(Fan et al., 2011)

1.4.2 Background and theory of diffuse reflectance

There are several published papers that have been reported using reflectance as a tool to analyze properties of blood and blood components in vitro and in vivo for decades (Johns, Giller, & Liu, 2001; Meinke, Gersonde, Friebel, Helfmann, & Müller, 2005; Schmitt, Meindl, & Mihm, 1986; Serebrennikova, Smith, Huffman, Leparc, & García-Rubio, 2008; Steinke & Shepherd, 1986; Steinke & Shepherd, 1988; Steinke & Shepherd, 1988; S. Zhang, Soller, Kaur, Perras, & Salm, 2000; Zijlstra, Buursma, & Meeuwsen-van der Roest, 1991; Zonios & Dimou, 2006). The main advantage to reflectance is that it is a non-destructive technique and can be used to directly analyze samples without the need for any preprocessing. Although there have been several reported papers that evaluate hematocrit in vivo and in vitro using reflectance these methods are only applicable to red blood cells in solution. An alternative approach would be required to determine the hematocrit in dried blood spot analysis. Dried blood spot analysis is more closely related to the analysis of surfaces which are commonly analyzed using diffuse reflectance techniques.

Diffuse reflectance has been used to analyze solid samples including; paint, soil, paper, coal food and biological samples (Subhash et al., 2006; Ulery, Drees, & Soil Science Society of America, 2008; Wesley & Wendlandt, 1966; Yu et al., 2008). The principle of diffuse reflectance is used to explain multiple interactions that reflected light has with a mat or rough surface (Ulery et al., 2008; Wesley & Wendlandt, 1966). When light is directed at a rough surface the light is variously reflected, scattered, refracted and absorbed. In order to better understand diffuse reflectance one must first understand the basics of specular reflectance. In the most simplistic case, when light is reflected from a smooth surface it can be defined by the Fresnel

equation, Equation 1.6, where the reflected light (R) is dependent on the incident radiant energy (I_o) and the intensity of the light reflected (I).

Fresnel equation: $$R = \frac{I}{I_o} = \frac{(n-1)^2 + n^2k^2}{(n-1)^2 + n^2k^2}$$ (Equation 1.6)

The radiant energy of the incident light and reflected light is dependent on the refractive index (n) of the medium and the absorption index (k) (Wesley & Wendlandt, 1966). The angle (θ_1) of the incident light from a normal perpendicular to the surface will be the same as the reflected angle (θ_2) (Figure 1.5). The intensity of the light reflected from the surface will be dependent on the amount of light absorbed by the sample (k) and the amount of light that penetrates the sample (n_2). Reflectance of smooth surfaces such as these are commonly performed to determine the absorption properties or refractive index of a material. However, the surface must be smooth and the crystalline structure of the material must not diffuse light.

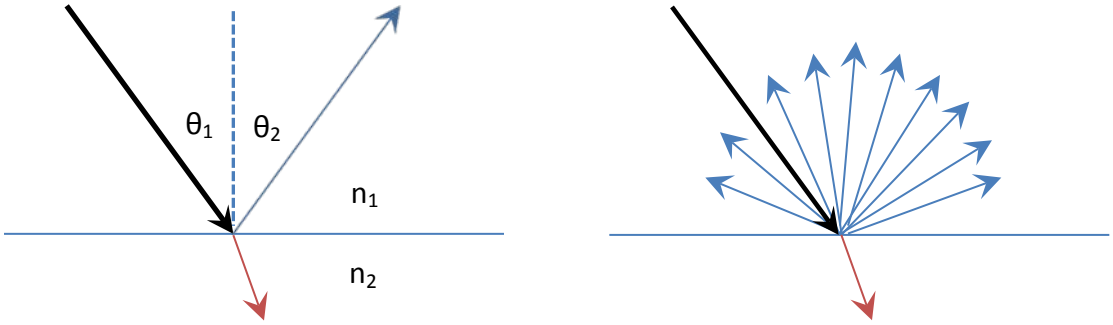


Figure 1.4: Diagram to show the difference between how light is reflected by specular reflectance and diffuse reflectance

Diffuse reflectance is more complex then spectral reflectance and no theories are completely valid for all cases (Wesley & Wendlandt, 1966). In diffuse reflectance light is scattered in multiple directions depending on the crystal structure of the material and/or the roughness of the surface (Figure 1.4). For a non-absorbing surface where the radiation is scattered homogeneously in all directions, the Lambert Cosine Law can be used, Equation 1.7 (Wesley & Wendlandt, 1966),

Lambert Cosine Law: $$B = (\mathrm{I}_o/\pi)\cos\alpha\cos\theta$$ (Equation 1.7)

where B is the intensity of the radiant light, α is the angle of the incident light and θ is the angle of the reflected light. The Lambert Cosine Law was shown to be valid for non-absorbing powders at certain angles, however at some angles the results were unacceptable (Wesley & Wendlandt, 1966). Absorbing materials add additional complexity to developing theoretical models, since some of the light is absorbed by the sample or penetrates into the sample altering the properties of the reflected light.

Reflectance properties of blood and tissue samples have been extensively investigated. One group investigated the reflectance properties of tissue samples indicated that the reflected light was dependent on the size and density of cellular components (Yu et al., 2008). It has also been reported that the optical absorbance of the red blood cells in vitro solutions affect the intensity of light that is reflected and light scattering properties were related to hematocrit (Lovell, Hebden, Goldstone, & Cope, 1999). Although these measurements were made in different media from a dried blood spot, it does show the potential to measure the intrinsic properties of blood using reflectance. Our approach is based on the phenomena that as the

hematocrit increases the red blood cell density increases across the surface of the dried blood spot and the surface will scatter and absorb light depending on the concentration of the cellular components.

1.4.3 Diffuse reflectance of dried blood spots

In order to correct sample volume for differences in hematocrit on dried blood spots, we have investigated the use of UV/NIR reflectance to relate hematocrit. By relating hematocrit to a UV/NIR measurement we can then estimate sample volume from a predefined punch size. We will test our approach with donor samples prepared at various hematocrit levels and fortified at known concentrations with acylcarnitines C0, C2, C3, C4, C5, C6, C8, C10, C12, C14, C16 and C18. Correcting for sample volume should improve the accuracy of newborn screening potentially improving the positive predictive value and will reducing errors associated with not correcting sample volume for high and low hematocrit levels.

CHAPTER 2

A QUANTITATIVE METHOD FOR ACYLCARNITINES AND AMINO ACIDS USING HIGH RESOLUTION CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY IN NEWBORN SCREENING DRIED BLOOD SPOT ANALYSIS

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2.1. Introduction

Newborn screening is claimed to be "one of the most successful public health programs" (Sahai & Marsden,2009) ever initiated and originated with Robert Guthrie screening for phenylketonuria (PKU) (Guthrie & Susi, 1963). Since its inception, additional markers have been identified for different disorders and added to the panel of screening tests. However, most of these methods required a separate analysis for each disorder. It wasn't until a method was developed by researchers at Duke University that multiple heritable metabolic disorders could be identified in a single test (Millington et al., 1990). This approach relied on a technique called flow injection in which samples were directly injected onto the mass spectrometer without chromatographic separation. Mass spectra were extracted from the data allowing for identification of metabolic disorders based on elevated levels of specific acylcarnitines. Over the years additional analytes were added to this screening method allowing for early detection of more than 20 different metabolism disorders in a single analysis (Chace et al., 2003;

Sweetman, 2001). Although this approach has proven to be successful and provides a rapid screening test for many disorders, the analysis is not fully quantitative (Sahai & Marsden, 2009), and screen positive results often need to be confirmed by a second-tier test (Lehotay et al., 2011).

One major disadvantage with this flow injection approach is its inability to differentiate isobaric species associated with different metabolic disorders resulting in additional testing to identify the specific disorder (Bennett, 2009; Matern et al., 2007; Sahai & Marsden, 2009; Sweetman, 2001). Chromatographic separation is required to separate these isobars, however current approaches involve lengthy analysis times (Maeda et al., 2007), use ion pair reagents (Piraud et al., 2005) or require additional instrumentation such as capillary electrophoresis (Chalcraft & Britz-McKibbin, 2009). Another potential problem for a flow injection approach is quantification using a single point response factor with stable isotope labeled internal standards. Assuming that the responses of the isotopically labeled internal standards are equivalent to their corresponding analyte can lead to inaccuracies in quantification and fluctuations in quantification limits. It has been reported, that the response factors of the analytes and internal standards must be evaluated to accurately quantify analytes (De Jesús, Chace, Lim, Mei, & Hannon, 2010). Chace and coworkers have reported the quantification of leucine can be impacted by unresolved isobaric species that have different response factors (Chace et al., 1995). Although this research allowed for the identification of MSUD, it also shows that response factors can be affected by unresolved isobars. Not previously discussed regarding newborn screening, is the potential for response factors to be affected by matrix effects. Although, stable isotope labeled internal standards reduce errors associated with

matrix suppression there are reports that show analyte to internal standard response ratios can be affected (Jemal, Schuster, & Whigan, 2003; Lindegardh et al., 2008; Trufelli, Palma, Famiglini, & Cappiello, 2011). In addition, since there is no chromatographic separation employed in the flow injection screening method, ionization can be reduced by the presence of matrix related components or by other analytes at higher concentrations (Trufelli et al., 2011). This could result in the misidentification of samples close to the cut-off value, if the matrix suppression reduces or enhances the ionization of an analyte differently than its corresponding internal standard.

The original and still widely used approach for flow injection analysis involves derivatization of acylcarnitines and amino acids with n-butanol and HCl to form butyl esters which enhances the sensitivity of certain analytes (Chace et al., 2003). However, the caustic nature of this derivatization is a safety hazard to laboratory personnel and hydrochloric acid is corrosive to laboratory equipment. More recently a method has been developed without derivatization, but this approach may result in lower intensity for specific dicarboxylic acid acylcarnitines (De Jesús et al., 2010). Although both of these techniques have been shown to provide acceptable results for screening purposes there is still the potential for a high false positive rate for some disorders (Matern et al., 2007; Sahai & Marsden, 2009). This has resulted in the need for implementation of second-tier tests to identify certain disorders (Matern et al., 2007; Sahai & Marsden, 2009). The current approach to confirm specific disorders is one second-tier test for each disorder, resulting in multiple second-tier tests that have to be used by newborn screening laboratories and/or physicians. To date there is not a comprehensive second-tier test able to quantify acylcarnitines and amino acids in a single

analysis. Common practice for quantitative analysis of acylcarnitines and amino acids profiles requires a second blood sample to be drawn from the infant and sent to a clinical laboratory for analysis. This can result in unnecessary parental anxiety for false positive cases and further delaying the time to diagnosis. Therefore, there is a need for a rapid second-tier test that could be used to improve identification of the confounding disorders associated with results obtained by flow injection newborn screening methods.

Typical methods employed by clinical laboratories for quantitative analysis of acylcarnitines and amino acids are not usually available within public health newborn screening laboratories. In addition, these methods are not commonly performed together, so samples are analyzed by separate procedures using different instruments. The reported methods for acylcarnitines are not practical for second-tier testing due to long analysis times (Maeda et al., 2007; Maeda et al., 2008; Zuniga & Li, 2011), poor chromatography (Ghoshal et al., 2005), or involve alternative instrumentation such as capillary electrophoresis (Chalcraft & Britz-McKibbin, 2009). There are many methods available for amino acid analysis; however, the majority involves derivatization of samples followed by HPLC-UV, FLD, LC/MS or GC/MS (Kaspar et al., 2009). These methods typically have analysis times longer than ten minutes and require costly derivatization techniques to enhance sensitivity and retention. One published method for the analysis of amino acids does not require derivatization; however the analysis time is 50 minutes (Langrock, Czihal, & Hoffmann, 2006), which would be unacceptable as a rapid second-tier test. Our method is the first comprehensive method capable of chromatographically separating twelve acylcarnitines and seven amino acids in 2.2 minutes, with a total injection to

injection cycle time of 3.1 minutes, a dramatic improvement over all other previously reported techniques.

The combination of high resolution chromatography and the unique selectivity of HILIC stationary phase are ideal for fast chromatographic separation of both amino acids and acylcarnitines without the need for derivatization or ion pair reagents. It has been reported that ultra-high pressure liquid chromatography using smaller particles and higher linear velocities are able to achieve high efficiency separations in faster analysis times compared to conventional HPLC separations (MacNair et al., 1997). In addition, polar stationary phases such as hydrophilic interaction liquid chromatography (HILIC) are better suited to retain diverse polarity analytes and offer improved sensitivity over reverse phase chromatography (Hemström & Irgum, 2006). The basic HILIC stationary phase consists of an unmodified silica particle with Si-OH as the functional group, however there are also several modified silica phases that are commercially available (Hemström & Irgum, 2006). Although the unmodified phase has been widely used for HILIC chromatography the HILIC amide phase provides better peak shape for basic analytes and takes less time to re-equilibrate between injections (Hemström & Irgum, 2006). Using both UHPLC and HILIC allowed us to developed a complementary rapid method that could be used by newborn screening laboratories to identify fatty acid oxidation disorders, amino acid metabolism disorders and organic acid disorders. In addition, the chromatographic separation resolves leucine, isoleucine and hydroxyproline allowing for confirmation of MSUD, which is not possible with the flow injection methods. This analysis is performed with the same sample extract used in the non-derivatized screening method to rapidly confirm abnormal screening results. In addition, quantification is based on a full calibration using reference

standards prepared in whole blood and dried on newborn screening cards. Alternative conditions were used to resolve butyrylcarnitine (C4) from isobutyrylcarnitine(ISO C4) along with valerylcarnitne(C5) from isolvalerylcarninte (ISO C5) which would allow for differential diagnosis for short chain acyl-coenzyme A dehydrogenase deficiency(SCAD) and isobutyryl-CoA dehydrogenase deficiency(IBCD) or isovaleric academia (IVA). This methodology provides newborn screening laboratories with a complementary rapid second-tier test which is able to confirm multiple metabolism disorders in a single analysis.

2.2 Materials and methods

2.2.1 Materials and reagents

Acylcarnitine reference standards (purity > 95%) were purchased from VU Medical Center Metabolic Laboratory, (Amsterdam, The Netherlands) and amino acid reference standards (purity > 96%) were purchased from Sigma Aldrich (Milwaukee, WI, USA). Stable isotope labeled amino acids and acylcarnitines reference materials (purity > 98%) were obtained from Cambridge Isotopes (Andover, MA, USA). Hydroxyproline obtained from ARCOS Organics (Morris Plains, NJ, USA) was used as a system check to confirm the retention time. HPLC grade acetonitrile, formic acid and ammonium formate were from Fisher Scientific (Pittsburgh, PA, USA). Control human sodium heparin whole blood was obtained from Biochemed (Winchester, VA, USA). Newborn screening cards, 903 Protein Saver, were obtained from Whatman (Piscataway, NJ, USA). A 22 Multiple Syringe Pump from Harvard Apparatus (Holliston, MA, USA) and a 250 µL Hamilton gas tight syringe from Fisher Scientific (Pittsburgh, PA, USA) was used for infusion tests.

2.2.2 Chromatographic instrumentation

Chromatographic separations were conducted with a Waters Acquity HILIC Amide column, 2.1 x 50 mm, 1.7 µm; Waters Corp. (Milford, MA, USA). Separation of C4 and C5 isomers was achieved by placing a Supelco Astec CYCLOBOND™ I 2000 Chiral HPLC guard column, 1.0 x 20 mm, 5 µm from Sigma Aldrich (Milwaukee WI, USA) prior to the HILIC Amide column. A Waters Acquity UPLC pump and sample manager were used for analysis and gradient separation. A Waters Premier triple quadrupole mass spectrometer (Milford, MA, USA) equipped with electrospray ionization (ESI) source in positive ion mode was used. Optimization of the instrument parameters including desolvation temperature, source temperature, desolvation gas flow, cone gas flow, capillary voltage, cone voltage and collision energy was performed by direct infusion of a solution containing acylcarnitines, amino acids and their labeled internal standards. Optimal sensitivity was achieved using 3.8 kV for the capillary, 390 ^{o}C desolvation temperature with nitrogen as the desolvation gas at a flow of 800 L/hr. Cone gas was nitrogen at a flow of 20 L/hr with a source temperature of 110 ^{o}C. Argon was used for the collision gas at a flow of 0.4 L/hr. Multiple reaction monitoring (MRM) was used for all analytes and internal standards. Specific transitions were used for each compound with optimized cone voltage and collision energy shown in Table 2.1. In order to monitor for all these transitions, MRMs were scheduled based on the retention time of each analyte to achieve a minimum 0.4 minute window around each peak with a 0.02 second dwell time and a 0.02 second inter-scan delay.

Table 2.1: Acylcarnitines and amino acids analyzed and the mass spectral settings for MRM mode

Analyte	ID	CV (V)	CE (eV)	MRM Transition	I.S.	I.S. MRM Transition	Time segment (min)
Carnitine	C0	22	20	161.9→84.6	C0-D9	170.9→84.6	1.73 to 2.03
Acetylcarnitine	C2	22	20	203.9→84.6	C2-D3	206.9→84.6	1.57 to 1.87
Propionylcarnitine	C3	22	20	217.9→84.6	C3-D3	220.9→84.6	1.43 to 1.73
Butyrylcarnitine	C4	22	21	231.9→84.6	C4-D3	235.0→84.6	1.31 to 1.61
Valerylcarnitine	C5	22	23	246.0→84.6	C5-D9	255.1→84.6	1.21 to 1.51
Hexanoylcarnitine	C6	22	23	260.0→84.6	C8-D3	291.1→84.6	1.10 to 1.40
Octanoylcarnitine	C8	22	23	288.1→84.6	C8-D3	291.1→84.6	0.97 to 1.27
Decanoylcarnitine	C10	22	28	316.1→84.6	C8-D3	291.1→84.6	0.80 to 1.10
Lauroylcarnitine	C12	22	28	344.1→84.6	C14-D9	381.2→84.6	0.63 to 1.01
Myristoylcarnitine	C14	22	28	372.1→84.6	C14-D9	381.2→84.6	0.63 to 1.01
Palmitoylcarnitine	C16	22	28	400.2→84.6	C16-D3	403.2→84.6	0.00 to 0.95
Stearoylcarnitine	C18	22	28	428.2→84.6	C16-D3	403.2→84.6	0.00 to 0.95
Phenylalanine	Phe	20	15	165.8→119.7	Phe-D6	171.8→125.7	1.37 to 1.67
Leucine/Isoleucine	Leu/Ile	18	11	131.8→85.7	Leu-D3	134.85→88.7	1.41 to 2.03
Methionine	Met	14	11	149.8→103.6	Met-D3	152.85→106.6	1.50 to 1.80
Tyrosine	Tyr	15	14	181.8→135.8	Tyr-D6	187.8→141.8	1.54 to 1.84
Valine	Val	20	8	117.8→71.8	Val-D8	125.8→79.7	1.60 to 1.90
Citrulline	Cit	16	10	175.8→158.7	Cit-D2	177.8→160.7	1.88 to 2.18

2.2.3 Preparation of dried blood spots

For the validation portion of this study, sodium heparinized human whole blood was fortified at 6 levels for a calibration curve and at 4 additional levels for quality control samples. Individually prepared reference standards containing acylcarnitines and amino acids were spiked into whole blood to fortify samples at the desired concentration to prepare the highest calibration standard and quality control sample. These samples were then serially diluted to prepare the additional calibration levels and quality control samples. Each dried blood spot was prepared by spotting 50 µL of sample onto a Whatman, 903 Protein Saver newborn screening card. These samples were allowed to dry for at least 2 hours at room temperature and then stored at 0-5 ^{o}C until analyzed.

2.3. Results and discussion

2.3.1 Response factor evaluation

In newborn screening, response factors are used to quantify analytes and set cut-off values to help identify children at risk of having a metabolic disorder. The analyte concentration is determined based on Equation 2.1 where the response factor is often not calculated and assumed to be equal to one. However, this assumption can lead to errors in calculated concentrations if the response factors deviate from one. We evaluated the response factors for all analytes using both external solutions and extracted samples to determine if the sample matrix had a significant effect on the response factors. The analyte response factors were initially determined for each analyte by flow injection analysis without derivatization using external standards containing both analytes and internal standards. External standards were prepared such that the final concentrations of the analytes and internal standards were

equivalent. Serial dilutions were made to achieve five-point response curves with concentrations above and below target concentration of internal standard in the final extract. For flow injection analysis, 3 replicate injections of an external standard containing analytes and internal standards were injected onto the LC/MS/MS system and the response from 50 scans were averaged, which is the maximum number of scans across the center of the elution plug. The responses of each analyte and their respective isotopically labeled internal standard were evaluated. Data for carnitine and acetylcarnitine were plotted to show the relationship between response and concentration, Figures 2.1A and 2.1B respectively. Equation 2.2 was used to calculate the response factors for each analyte and its isotopically labeled internal standard at various concentrations. The response factors for all the analytes were plotted versus concentration to evaluate if the response factors were consistent across the concentration range. Example plots for response factors for carnitine and acetylcarnitine are shown in Figures 2.2A and 2.2B respectively. The average response factors were calculated by averaging the response factor across all concentrations and results are reported in Table 2.2.

Equation 2.1: Conventional calculation for concentration for analytes extracted from dried blood spots

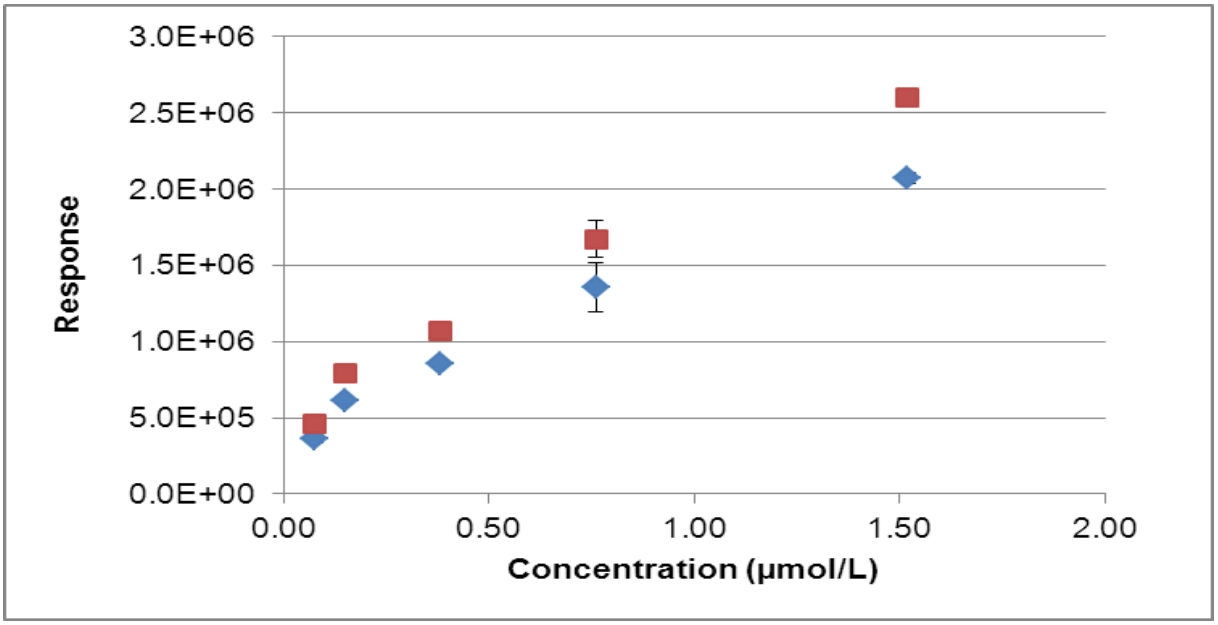
$$Analyte\ Conc. = \frac{Analyte\ Response}{I.S.\ Response} \times Conc.\ I.S.\ \times Response\ Factor\ \times \frac{Extraction\ Volume}{Sample\ Volume}$$

Equation 2.2: Response factor determined using the instrument response for an analyte and internal standard analyzed at identical concentrations

$$Response\ Factor\ = \frac{Internal\ Standard\ Response}{Analyte\ Response}$$

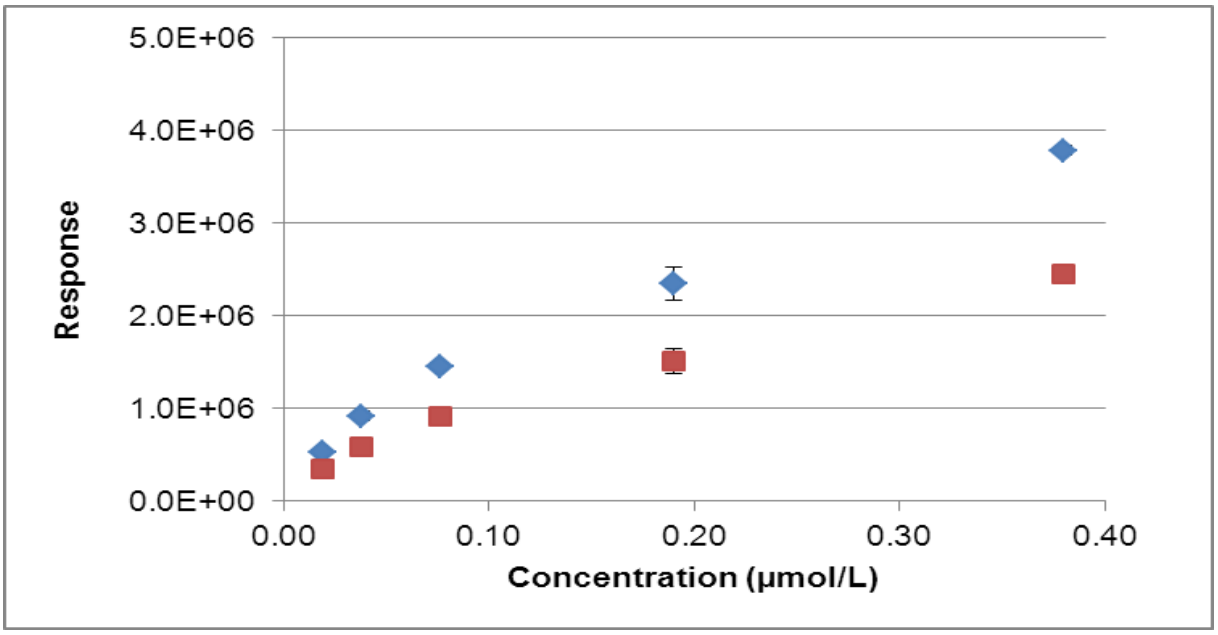
Equation 2.3: Calculation of accuracy for the response factor determined using internal calibration curve

$$\%\ Accuracy_{RF} = \frac{Calculated\ concentration\ determined\ with\ calibration\ curve}{Calculated\ concentration\ using\ IS\ with\ RF\ of\ 1}\ x\ 100\%$$



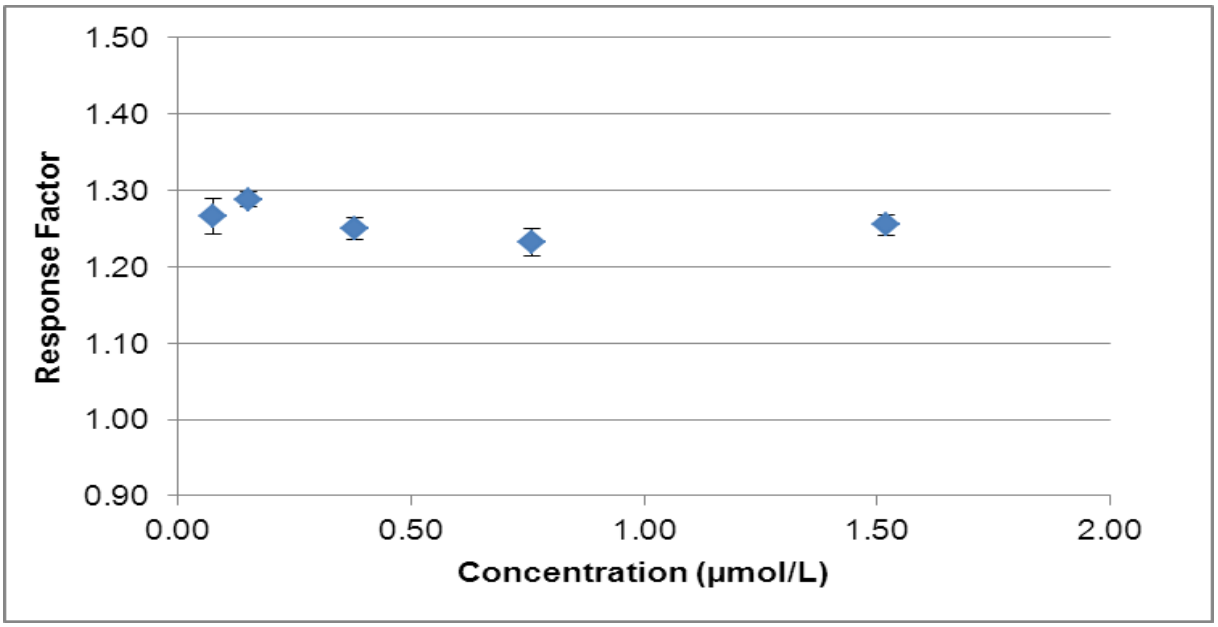
Legend: Carnitine (squares) and carnitine-D9 (diamonds) for external standards using flow injection analysis. Each sample analyzed in triplicate, error bars represent 1 S.D..

Figure 2.1A: Plot of external standard response (n =3) versus concentrations for carnitine and carnitine-D9, samples analyzed by LC/MS flow injection analysis with precursor ion scan mode for the production of 85 m/z



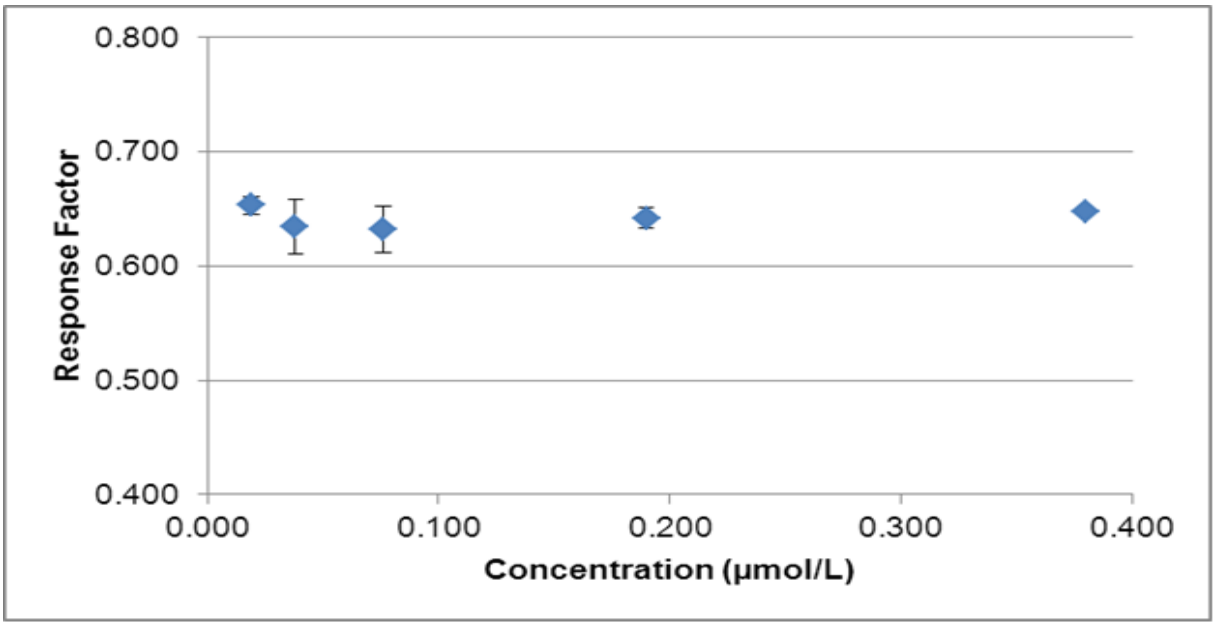
Legend: Acetylcarnitine (squares) and acetylcarnitine-D3 (diamonds) for external samples using flow injection analysis. Each sample analyzed in triplicate, error bars represent 1 S.D..

Figure 2.1B: Plot of external standard response (n=3) versus concentration for acetylcarnitine and acetylcarnitine-D3, samples analyzed by LC/MS flow injection analysis with precursor ion scan mode for the production of 85 m/z



Legend: Each sample analyzed in triplicate, error bars represent 1 standard deviation

Figure 2.2A: Plot of calculated response factors (n=3) for external standards containing carnitine and carnitine-D9, evaluated at five different concentrations, samples analyzed by LC/MS flow injection analysis with precursor ion scan mode for the production of 85 m/z



Legend: Each sample analyzed in triplicate, error bars represent 1 standard deviation

Figure 2.2B: Plot of calculated response factors (n=3) for external standards containing acetylcarnitine and acetylcarnitine-D3, evaluated at five different concentrations, samples analyzed by LC/MS flow injection analysis with precursor ion scan mode for the production of 85 m/z

The response factors in extracted dried blood spot samples were evaluated using the flow injection analysis technique described above. Internal standard stock solutions were prepared in the appropriate solvent identified by Cambridge Isotopes and diluted 200-fold in the extraction solvent to achieve the appropriate concentration used in the established newborn screening method. A 3.0 mm sample was punched from a dried blood spot quality controls sample and placed in a 96-well plate. Three replicates of each sample were extracted in 100 µL of methanol with 0.1% formic acid containing labeled internal standards. Samples were vortexed for 30 minutes vigorously and then 70 µL was transferred to a clean 96-well plate for analysis. These samples were analyzed by flow injection analysis for acylcarnitines and amino acids. The calculated concentration was determined using the response ratios with the appropriate labeled internal standard and then multiplying by the concentration of the internal

standard and the dilution factor as shown in Equation #2.1. In addition, these samples were quantitated using an internal calibration curve and the back-calculated concentrations were used to determine the actual concentration of the analyte in the sample. Using Equation #2.3, the accuracy of the response factor of 1 was determined for each analyte. The accuracy value can be then be multiplied by 1 to obtain the actual response factor for the extracted samples which is reported in Table 2.2.

Table 2.2: Evaluation of response factors for external standards and extracted samples

Analyte	Response Factors for External Standards	S.D.	%RSD	p-value*	Response Factor for Extracted Samples	S.D.	%RSD	p-value*
C0	1.26	0.0238	1.89%	< 0.05	1.09	0.0649	5.94%	< 0.05
C2	0.642	0.0151	2.35%	< 0.05	1.53	0.137	8.95%	< 0.05
C3	0.792	0.0530	6.69%	< 0.05	1.26	0.148	11.7%	< 0.05
C4	0.906	0.0268	2.96%	< 0.05	1.31	0.0616	4.70%	< 0.05
C5	1.13	0.0344	3.05%	< 0.05	1.27	0.0851	6.70%	< 0.05
C6	1.19	0.0361	3.02%	< 0.05	1.13	0.0772	6.83%	< 0.05
C8	0.905	0.0267	2.95%	< 0.05	0.931	0.120	12.9%	**0.249**
C10	0.728	0.0283	3.89%	< 0.05	0.713	0.119	16.7%	< 0.05
C12	0.963	0.0287	2.98%	< 0.05	0.883	0.129	14.6%	< 0.05
C14	0.897	0.0251	2.80%	< 0.05	0.829	0.117	14.1%	< 0.05
C16	1.01	0.0299	2.95%	**0.0742**	0.976	0.114	11.7%	**0.315**
C18	1.10	0.0471	4.26%	< 0.05	1.16	0.141	12.1%	< 0.05
Phe	0.931	0.00694	0.746%	< 0.05	1.29	0.0841	6.50%	< 0.05
Leu/Ile	0.932	0.00461	0.495%	< 0.05	1.22	0.105	8.62%	< 0.05
Tyr	1.16	0.0626	5.42%	< 0.05	1.82	0.120	6.60%	< 0.05
Val	1.15	0.0249	2.16%	< 0.05	1.38	0.0893	6.46%	< 0.05
Cit	1.09	0.0282	2.58%	< 0.05	1.67	0.0503	3.00%	< 0.05
Met	1.08	0.0465	4.32%	< 0.05	1.43	0.0214	1.49%	< 0.05

Three replicates at 5 levels were analyzed (n=15) for externals. Three replicates at 4 levels were analyzed (n=12) for extracted samples. *p-value was used to test if the mean is different than 1.0 using JMP 9.0.0 software. Bold p-values indicate significant differences

From our results, it is apparent that the response factors determined for external standards are different than the response factors determined for extracted samples for all

analytes except C6, C8, C10, C16 and C18, indicating that matrix does affect the response factors. In addition, the response factors for most analytes and internal standards are significantly different from one using a two tailed t-test ($p < 0.05$). Overall, the response factors for external samples appear to be very consistent with %RSDs less than 6.69%; however, response factors for extracted samples are much more inconsistent with %RSDs as high as 16.7% for some analytes. Since the responses are not equal for analytes and internal standards, concentrations of analytes would not be proportional to the internal standard at the same concentration, thus resulting in values that are lower or higher than the actual concentration in the sample. Further, if the slope of the analyte response versus concentration is significantly steeper than that of the internal standard, then small changes in analyte concentration may result in relatively large quantification errors. Therefore, when samples are analyzed by flow injection analysis using only response factors, there is a deviation from the actual value when analyte response factors are different from their internal standard. For instance, the response factor for C5 was determined to be 1.27 so the calculated concentration is 21.3% lower than the actual concentration. This indicates that using the concentration of the internal standard has the potential to inaccurately quantify analytes when the response factors are significantly different from one.

2.3.2 Flow injection analysis ionization suppression evaluation

One major problem reported with electrospray ionization is matrix related ionization suppression (Matuszewski, Constanzer, & Chavez-Eng, 2003; Trufelli et al., 2011). As shown previously, the response factors for external samples are different from extracted samples, which is a result of competing ionization between the analytes and other components found in

the extracted sample. When analyzing samples by flow injection analysis there is no separation between matrix components and the analytes. In addition analytes at higher concentrations compete for ionization with other analytes reducing their response which could potentially cause an analyte to be undetected. Using labeled internal standards reduces problems with quantification related to matrix effects; however sensitivity can be dramatically affected (Trufelli et al., 2011).

In order to evaluate ionization suppression or enhancement, a post injection infusion experiment for acylcarnitines and amino acids was conducted using a standard technique (Matuszewski et al., 2003; Trufelli et al., 2011). First a sample was injected into the mobile phase flow without post column infusion to determine the lag time between the injection and the start of the sample plug and to determine time duration of the flow injection profile. The lag time is related to the flow rate and dead volume of the system, which is dependent on the tubing length and the tubing diameter. The flow injection profile of the sample appears as a broad peak since there is not any chromatographic separation. After determining the profile for the flow injection analysis; matrix effects could be evaluated by infusing an external standard containing acylcarnitines and amino acids labeled internal standards(20 µL/min), at a 200-fold dilution of the stock solution, in the flow path to the mass spectrometer prior to the electrospray source. This provides an elevated baseline response for transitions associated with each labeled internal standard. The labeled internal standards were used to assess matrix effects, since all blood samples will contain endogenous levels of amino acids and acylcarnitines which could artificially elevate or suppress the baseline. Next, dried blood spot sample extracted in methanol with 0.1% formic acid without internal standard are injected onto the

system. Shifts in the baseline would indicate regions of ion suppression (a negative response shift) or ion enhancement (a positive shift in the baseline). Matrix effects were evaluated for six donor blood samples containing endogenous levels of acylcarnitines and amino acids and at 3 fortified levels. To quantify the degree of ion suppression, the average internal standard responses were evaluated during the flow injection profile and compared to the average internal standard response obtained by direct infusion with mobile phase.

A representative extracted unfortified sodium heparin human dried blood spot sample analyzed by flow injection without infusion of internal standard solution is shown in Figure 2.3A. This represents the flow injection profile for a typical sample. A representative extracted unfortified sodium heparin human dried blood sample analyzed by flow injection with infusion of internal standard solution is shown in Figure 2.3B. It is apparent from this experiment that there is a dramatic decrease in ionization during the time that the plug of sample is being analyzed. The overall decrease in ionization was calculated for each analyte at 4 different levels in triplicate and average overall suppression was determined to be 63%. For some analytes the matrix suppression was as high as 78% which could dramatically affect the detectability of analytes by flow injection resulting in potential misidentification of a metabolism disorder. In order to reduce this matrix effect and improve sensitivity of this analysis, some form of chromatographic separation or sample clean-up is necessary.

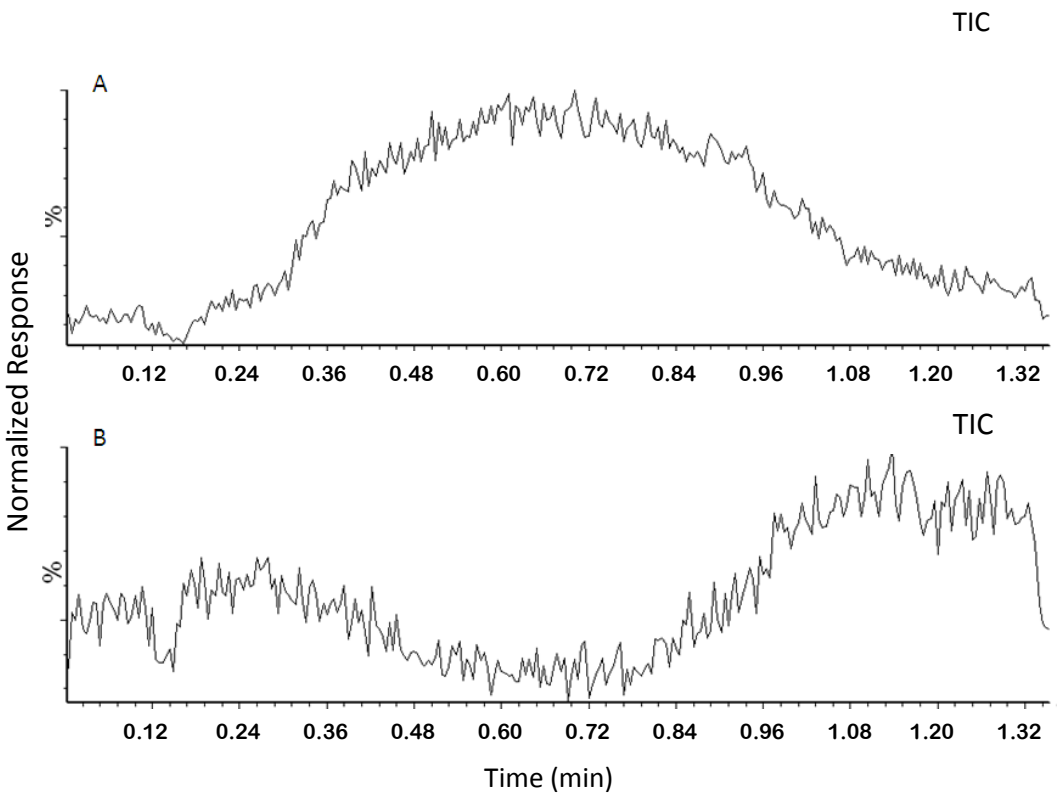


Figure 2.3: TIC of a precursor ion scan for production of 85 m/z, by flow injection analysis of extracted dried blood spots, A – Flow injection analysis of an unfortified sodium heparin blood sample without infusion of internal standards, B – Flow injection analysis of a unfortified sodium heparin blood sample with infusion at 20 µL/min of labeled internal standards solutions at a 200-fold dilution to assess matrix effects

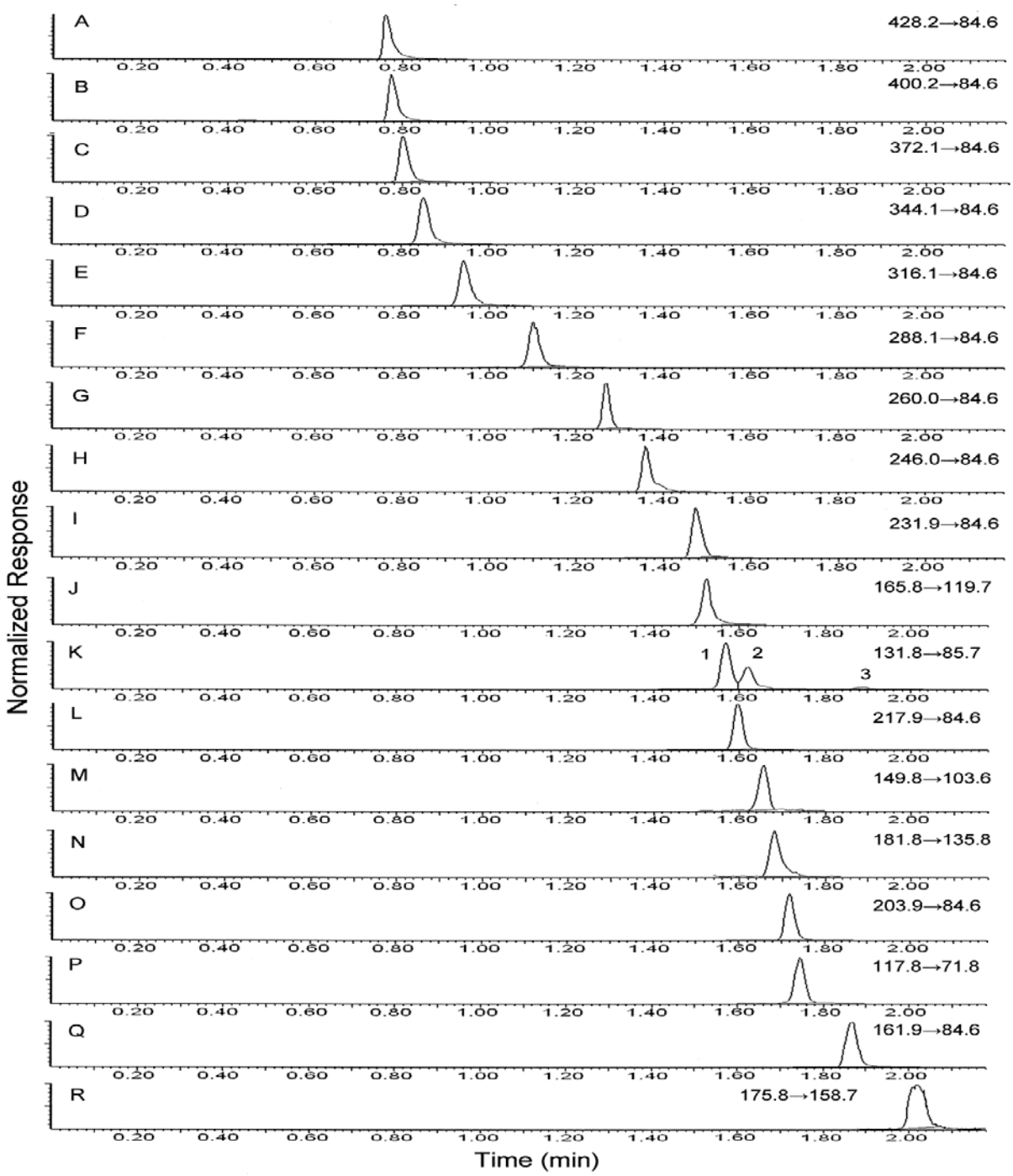
2.3.3 Chromatographic separation of amino acids and acylcarnitines

Preliminary investigations involved optimization of chromatographic conditions to achieve acceptable retention of all analytes in a 3.1 minute total injection cycle time. Mobile phase A contained water with 0.1% formic acid and mobile phase B contained a mixture of 90% acetonitrile and 10% water, such that the final buffer concentration was 10 mM ammonium formate. A gradient elution was employed from 96% mobile phase B to 55% mobile phase B over 1.65 minutes (Table 2.3). An Acquity UPLC pump was used to control the mobile phase flow at 0.3 mL/min and mobile phase composition allowing for the gradient elution of analytes.

Representative chromatograms of a single injection of an extracted sample using this gradient elution is presented in Figure 2.4. Scheduled MRMs were set based on the retention times of the analytes and then allowing for 0.2 min retention time shift in either direction. Acceptable separation of the isomers leucine, isoleucine and hydroxyproline was achieved; however C4 and C5 isomers were not separated under these conditions.

Table 2.3: Chromatographic conditions for separation of acylcarnitines and amino acids

Time (min)	Flow Rate (mL/min)	% A	% B
Initial	0.30	4	96
1.65	0.30	45	55
1.66	0.30	4	96
3.10	End		



Legend: A: C18, B:C16, C:C14, D:C12, E:C10, F:C8, G:C6, H:C5, I:C4, J: Phenylalanine, K: (1)Leucine, (2)Isoleucine, (3) Hydroxyproline, L:C3, M:Methionine, N:Tyrosine, O:C2, P:Valline, Q:Carnitne, R:Citrulline

Figure 2.4: Chromatographic separation of 12 acylcarnitines and 8 amino acids with using gradient conditions shown in Table 2.3 and scheduled MRM transitions for each analyte

2.3.4 Chromatographic separation of C4 and C5 isomers

Additional work was conducted to resolve the C4 and C5 isomers, since we were unsuccessful in achieving separation on the HILIC Amide column alone. Several different chiral stationary phases were evaluated to improve this separation; however the cyclodextrin phases provided the best resolution. Cyclodextrin phases provide unique selectivity in which interaction is based on inclusion complexation (Singh et al., 2010). Therefore, smaller analytes with less steric hindrance will form a more stable complex with the cyclodextrin, whereas larger analytes with more steric hindrance will not complex as strongly.

Separation of the C4 and C5 isomers was optimized using the Astec cyclodextrin guard column. The percent aqueous was varied from 0 to 10% and with buffer solutions prepared at a basic pH and an acidic pH. The resolution between each isobar was determined for each condition using an external standard containing the individual isobars. Figures 2.5 and 2.6 shows the resolution that was determined for each condition for C4 and C5 isomers respectively. It was apparent that 2-4% mobile phase A provided the best resolution and a buffer prepared at pH 8.7 was better than a buffer at pH 4.5. The best resolution obtained for C4 isobars was 0.38 which is not sufficient for baseline resolution. The resolution achieved for C5 isobars was 0.8 which was better but still not sufficient for baseline resolution. Longer guard columns could be used however this would increase the analysis time making it impractical for a rapid second-tier test. Better resolution may have been achievable with smaller particles or with alpha cyclodextrin instead of the beta cyclodextrin, however these columns are not commercially available. Therefore it was decided to use the optimized conditions obtained with the current configuration for the additional research.

Figure 2.5: Plot of resolution versus % mobile phase A, evaluated for separation of C4 isomers on an Astec CYCLOBOND™ I 2000 guard column using isocratic elution at different percentages of mobile phase A and with mobile phase B prepared at pH 8.7 and pH 4.5

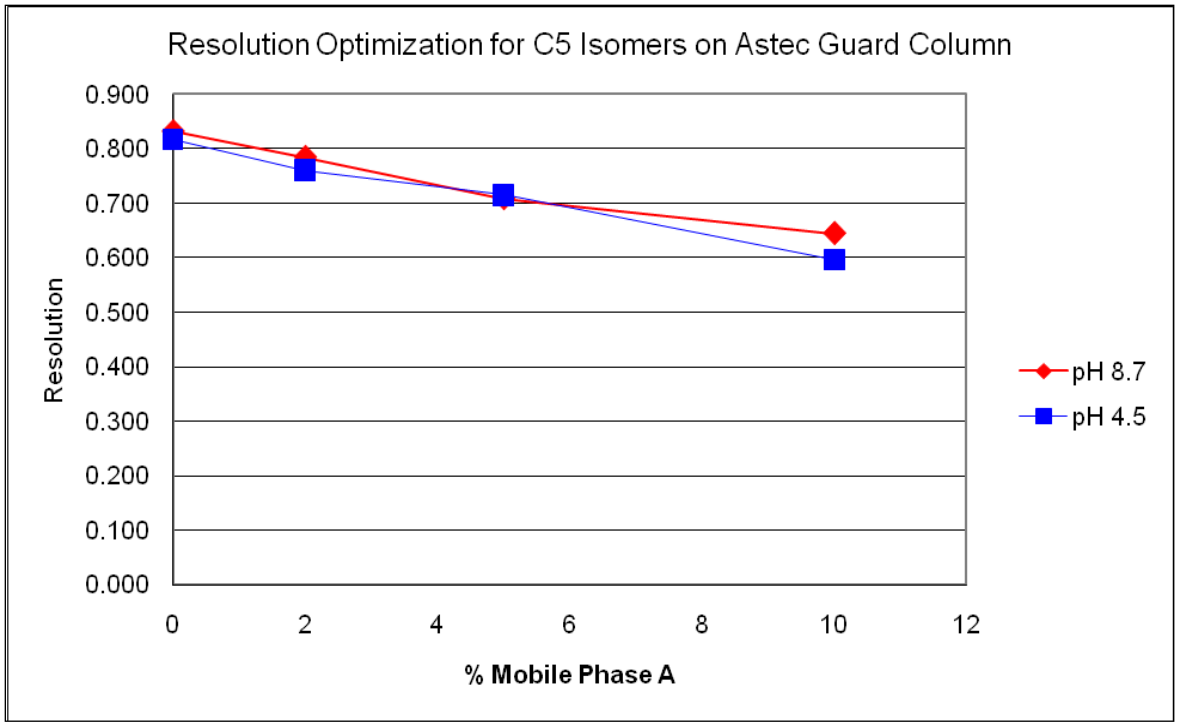
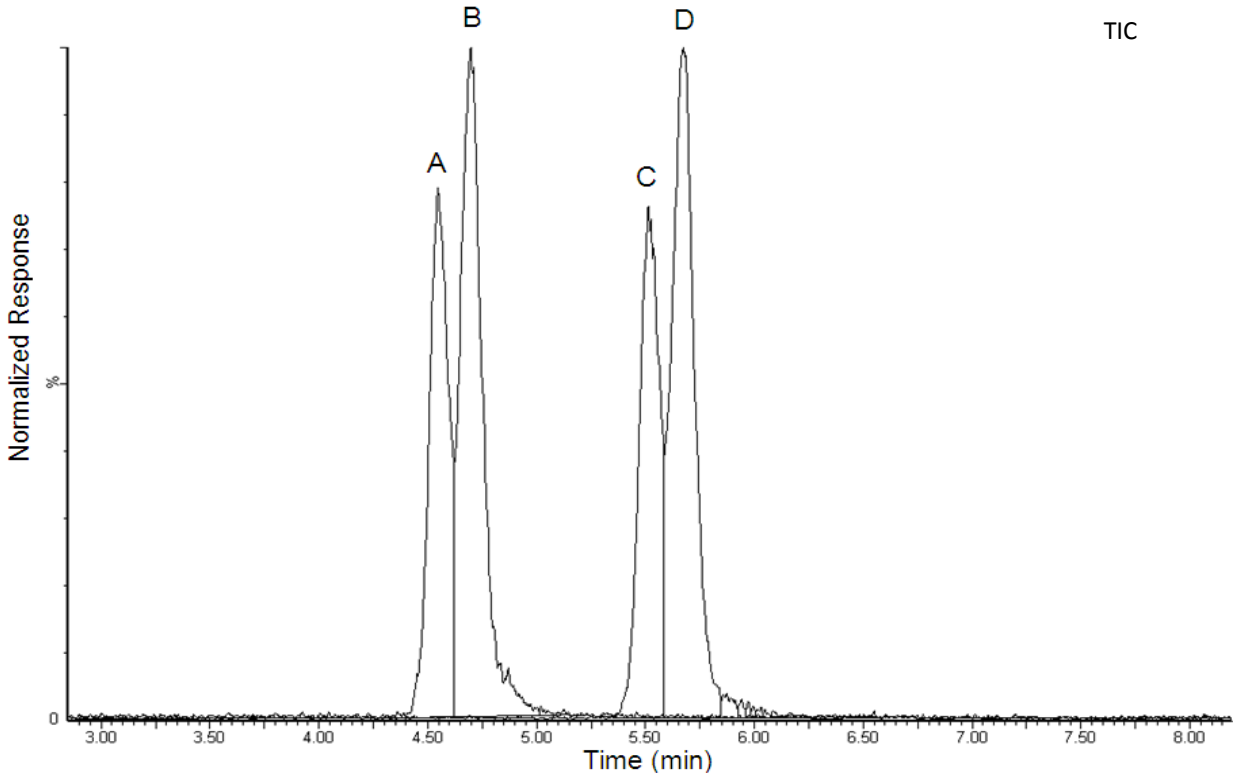


Figure 2.6: Plot of resolution versus % mobile phase A, evaluated for separation of C5 isomers on an Astec CYCLOBOND™ I 2000 guard column using isocratic elution at different percentages of mobile phase A and with mobile phase B prepared at pH 8.7 and pH 4.5

Combining the cyclodextrin phase and HILIC Amide phase provides resolution of 0.83 for the C5 isobars and a resolution of 0.97 for the C4 isobars. Baseline resolution is defined as a resolution of 1.5, however a resolution of 1 is acceptable for quantitative analysis (Miller, 2005). The same mobile phase was used for this separation as used for the separation in the first part with a modified gradient from 0% to 5% mobile phase A over 10 minutes with a flow rate of 0.3 mL/min. The chromatographic separation of C4 and C5 isomers in an extracted sample that contains butyrylcarnitine, isobutyrylcarnitine, valerylcarnitine and isovalerylcarnitine is shown in Figure 2.7. Although baseline resolution was not achieved, the separation described would allow identification and quantification of the analytes. As stated previously, longer columns could be used to achieve full resolution of these isomers; however

the run time would be significantly longer. It was decided to employ the current separation, because a longer analysis time would not be desirable for a rapid second-tier test. Separation of these isomers allows for differential diagnosis for short chain acyl-coenzyme A dehydrogenase deficiency (SCAD) and isobutyryl-CoA dehydrogenase deficiency(IBCD) or isovaleric academia (IVA), which is not possible with flow injection analysis.



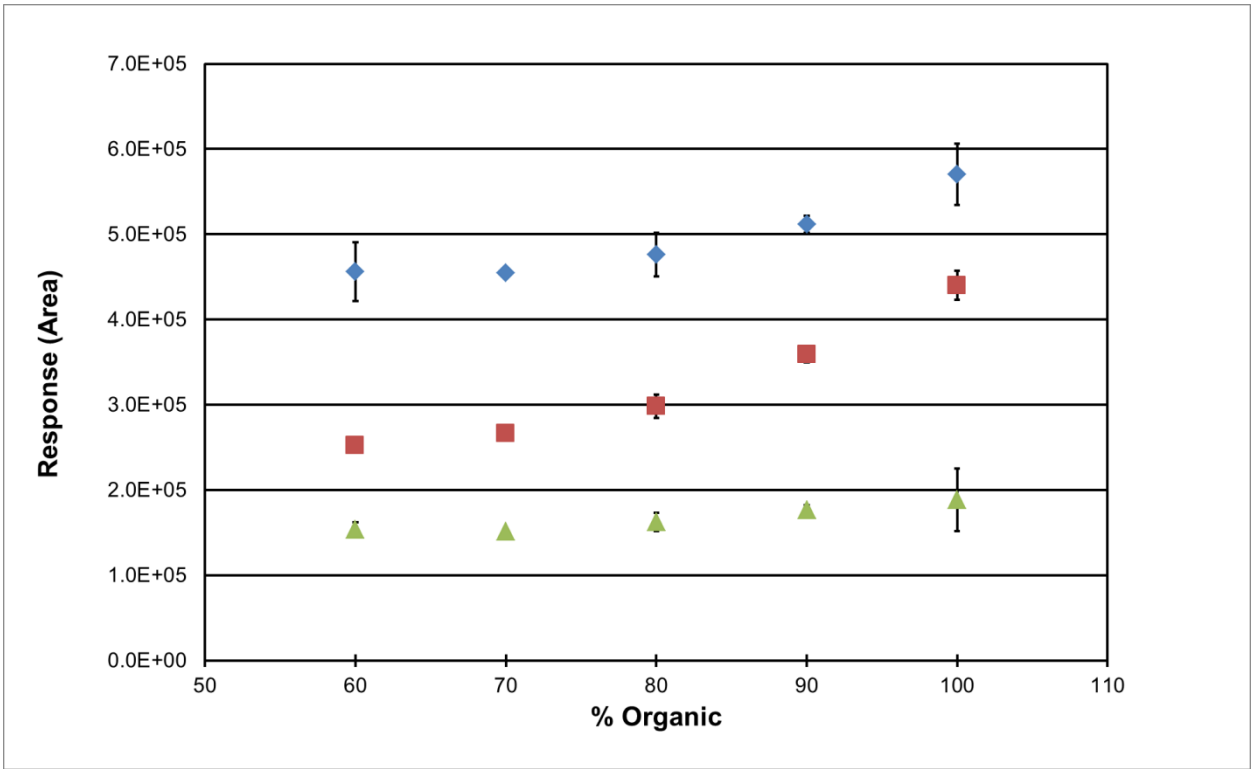
Legend: A:ISO C5, B:C5, C:ISO C4,D:C4.

Figure 2.7: Chromatographic separation of an extracted dried blood spot sample containing butylcarnitine (C4) and isobutyrylcarnitine (ISO C4) at 1.2 µmol/L and valerylcarnitine (C5), isovalerylcarnitine (ISO C5) at 0.96 µmol/L using modified gradient conditions with HILIC Amide and Astec CYCLOBOND™ I 2000 guard column

2.3.5 Extraction optimization

An extraction optimization experiment was conducted in which samples were extracted using various concentrations of methanol and water containing 0.1% formic acid. Additional

experiments were performed using acetonitrile and water containing 0.1% formic acid, and acetonitrile and water containing 10 mM ammonium acetate. A 3.1 mm spot was punched from a control sample and extracted in 100 µL of each extraction solution. Three replicates were analyzed for each extraction solution evaluated. The samples were injected using the chromatographic conditions described earlier. The response ratios with internal standard were plotted for each analyte and extraction condition, typical extraction profile curves for acetylcarnitine (C2), palmitoylcarnitine (C16) and phenylalanine (Phe) using methanol containing 0.1% formic acid are shown in Figure 2.8. The extraction profiles for all the other analytes are very similar to these three profiles and are therefore not presented. From these plots it is apparent that 100% methanol with 0.1% formic acid demonstrated the highest recovery as compared to the other conditions tested and is a common extraction solution used for flow injection analysis (Chace, 2001; Chace, 2007). Although there was not a significant difference between some of the other extraction solutions, it was observed that more aqueous in the extraction solution caused the samples to appear cloudy. The samples extracted in acetonitrile mixtures were visibly clearer than the samples extracted with methanol mixtures; however 20% aqueous is required in order to achieve comparable recovery to 100% methanol containing 0.1% formic acid. Since methanol containing 0.1% formic acid is commonly used in newborn screening analysis, we elected to employ this solvent for further investigations.



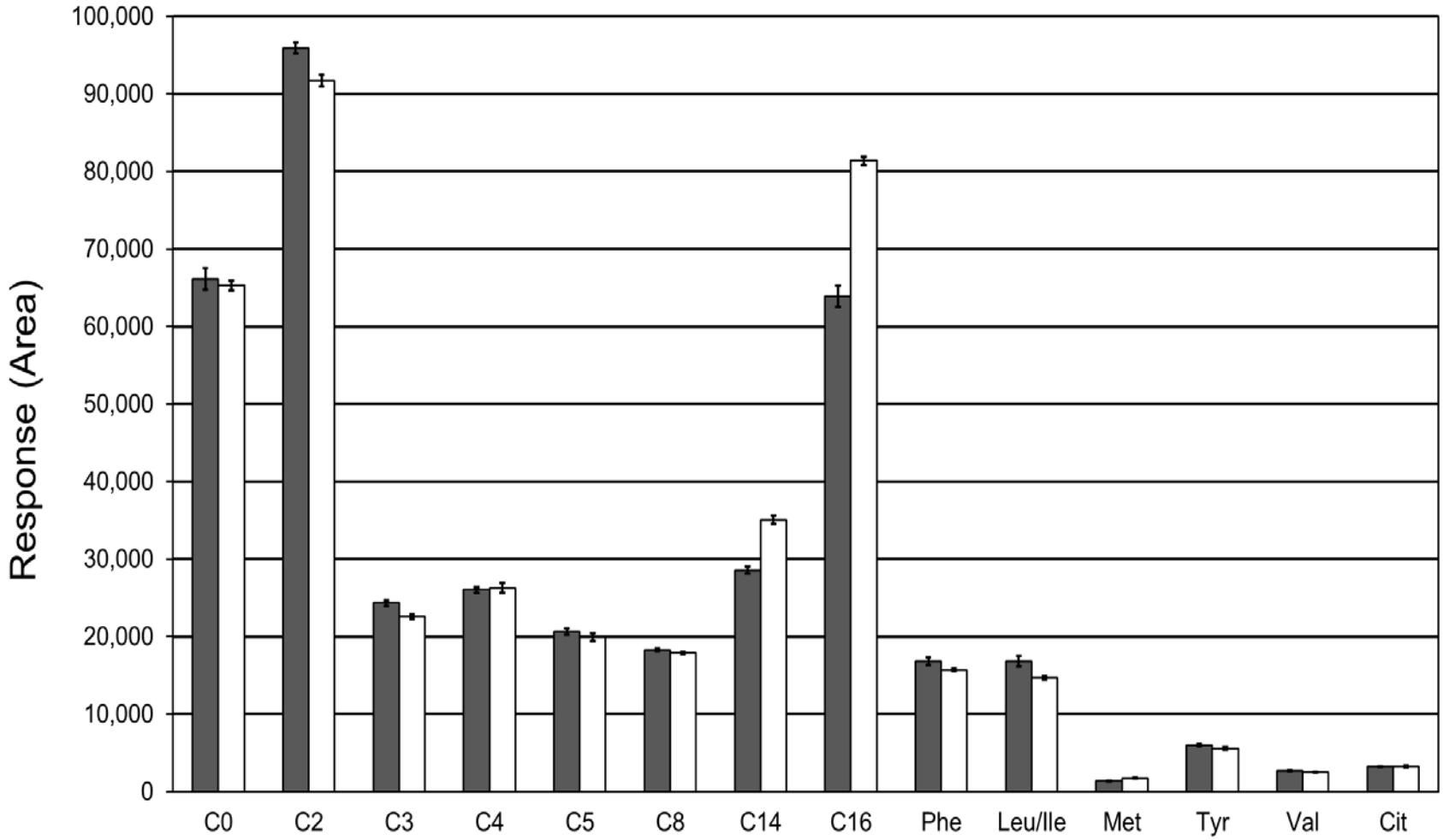
Legend: C2-blue diamonds, C16-red squares, phenylalanine-green triangles. Values for phenylalanine were multipled x2 for graphical purposes. Error bars represent 1 standard deviation for 3 replicates.

Figure 2.8: Plot of response versus % organic to show extraction efficiency of C2, C16 and phenylalanine from dried blood spots, sample were extracted in different percentages of methanol and water containing 0.1% formic acid (n=3) and analyzed using 3.1 minute chromatographic separation and scheduled MRMs

2.3.6 Ionization suppression evaluation for chromatographic separation

Ionization suppression was also evaluated for the chromatographic separation using the same technique as described earlier. As discussed earlier it is difficult to assess matrix affects for samples that contain endogenous levels of the analytes of interest. Therefore the labeled internals were used to assess matrix effects. Dried blood spot samples were extracted in 100 µL of methanol containing 0.1% formic acid and injected using the chromatographic conditions that were described in section 2.3.3. In order to evaluate matrix effects the labeled internal standards were infused post column creating an elevated baseline. While it was apparent that

some regions in the chromatogram showed some ionization suppression, the complexity of the chromatogram makes it difficult to quantify the degree of matrix suppression using this approach. Therefore, an additional experiment was conducted by comparing internal standard responses in extracted samples to external samples. This experiment was performed for dried blood spots prepared from six different individuals. Dried blood spot samples were extracted in triplicate for each individual using an internal standard working solution prepared in methanol containing 0.1% formic acid. The samples were transferred to a clean 96-well plate and injected onto the LC/MS/MS system. The responses for the internal standard in extracted samples were compared to injections of the working internal standard solution. The results for this experiment showed minimal matrix effects based on the percent differences in responses of the internal standards in extracted and external samples, shown in Figure 2.9. Overall there was less than 20% matrix suppression observed for all the compounds except C16 which showed ionization suppression of 21.5%. All analytes demonstrated less than 5% relative standard deviations from triplicate analysis of 6 different lots of sodium heparin human whole blood samples. This is a dramatic improvement over the flow injection analysis in which as much as 63% ion suppression was observed. There was also a significant improvement for the relative standard deviation for this chromatographic analysis compared to the flow injection approach, which could further improve the positive predictive value (PPV) for metabolism disorders associated with elevated levels amino acids and acylcarnitines.



Legend: Black bars are extracted samples white bars are external standards. Error bars represent 1 standard deviation.

Figure 2.9: Bar graph to show matrix suppression evaluated by comparison of internal standard responses in external standards with extracted dried blood spot samples, samples analyzed in triplicate using 3.1 minute chromatographic separation and scheduled MRMs

2.3.7 Validation of analysis with chromatographic separation

Validation of the chromatographic method included precision and accuracy, linearity and recovery. Three replicate samples were analyzed on three separate days in order to assess inter assay precision and accuracy. Prior to validation six different lots of sodium heparin human whole blood were analyzed to select a matrix that contained the lowest endogenous levels of analytes and to ensure the levels of analytes were below the reported cut-off values (McHugh et al., 2011) . The endogenous levels of all analytes were determined using the method of standard addition to create a four point curve from the amount fortified versus the response ratios obtained. Acceptable linearity with correlation coefficients greater than 0.9950 were observed for all analytes using linear regression. From the linear regression $y = mx + b$, where "y" is the analyte response ratio, "x" is the analyte concentration, "m" is the slope and "b" is equal to the y-intercept. Using this equation, the concentration in the unfortified sample can be obtained at the x-intercept where $y = 0$, $x = -b/m$. After establishing concentration in the blank sample the calibration range for each analyte was established by adding the amount fortified to the blank concentration producing a six point calibration curve. A larger range was used for calibration curves to allow for quantification above and below the targeted cut-off values for each analyte. Different regression models and weighting factors were assessed for each analyte and the best model was chosen based on having the best correlation coefficient and lowest average percent difference from theoretical. After evaluating multiple models it was determined that a quadratic fit model with a 1/concentration weighting factor provided the best results for all analytes. The final calibration range based on the adjusted concentrations is

shown in Table 2.4. Correlation coefficients for all runs were greater than 0.9950 for all analytes.

Table 2.4: Calibration range with average correlation coefficients from 3 days

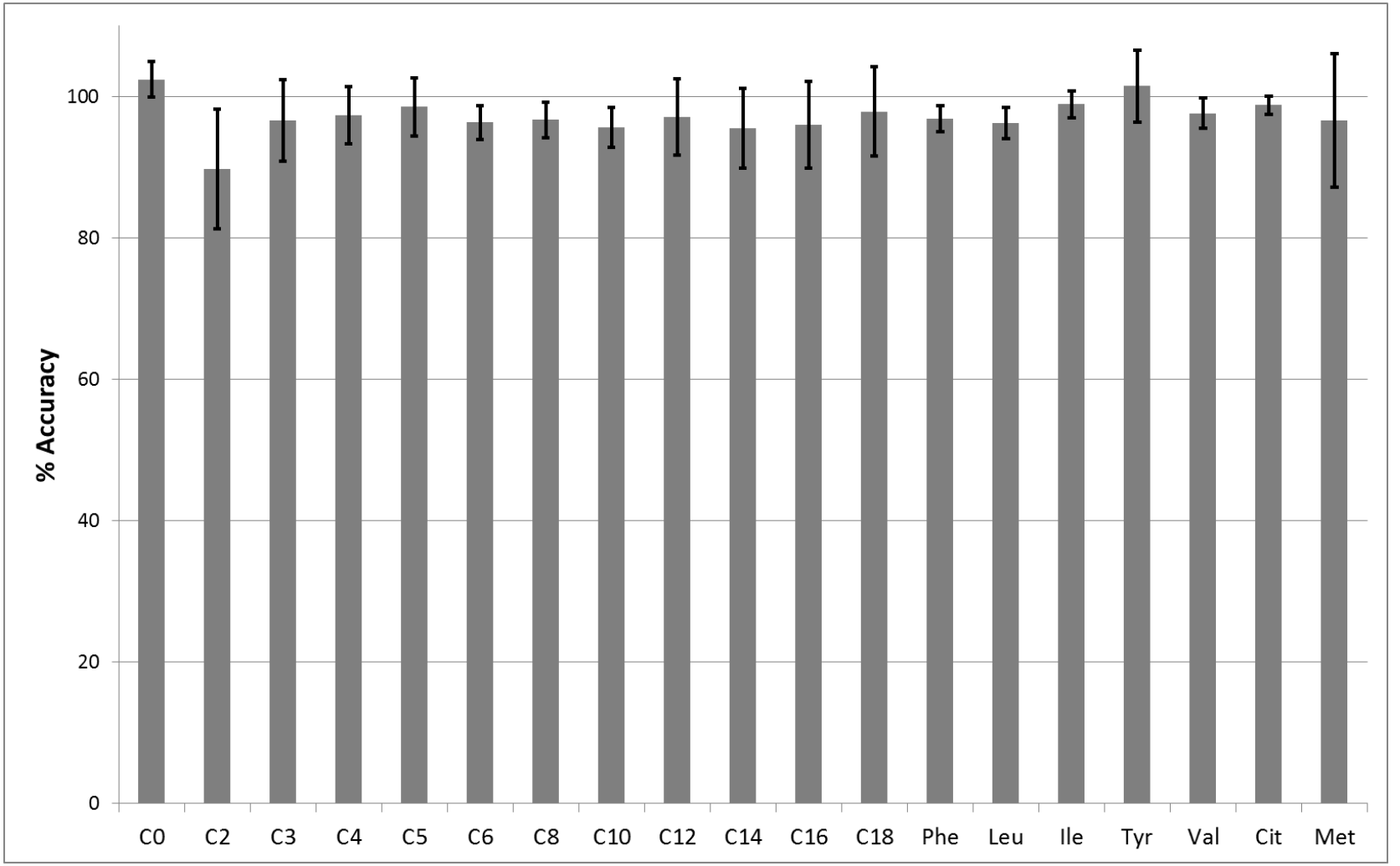
	Calibration Range (µmol/L)	Average Correlation Coefficient
C0	36.4 – 636	0.9990
C2	16.7 – 257	0.9993
C3	1.28 – 41.3	0.9989
C4	0.210 – 10.2	0.9994
C5	0.160 – 8.16	0.9988
C6	0.048 – 8.05	0.9982
C8	0.090 – 8.09	0.9967
C10	0.090 – 8.09	0.9965
C12	0.055 – 10.1	0.9968
C14	0.052 – 8.052	0.9958
C16	0.076 – 40.8	0.9954
C18	0.570 – 16.6	0.9951
Phe	91.3 -1291	0.9973
Leu	115 – 715	0.9952
Ile	64.4 -664	0.9982
Tyr	61.4 -1261	0.9971
Val	180 -1180	0.9980
Cit	42.8 -443	0.9966
Met	15.1 -615	0.9973

The precision and accuracy for all the analysis of acylcarnitines and amino acids extracted from dried blood spots were calculated at each of the four concentrations in triplicate over 3 separate days, the precision was within 9,98% and the accuracy ranged from 85.7% to 108%, as shown in Table 2.5. However, the highest quality control sample for acetylcarnitine had an accuracy of 78.2%, which is outside the acceptable limit of $\pm$ 15% based on FDA guidance for bioanalytical method validation. The overall average accuracy was evaluated for each analyte by averaging the results from the 4 levels reported in Table 2.5 and is plotted in Figure 2.10.

Table 2.5: Precision and accuracy over three days using chromatographic separation and MRM transitions

	Level 1		Level 2		Level 3		Level 4	
	Mean (µmol/L)	%RSD	Mean (µmol/L)	%RSD	Mean (µmol/L)	%RSD	Mean (µmol/L)	%RSD
C0	59.4 (101)	3.10%	87.1 (99.6)	2.97%	240 (104)	3.03%	545 (105)	3.25%
C2	23.7 (98)	9.85%	33.7 (93.4)	7.89%	86.0 (89.5)	7.97%	169 (78.2)	5.05%
C3	2.50 (103)	10.9%	4.14 (98.3)	7.39%	12.7 (95.8)	4.83%	28.0 (89.6)	2.91%
C4	0.529 (103)	8.90%	0.934 (97.2)	3.42%	3.08 (95.8)	3.19%	7.20 (93.4)	4.23%
C5	0.412 (104)	8.48%	0.747 (98.9)	4.51%	2.47 (96.8)	2.65%	5.81 (94.4)	3.40%
C6	0.291 (99.8)	2.16%	0.617 (94.7)	0.29%	2.34 (95.4)	2.69%	5.77 (95.3)	2.17%
C8	0.333 (97.2)	6.00%	0.654 (93.0)	4.64%	2.47 (98.7)	2.92%	5.97 (97.8)	3.68%
C10	0.338 (95.3)	7.14%	0.658 (92.2)	3.64%	2.41 (95.9)	3.50%	6.06 (99.1)	3.36%
C12	0.335 (93.9)	3.62%	0.740 (91.4)	2.25%	3.07 (100)	2.07%	7.80 (103)	0.964%
C14	0.283 (91.4)	8.72%	0.604 (90.2)	3.35%	2.43 (98.4)	3.51%	6.18 (102)	1.57%
C16	2.43 (94.7)	13.1%	4.17 (88.4)	7.35%	15.2 (97.8)	1.53%	38.2 (103)	1.48%
C18	1.23 (97.1)	13.2%	1.78 (89.4)	8.43%	5.64 (101)	4.73%	13.3 (104)	2.48%
Phe	123 (98.9)	6.38%	170 (96.3)	5.62%	426 (97.7)	3.45%	903 (94.6)	4.89%
Leu	130 (99.3)	3.47%	151 (96.4)	2.25%	272 (95.2)	1.75%	513 (94.1)	3.87%
Ile	86.7 (101)	5.91%	111(98.8)	5.10%	233 (96.4)	2.46%	498 (99.4)	4.91%
Tyr	124 (97.6)	4.35%	213 (97.3)	8.84%	719 (108)	5.90%	1614 (103)	2.50%
Val	233 (99.6)	7.38%	270 (97.3)	9.07%	488 (98.9)	6.22%	877 (94.7)	5.83%
Cit	51.1 (100)	6.77%	66.7 (97.7)	4.48%	154 (99.8)	3.09%	320 (97.6)	2.96%
Met	31.3 (91.6)	4.98%	51.5 (85.7)	3.79%	197 (104)	5.03%	473 (105)	7.80%

3 replicates for each level on 3 days (n=9). Percent (%) of target values indicated in parentheses



Legend: Error bars represent 1 standard deviation for the average of the 4 different levels

Figure 2.10: Bar graph for overall average accuracy determined for 12 acylcarnitines and 7 amino acids at 4 levels over three days, dried blood spots analyzed with 3.1 minute chromatographic separation and scheduled MRM transitions for each analyte

2.4 Conclusions

In conclusion, a high speed separation for quantification of twelve acylcarnitines and seven amino acids using the same extracted sample that is analyzed with the non-derivatized newborn screening method was developed. The method was validated with respect to linearity, precision and accuracy and recovery. Matrix related ionization suppression evaluated by post column infusion showed less that and average of 8.24% difference in the response of the labeled internal standards when comparing the response in extracted dried blood spots to the response in external standards. However, the responses for C14-d9, C16-d3 and valine-d8 were approximately a 20% lower in extracted samples compared to external standards. Overall the matrix suppression was dramatically better with the chromatographic separation than for the flow injection analysis method. Furthermore, the chromatographic separation allows for assessment of MSUD by separating leucine, isoleucine and hydroxyproline. Alternative chromatographic conditions can be used to adequately resolve C4 and C5 isomers allowing for differential identification of additional disorders SCAD, IVA IBMC, which is not possible in the current newborn screening methods. The overall chromatographic separation can be conducted in approximately the same time frame as the established flow injection analysis and with the same sample, eliminating the need for additional sample preparation or instrumentation (Sweetman, 2001). This method is a major improvement over the current newborn screening method allowing for differentially diagnoses of multiple disorders and improved quantification by resolving matrix related interferences. In addition, our method does not require derivatization of samples and the injection cycle time is approximately the same as for the flow injection analysis.

2.5 Acknowledgements

We would like to thank the Virginia Division of Consolidated Laboratory Services for supplying us with the internal standard reference material and Altria Client Services for providing additional supplies and LC/MS/MS instrumentation.

CHAPTER 3

DIRECT ANALYSIS OF DRIED BLOOD SPOTS BY IN-LINE DESORPTION COMBINED WITH HIGH RESOLUTION CHROMATOGRAPHY AND MASS SPECTROMETRY FOR QUANTIFICATION OF MAPLE SYRUP URINE DISEASE BIOMARKERS LEUCINE AND ISOLEUCINE

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3.1 Introduction

Direct analysis of dried blood spots (DBS) for newborn screening (NBS) could significantly improve the efficiencies of screening laboratories, allowing for reduced sample handling, decreased material costs and faster analysis. Further, there is the potential for linking the data analysis directly to the patient, improving data integrity by minimizing any errors associated with improper sample identification and handling. During our research, devices based on different technology for automated DBS analysis have been proposed and are now past the prototype stage (Abu-Rabie & Spooner, 2009; Déglon et al., 2011). In addition, Leap Technologies has recently released an automated device which allows for in-line desorption of dried blood spots, similar to the device that we published; however we are not aware of any studies that have been published using their device. The Leap Technologies device is similar to our device in that the DBS card is placed in-line with the mobile phase flow from a HPLC system,

allowing for desorption of analytes from the DBS followed by chromatographic separation. Other work has shown that direct analysis of DBS is sensitive and practical for analysis of drugs and their metabolites for clinical pharmacokinetic studies (Déglon et al., 2011; Thomas et al., 2010; Abu-Rabie & Spooner, 2009; Van Berkel & Kertesz, 2009), however they still require the addition of internal standard (IS) either into the sample matrix prior to analysis or require a predetermined disk to be punched from the filter paper card. Other direct sampling approaches have been reported including the analysis of DBS on filter paper by desorption electrospray ionization (DESI) (Takáts, Wiseman, & Cooks, 2005) or the analysis of biological fluids on glass rods by direct analysis in real time (DART) (Cody, Laramee, & Durst, 2005), but both lack the ability to separate isomers and avoid matrix effects since no chromatographic separation is involved. To date there has been no reported applications using direct sampling with chromatographic separation for biomarker analysis related to metabolism disorders.

NBS for metabolism disorders has involved the analysis of DBS for nearly 50 years, a sample collection technique originally proposed by Robert Guthrie for the analysis of phenylalanine to identify newborns that suffered from phenylketonuria (Guthrie & Susi, 1963). Currently more than 50 different disorders can be identified from a NBS card including metabolism disorders, endocrinopathies, hemoglobinopathies and infectious diseases (Sahai & Marsden, 2009). Within the first week of life, all newborns have blood collected by a heel prick and spotted on special filter paper cards. These cards are dried and then shipped to NBS laboratories for testing. This sample collection technique offers some distinct advantages including minimal sample collection volume and reduced costs associated with shipping and storage; it is less invasive than collecting venous samples and minimizes risks associated with

transmission of some infectious diseases such as HIV (Li & Tse, 2010; Parker & Cubitt, 1999). Depending on birthrate, a NBS laboratory receives between 350-1500 samples per day and due to the time sensitive nature of some of these disorders, they must report results as soon as possible (Chace, 2009). Due to the large workload there is need for automated techniques and rapid screening methods to quickly identify affected children.

Although the NBS methods for amino acids and acylcarnitines have proven to be acceptable for identification of several metabolism disorders, there have been reports of a high number of false positives results (Sahai & Marsden, 2009), additional confirmation testing required (Matern et al., 2007) and an inability to differentially identify certain disorders (Chace et al., 2003). The widely used analysis of acylcarnitines and amino acids employs an extraction with butyl esterification followed by flow injection LC/MS/MS analysis. Even though this technique allows for rapid screening, it lacks the ability to differentiate isobaric biomarkers (Sweetman, 2001; Matern et al., 2007; Bennett, 2009). Maple Syrup Urine Disease (MSUD), a condition that results in the accumulation of branched-chain amino acids, is often misdiagnosed by newborns screening method since leucine, isoleucine and hydroxyproline are unresolved and a second-tier test is required for confirmation of this specific disorder (Matern et al., 2007). It has been reported that nearly 0.1% of all NBS samples have elevated levels of leucine/isoleucine/hydroxyproline and require additional testing for proper diagnosis (Matern et al., 2007). Amino acid analysis typically involves some form of derivatization and chromatographic separation to resolve these isobaric species by either HPLC UV (Fekkes, 1996; Pappa-Louisi, Nikitas, Agrafiotou, & Papageorgiou, 2007), UPLC/MS (Boogers, Plugge, Stokkermans, & Duchateau, 2008), CE-MS (Soga, Kakazu, Robert, Tomita, & Nishioka, 2004) or

GC/MS (Kaspar, Dettmer, Gronwald, & Oefner, 2008), thus requiring the need for additional instrumentation, operators and costly consumables.

We have developed a rapid in-line analysis of dried blood spots using a novel device coupled with a HILIC Amide chromatographic separation that is capable of resolving leucine, isoleucine and hydroxyproline in a 5 minute total analysis time. Direct analysis is performed without any offline sample preparation, eliminating the need to punch disks from the filter paper card or adding internal standard to the dried blood spot. HILIC Amide is a polar stationary phase with an amide functional group bonded to the silica that allows for better retention of hydrophilic species than conventional reverse phase columns. This chromatographic separation eliminates the need for derivatization to resolve these isomers and mass spectrometry detection provides enhanced selectivity over HPLC UV and HPLC FLD. Our technique allows for the assessment of MSUD by direct analysis of the original dried blood spot, reducing analysis time and eliminates the need for collection of additional samples from the newborn. Further, this methodology shows the ability to directly analyze dried blood spots for quantification of biomarkers related to metabolism disorders, an advancement that could greatly improve the efficiencies of newborn screening.

3.2 Materials and methods

3.2.1 Materials and reagents

Amino Acid Reference Standard, 1 nmol/µL in 0.1 N HCl was purchased from Agilent Technologies (Santa Clara CA, USA). Stable isotope labeled amino acid reference material was obtained from Cambridge Isotopes (Andover, MA, USA). Hydroxproline was obtained from ARCOS Organics (Morris Plains, NJ, USA). HPLC grade acetonitrile, formic acid and ammonium

formate were from Fisher Scientific (Pittsburgh, PA, USA). Dye solution part # 716000765-1 was obtained from Waters Corp. (Milford, MA, USA). Control human sodium heparin whole blood was obtained from Biochemed (Winchester, VA, USA). Newborn screening cards, 903 Protein Saver, were obtained from Whatman (Piscataway, NJ, USA). A 22 Multiple Syringe Pump from Harvard Apparatus (Holliston, MA, USA) and a 250 µL Hamilton gas tight syringe from Fisher Scientific (Pittsburgh, PA, USA) was used for infusion tests. Digital images and measurements for seal integrity evaluation were made with a VHX Digital microscope from Keyence (Itasca, IL USA).

3.2.2 Chromatographic and instrumentation

Chromatographic separations were conducted with a Waters Acquity HILIC Amide column (2.1 x 100 mm, 1.7 µm) Waters Corp., (Milford, MA, USA). A Waters Acquity UPLC pump and sample manager were used for analysis for gradient separation and for controlling a Rheodyne, 2 position 10-port switching valve purchased from Analytical Sales (Pompton Plains NJ, USA). The prototype direct sampling device was machined to hold two end caps from a pre-column filter holder and two 0.5 µm frits from Chrom Tech Inc. (Apple Valley, MN, USA). End caps were tightened using a Proto 6169A dial torque wrench from Stanley Proto Industrial Tools (Conyers, GA, USA).

3.2.3 Mass spectrometry conditions

A Waters Premier triple quadrupole mass spectrometer (Milford, MA, USA) equipped with an electrospray ionization (ESI) source in the positive ion mode was used. Optimization of

the instrument parameters including desolvation temperature, source temperature, desolvation gas flow, cone gas flow, capillary voltage, cone voltage and collision energy was performed by direct infusion of a solution containing leucine, isoleucine and leucine-d3. Optimal sensitivity was achieved using 4.0 kV for the capillary, 18.0 V for the cone, and 14 eV for the collision energy. The desolvation temperature was 375 ^{o}C using nitrogen as the desolvation gas at a flow of 800 L/hr. Cone gas was nitrogen at a flow of 20 L/hr with a source temperature of 110 ^{o}C. Argon was used for the collision gas at a flow of 0.2 L/hr. Multiple reaction monitoring (MRM) from 131.6 m/z to 85.75 m/z was used for leucine and isoleucine and 134.6 to 88.8 m/z was used for leucine-d3 internal standard. A 0.1 second dwell time was used for each transition with a 0.02 second inter-scan delay for a total cycle time of 0.27 seconds.

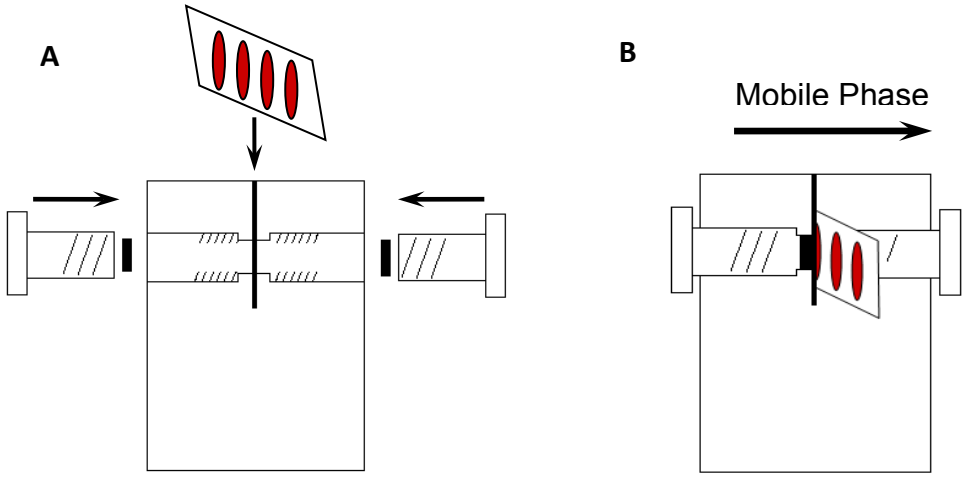
3.2.4 Preparation of dried blood spots

For the validation portion of this study, sodium heparin human whole blood was fortified at 3 levels (100 nmol/mL, 150 nmol/mL and 250 nmol/mL) using a 1.0 nmol/µL Amino Acid Reference Standard purchased from Agilent Technologies. Fortified samples were prepared in volumetric flasks by diluting the appropriate volume of amino acid reference standard (1.0 nmol/µL) with whole blood and thoroughly mixed. For the validation 55 µL of each fortified sample along with a blank was spotted onto Whatman, 903 Protein Saver newborn screening cards. Samples were allowed to dry at room temperature for 2 hours and stored at 0-5 ^{o}C prior to analysis.

3.3 Results and discussion

3.3.1 Design of device

This device allows for direct sampling of NBS cards without any offline preparation, eliminating the need to add internal standard or punching a predetermined-size disk from the dried blood spot. Direct analysis of NBS cards would allow for sample identification to be directly linked to sample analysis via a bar code reader, with the bar code being on the card itself, minimizing identity errors associated with transfer of the punched out spot to a tube or well if the disks are removed from the screening card. NBS cards are placed directly in the flow path of the liquid chromatograph eluent so that analytes can be desorbed from the card and chromatographically resolved on an analytical column. The prototype device was machined to hold two in-line frit end caps such that they could be placed on either side of the newborn screening card and tightened, forming a leak free seal. In addition this device allows for different diameter frits to be used so that the sample size could be varied if needed, however for our experiments we used a 1.5 mm x 0.5 µm frit. Figure 3.1 shows the prototype device that was used.



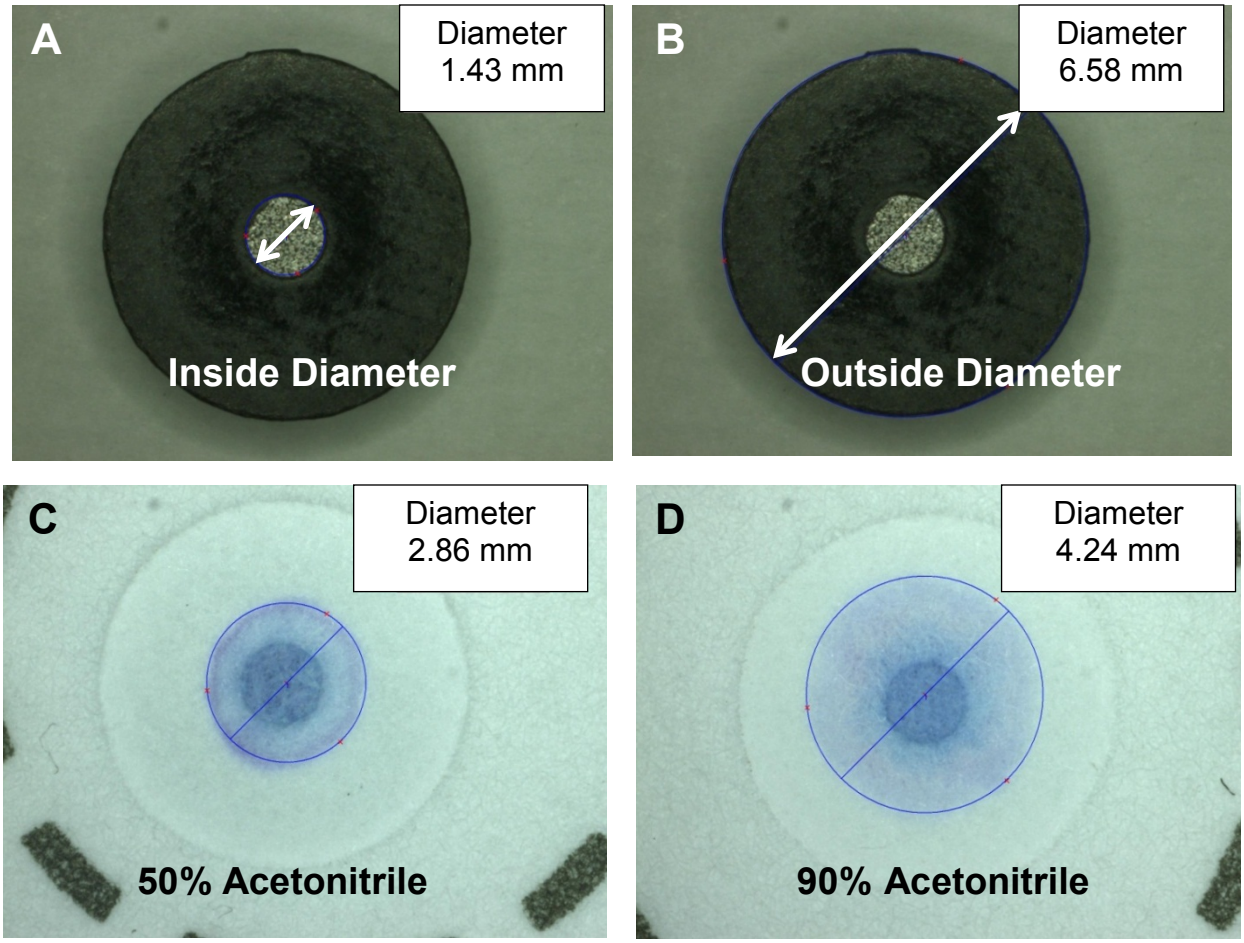
Legend: **A:** two in-line frit end caps are used with 0.5 µm frits and a slit allows for screening cards to be placed in the flow path, **B:** Frit end-caps are tightened forming a leak free seal so that eluent can be used to elute analytes from the filter paper card

Figure 3.1: Schematic of prototype in-line desorption device showing direct analysis of dried blood spots

3.3.2 Seal integrity investigation

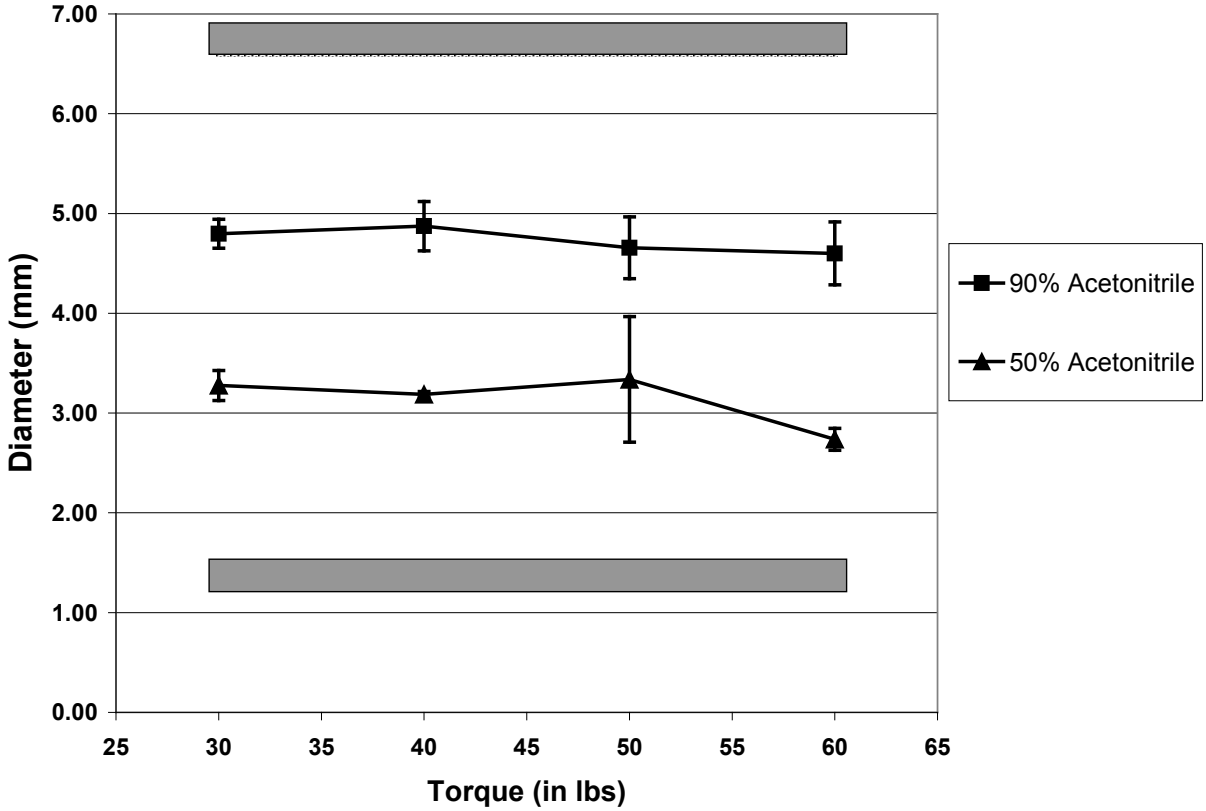
Infusion of a dye solution through the device containing a blank card was used to assess the seal integrity of the device. The end caps containing the in-line frits were tightened to various applied torques to determine how pressure influences the sealing attributes. In addition we evaluated high and low concentrations of organic solvent in the solution to determine if there was any impact related to solvent viscosity. A Harvard Apparatus syringe pump, with a 250 µL Hamilton gas tight syringe, was used to control the flow of the dye solution through the device. The solution was infused through the device at a 100 µL/min flow rate with either a 30 second or 1 minute analysis to simulate the intended operation conditions. Dye solutions of 50% acetonitrile or 90% acetonitrile were evaluated. End caps were tightened using a dial torque wrench at various torques from 30 to 60 in-lbs. A torque of 30 in-lbs was the minimum torque that could be applied to firmly hold the card in place and a torque of 60 in-lbs

was the maximum torque that could be applied without damaging the card from shearing forces. Therefore, it was decided to use this range to evaluate the seal integrity of the device. A digital microscope was used to take measurements of the inside and outside diameters of the frit, along with the diameter of bleed that appeared on the filter paper. Images shown in Figures 3.2A and 3.2B present the dimensions for the 0.5 µm in-line frit and images 3.2C and 3.2D were obtained when infusing a 50% acetonitrile solution or a 90% acetonitrile solution, respectively. Three replicates were analyzed under each condition at various torque settings, to evaluate reproducibility of this technique; data is plotted showing the bleed diameter for both dye solutions, Figure 3.3. The measurements for the inner and outer diameters of the frit are represented by gray boundary lines shown on the graph.



Legend: **A, B** Digital microscope images of 0.5 µm inline frit used and representative images for a solution with **C** 50% acetonitrile and **D** 90% acetonitrile showing the bleed diameters obtained

Figure 3.2: Digital microscopic images for 0.5 µm in-line frit and filter paper samples showing the bleed diameter for a dye solution containing either 50% acetonitrile or 90% acetonitrile infused at 100 µL/min for 1 minute



Legend: Top and bottom boundary lines in grey indicate the inside and outside diameter of the inline filter. Error bars represent 1 S.D. from three replicates.

Figure 3.3: Plot of the bleed diameter by direct infusion of a dye solution containing either 50% acetonitrile or 90% acetonitrile through the in-line desorption device at 100 µL/min for 1 minute

ANOVA with a student t-test was used to compare the variances and means for the bleed diameters of each solution at the different applied torques. The results indicate that there is not a significant difference in bleed diameter for different applied torques; 90% acetonitrile $p > 0.057$ and 50% acetonitrile $p > 0.2395$. The average bleed diameter observed for 50% acetonitrile solution was 3.13 mm and for the 90% acetonitrile solution it was 4.73 mm. Although the bleed diameter was larger for the solution containing 90% acetonitrile, all of the results were within the outside diameter of the frit indicating that a leak free seal was achieved.

In addition, the results showed acceptable reproducibility with relative standard deviations (RSD) less than 6.86% for all experiments except for the 50% acetonitrile at 50 in-lbs which demonstrated a RSD of 18.9%. The results indicate that an acceptable seal was achieved and no bleed exceeded the outside diameter of the frit with the values of torque studied. Since the bleed was contained within the dimensions of the frit and did not disturb the remaining portion of the sample, multiple analyses can be performed from a single dried spot. Based on the 6.6 mm outside diameter of the frit and the 13 mm dried blood spots, three measurements can be made from a single dried blood spot. In addition, the Whatman, 903 Protein Saver newborn screening cards appear to withstand the solvents used and do not deteriorate under these conditions.

3.3.3 Elution profile for leucine and isoleucine from dried blood spots

In order to determine the optimal elution conditions for leucine and isoleucine, by direct analysis of the DBS, various mixtures of eluent were evaluated using a flow rate of 0.1 mL/min. In addition, the elution solvent needed to be appropriately matched to the chromatographic separation, in order to achieve acceptable retention of leucine and isoleucine. Hydrophilic interaction chromatography (HILIC) employs a polar stationary phase with a normal phase separation mechanism in which the aqueous mobile phase is the stronger elution solvent and the organic mobile phase is the weaker elution solvent. Therefore, a higher concentration of aqueous will result in less retention of the analytes on the HILIC analytical column reducing the chromatographic performance of the column. Optimal retention of compounds is achieved with organic concentration greater than 50%. Mobile phase A contained 10 mM ammonium

formate with 0.1% formic acid and mobile phase B contained a mixture of 90% acetonitrile and 10% water, such that the final buffer concentration was 10 mM ammonium formate. For elution optimization, we evaluated various percentages of mobile phase A, the aqueous component, from 5% to 35% to determine elution profiles of leucine and isoleucine. No chromatographic separation was used for this portion so that the elution profile could be analyzed independent of chromatographic performance. The 2-position 10-port switching valve was switched to elute the analytes at 0.25 minutes and data was collected for 3 minutes using multiple reaction monitoring (MRM) of the 131.7 to 85.8 m/z, the specific mass transition for leucine and isoleucine. Figure 3.4 shows the elution profiles obtained under the various conditions tested.

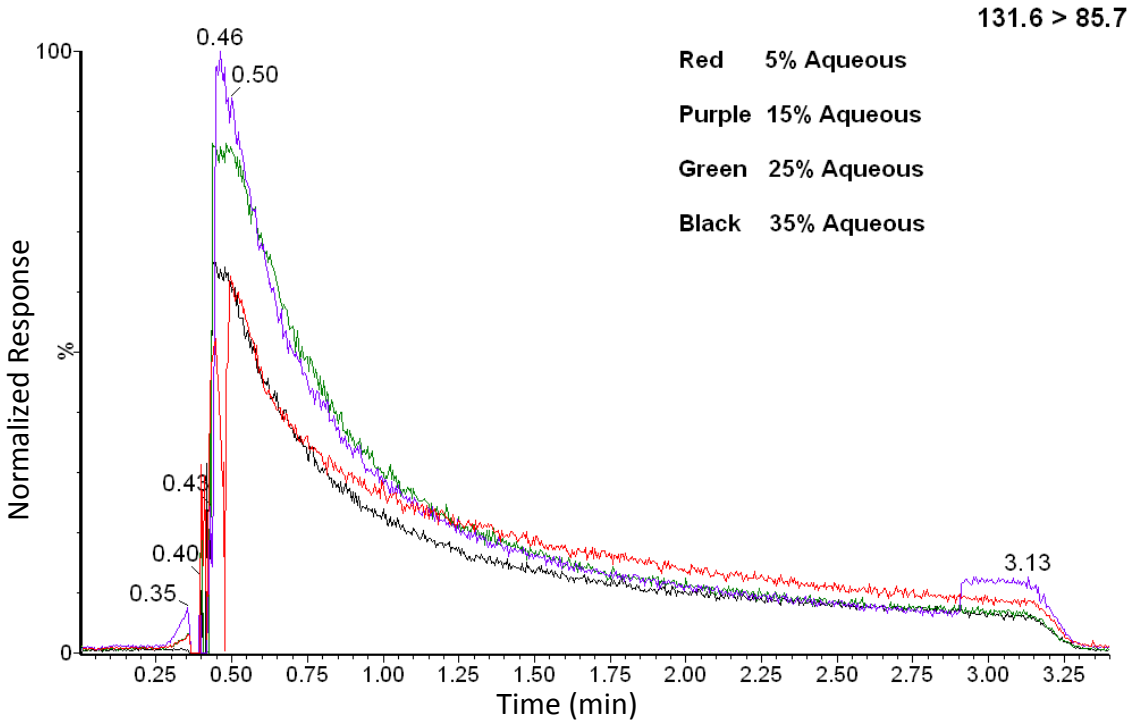


Figure 3.4: Elution profiles for leucine and isoleucine from dried blood spots using in-line desorption device, mobile phase flow rate set to 0.1 mL/min and evaluated isocratic elution with 5%, 15% , 25% and 35% aqueous

The plots indicate that the majority of the analytes elute within the first minute, from 0.25 minutes to 1.25 minutes, under all elution solvents tested. However, the plots show that some residual analyte continues to elute out past 3 minutes. Further evaluation of the profiles show differences in the absolute response varied between the elution solvents. There are several possible factors that could contribute to these differences, such as solvent desorption strength, matrix suppression and ionization efficiency. Overall the elution profiles appeared to be similar and only slight differences are seen in the residual level of analyte eluted after 3 minutes. Based on these results a one minute elution would be acceptable and additional elution time does not provide significant gains. Further elution time impacts the loading

volume placed on the chromatographic column, increasing this volume can dramatically impact chromatographic performance and in order to minimize the overall analysis time the elution time should be as short as possible.

3.3.4 Chromatographic optimization

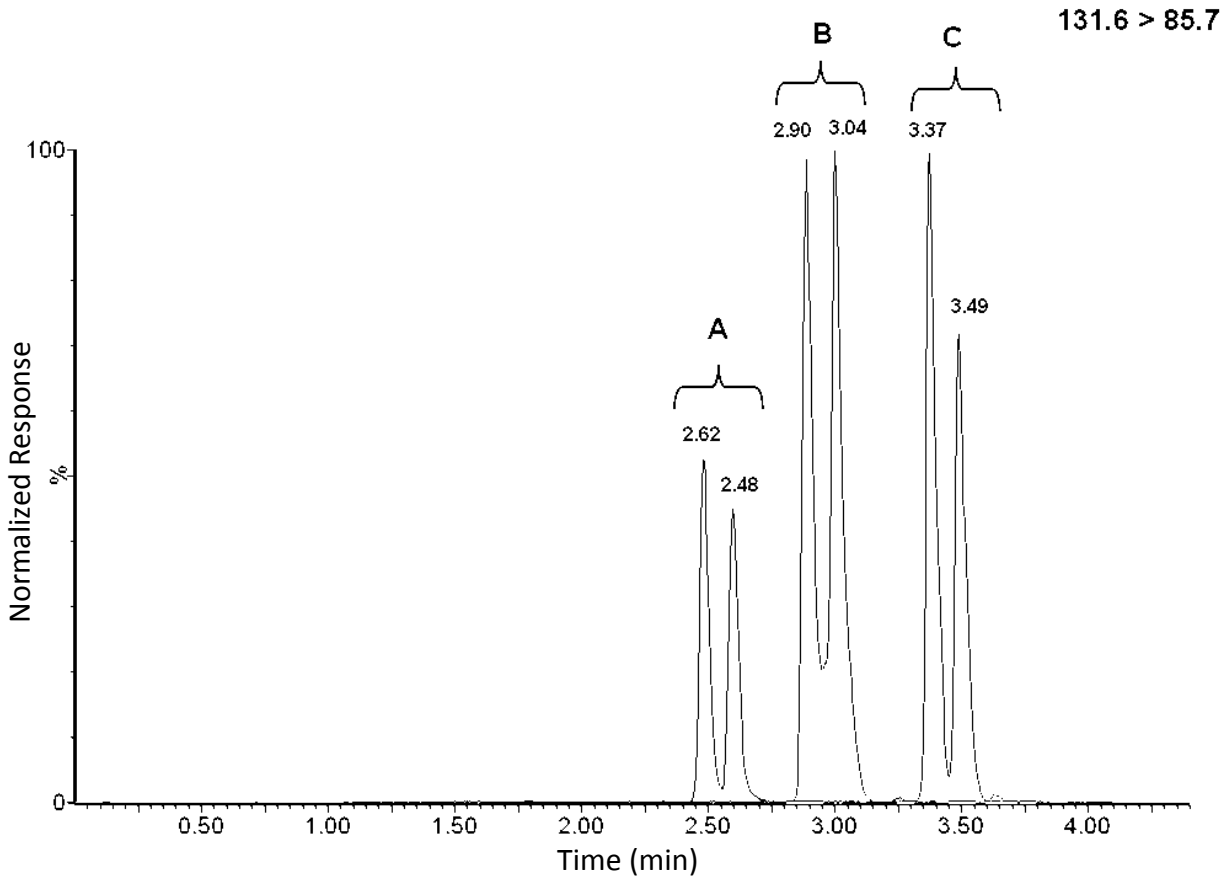
Preliminary investigations involved optimization of chromatographic conditions to achieve separation between leucine, isoleucine and hydroxyproline. Mobile phase composition was the same as described above, but for this a gradient elution was employed from 90% mobile phase B to 75% mobile phase B over 1.6 minutes shown in Table 3.1. An Acquity UPLC pump was used to control the mobile phase flow and mobile phase composition allowing for a gradient elution of analytes. Resolution was assessed for the separation of leucine and isoleucine and was determined to be approximately 1.2. Separation between two closely eluting peaks is determined to be acceptable when the resolution is greater than 1 (Miller, 2005).

Table 3.1: Initial Chromatographic conditions without in-line desorption device

Time (min)	Flow Rate (mL/min)	% A	% B
Initial	0.35	10	90
1.60	0.35	25	75
2.70	0.35	25	75
2.75	0.35	5	95

The next step was to incorporate the on-line desorption device prior to the HILIC Amide separation to assess chromatographic performance for direct analysis of DBSs. In order to include the desorption device the gradient was modified to include both the desired switching interval, elution time and ratios of mobile phase A and B. The same gradient profile and slope

used to chromatographically elute the analytes was adjusted based on elution time either with a 30 or 60 second delay. Three different elution conditions were evaluated to determine impact of elution time and aqueous concentration on chromatographic performance. We evaluated a 30 second elution with 90% mobile phase B, a 60 second elution with 90% Mobile phase B and a 60 second elution with 95% mobile phase B; the results are shown in Figure 3.5. The resolution for the three conditions was evaluated and was determined to be 1.2 for both the 30 second elution and 90% mobile phase B and for 60 second elution and 95% mobile phase B. The resolution was 1.1 for the 60 second elution and 90% mobile phase B elution. Although acceptable resolution was achieved for a 30 second elution with 90% mobile phase B, the response was approximately 40% lower than with a 60 second elution time. However, resolution between leucine and isoleucine was not as good with a 60 second elution with 90% mobile phase B. The best resolution and response was obtained for a 60 second elution with 95% mobile phase B, Table 3.2 shows the final gradient program used.



Legend: A: 30 second elution 90% mobile phase B; **B:** 60 Second elution 90% mobile phase B; **C:** 60 second elution 95% mobile phase B

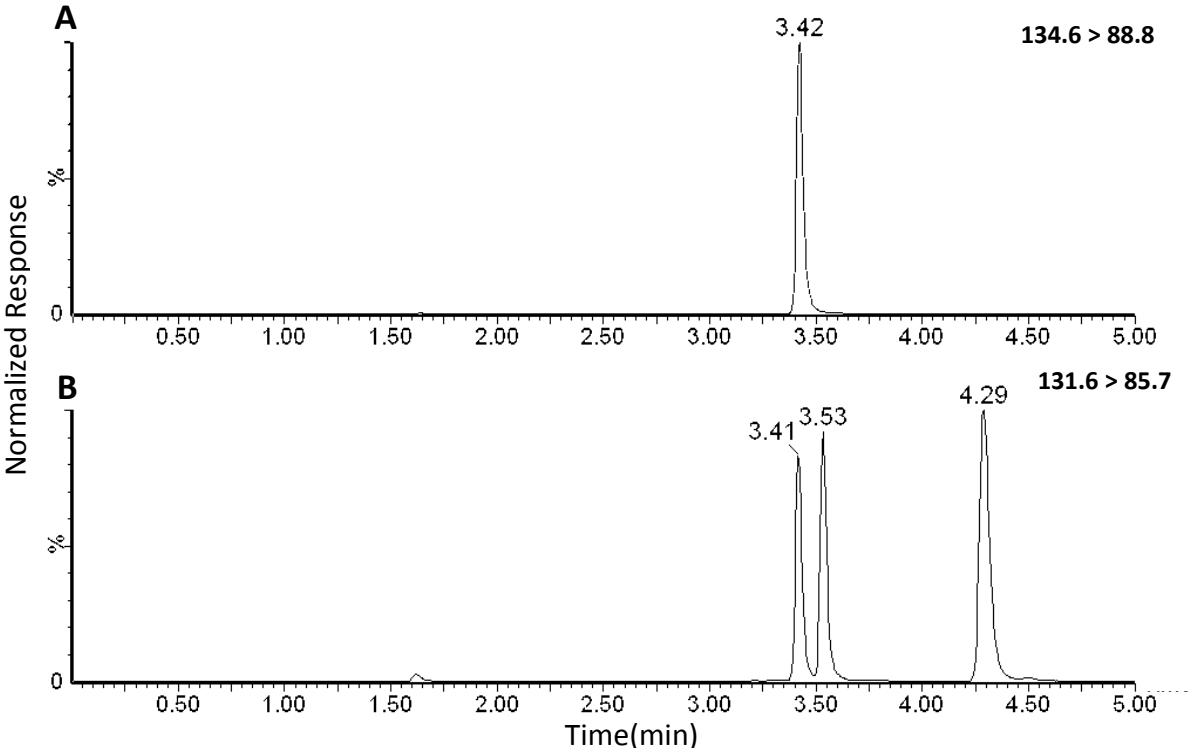
Figure 3.5: Chromatographic separation on HILIC Amide column for leucine and isoleucine following in-line desorption from a dried blood spot using different elution times and solvent strengths

Table 3.2: Final chromatographic conditions with in-line desorption device

Time (min)	Flow Rate (mL/min)	% A	% B	Valve Position	Description
Initial	0.1	5	95	1	Bi-pass
0.04	0.1	5	95	2	Load
1.05	0.1	5	95	1	Bi-pass
1.08	0.35	5	95	1	Bi-pass
2.50	0.35	20	80	1	Bi-pass
3.70	0.35	20	80	1	Bi-pass
3.75	0.35	5	95	1	Bi-pass

For quantification of leucine and isoleucine an Acquity UPLC autosampler was used to inject the internal standard working solution which passed through the screening card at the same time as the analytes are eluted. This timing was optimized by varying the initial switching time and evaluating the response of the internal standard. The optimal switching time is based on the dead volume of the system. The dead volume of the system is a measure of the system volume, which includes all the tubing, including the injection loop between the pump and the column. This volume can vary dramatically from system to system depending on the pump type, mixing valves and injection loops. As a hypothetical example; if the dead volume of the system was 1 mL and the flow rate was 1 mL/min then it would take approximately 1 minute for the mobile phase to go from the pump to the column. However, if the dead volume was 2 mL then it would take 2 minutes for the mobile phase to reach the column. For our purposes we were concerned about the volume between the autosampler and the device, so we optimized the switching time so that the internal standard would reach the device at the same time as the valve was switched. Systems with a larger dead volume will require additional time for the internal standard to reach the device and systems with a smaller dead volume will not

require as much time. This technique allows for quantification of leucine and isoleucine without pretreatment of the NBS card with internal standard. For the analysis, 10 µL of internal standard working solution, 6.25 nmol/mL, was injected onto the LC/MS system allowing for 4 times as many samples to be processed with the internal standard working solution compared to the current newborn screening method, resulting in significant cost savings. Figure 3.6 shows the final chromatographic separation employing the on-line desorption device and with elution using the internal standard. The internal standard, Leucine-d3, elutes at approximately the same time as leucine.

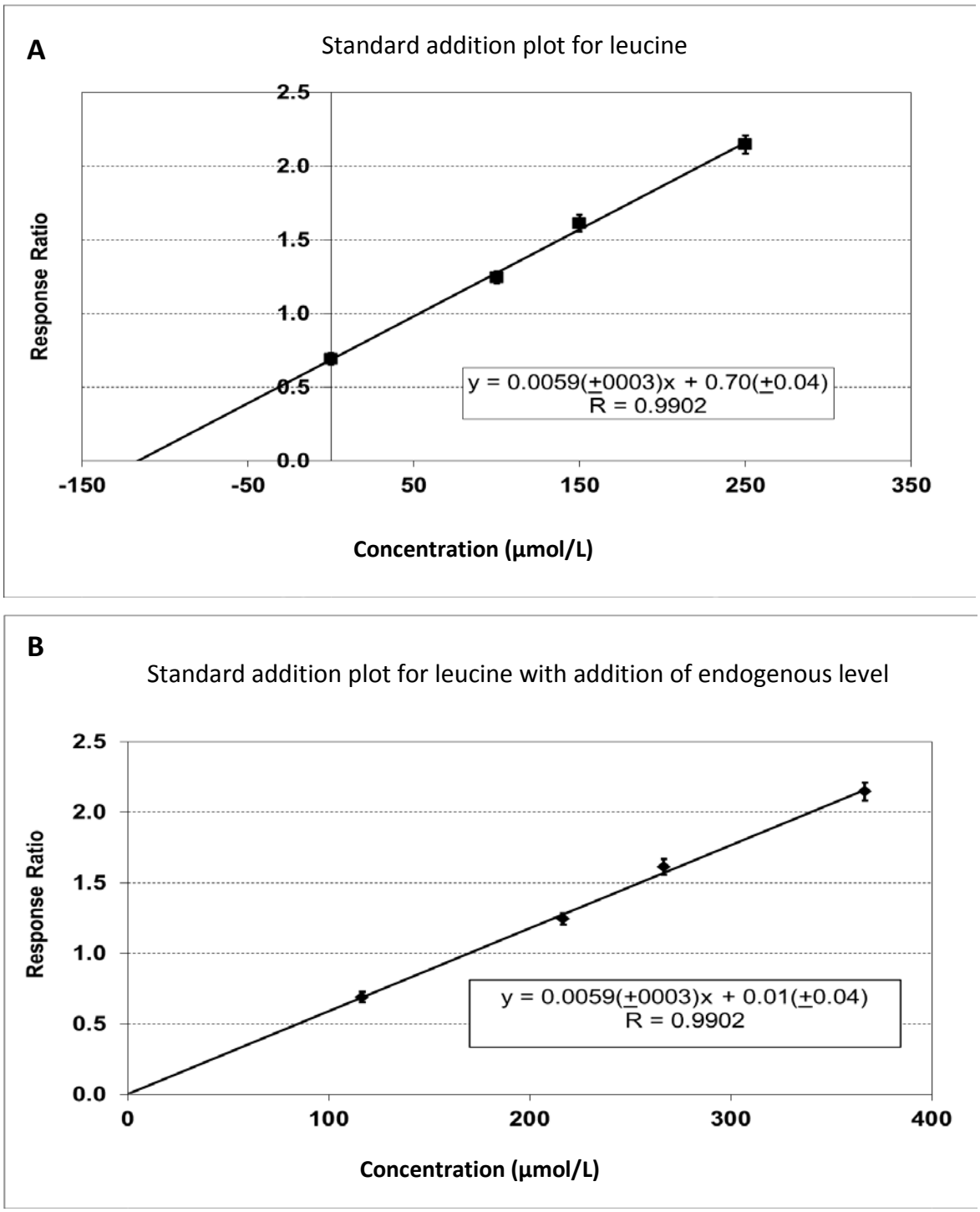


Legend: **A:** leucine-d3 at 3.42 min; **B:** leucine at 3.41 min, isoleucine at 3.53 min and hydroxyproline at 4.29 min.

Figure 3.6: Final chromatographic separation for leucine, isoleucine, hydroxyproline and leucine-d3 using in-line desportion device and a HILIC Amide column

3.3.5 Validation of device for quantification of leucine and isoleucine

Validation included both intra spot precision and accuracy, inter spot precision and accuracy, linearity, recovery and post column infusion for matrix suppression. Three replicate samples were analyzed from a single spot across three separate days in order to assess intra spot precision and accuracy. Inter spot precision was assessed by analyzing one replicate from 3 different spots on a single day. Since human whole blood contains endogenous levels of leucine and isoleucine, the concentration in the blank was determined first. Quantification of the endogenous levels was performed using the method of standard addition to create a curve from the amount fortified versus the response ratios obtained, shown in Figure 3.7A.



Legend: A: Plot based on amount fortified to blood, x-intercept is used to calculate endogenous level found in whole blood, **B:** Calculated concentration for a blank was used to determine concentration of fortified levels. Error bars represent 1 Standard deviation

Figure 3.7: Standard addition plots for leucine generated by the analysis of dried blood spot samples fortified with leucine at three levels and without fortification in order to quantify the endogenous level present in samples, all samples analyzed in triplicate

Regression analysis was evaluated over three days using analyte response ratios with internal standard (leucine-d3). The response ratios for the unfortified samples and samples fortified at three concentrations for leucine and isoleucine were plotted versus the fortified concentration. Linear regression y = mx + b was used to determine the unfortified sample concentration, where "y" is the analyte response ratio, "x" is the analyte concentration, "m" is the slope and "b" is equal to the y-intercept. The concentration in the unfortified sample can be obtained at the x-intercept where y = 0, x = -b/m. Using this concentration we can then calculate the amount in each fortified level, for example level 1 would be 100 nmol/mL + blank concentration. The data is then plotted using the calculated theoretical levels for the blank and each fortified level versus the response ratios, Figure 3.7B. A linear regression was used with a weighing factor of 1/concentration and back calculated values are determined for each level. The final calibration range based on the adjusted concentrations were from 128 to 383 nmol/mL for leucine and from 59.9 to 324 nmol/mL for isoleucine. Correlation coefficients (r) for all runs were greater than 0.9805, except for one run the correlation coefficient was 0.9706 for isoleucine. Precision and accuracy were calculated for each calibration standard on three separate days for inter spot variability and on one day for intra spot variability. Overall, intra spot precision ranged from 3.21% to 5.84% for leucine and 4.43% to 9.14% for isoleucine with accuracy of 92.3% to 105% for leucine and 90.9% to 108% for isoleucine (Table 3.3). Inter spot precision ranged from 2.38% to 9.98% for leucine and 3.26% to 7.33% for isoleucine with accuracy of 89.7% to 107% for leucine and 88.0% to 110% for isoleucine (Table 3.4).

Table 3.3: Intra spot precision and accuracy for leucine and isoleucine determined by 3 replicates analyzed on 3 different days

Fortified Conc.	Blank 0 nmol/mL	Low Level 100 nmol/mL	Mid Level 150 nmol/mL	High Level 250 nmol/mL
Leucine				
Average Conc. (nmol/mL)	128	205	274	383
Average Precision	5.84%	5.05%	3.21%	4.09%
Average Accuracy	105%	92.3%	101%	104%
Isoleucine				
Average Conc. (nmol/mL)	59.9	141	202	324
Average Precision	9.14%	8.64%	6.17%	4.43%
Average Accuracy	108%	90.9%	98.4%	106%

Table 3.4: Inter spot precision and accuracy for leucine and isoleucine determined by 1 replicate on 3 different spots analyzed on one day

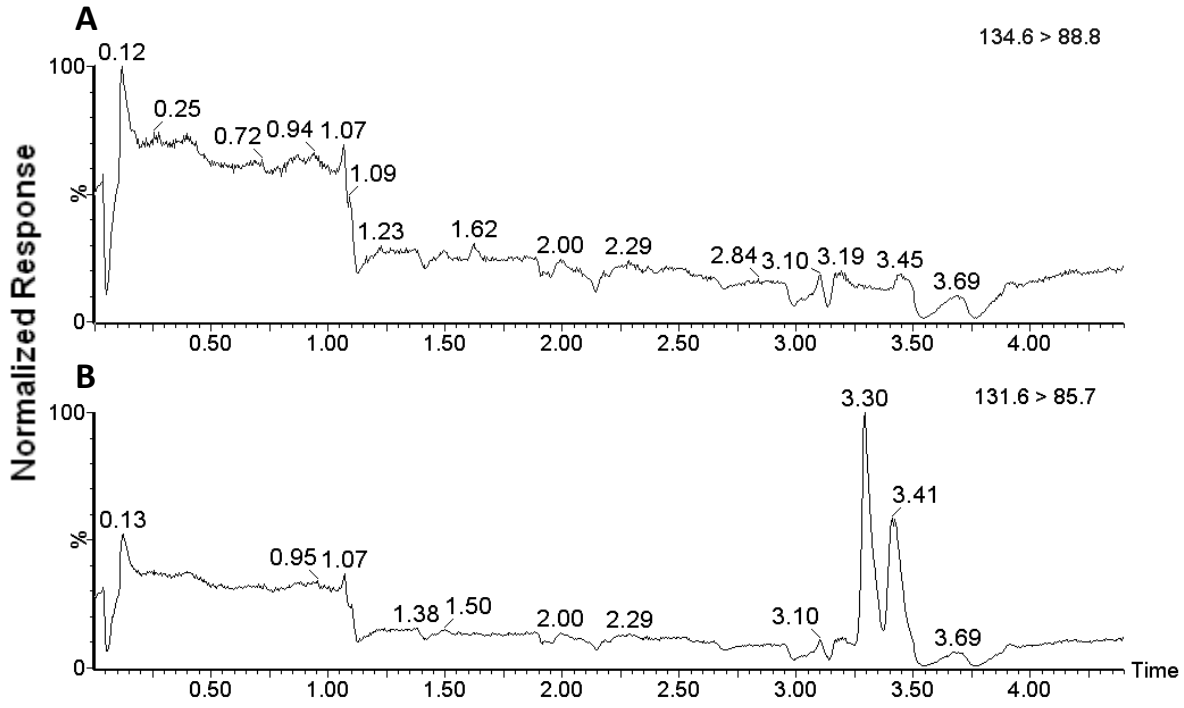
Fortified Conc.	Blank 0 nmol/mL	Low Level 100 nmol/mL	Mid Level 150 nmol/mL	High Level 250 nmol/mL
Leucine				
Average Conc. (nmol/mL)	128	198	275	383
Average Precision	2.38%	9.98%	8.19%	6.46%
Average Accuracy	107%	89.7%	102%	104%
Isoleucine				
Average Conc. (nmol/mL)	67.8	142	208	333
Average Precision	3.26%	7.33%	3.27%	7.21%
Average Accuracy	110%	88.0%	98.3%	107%

Three replicates of a mid-level fortified sample were used to assess recovery. Recovery was performed by conducting six replicate injections from a single position on a DBS. The peak areas from each individual injection were summed to provide a total area eluted which was used as 100% recovery. From this recovery of the first elution could be calculated. Recovery was determined to be 63.6% for leucine and 65.7% for isoleucine with RSDs within 4.34%.

3.3.6 Matrix suppression evaluated using a post-column infusion technique

A post-column infusion experiment for leucine, isoleucine and leucine-d3 was conducted to evaluate ion suppression or enhancement due to the matrix (Matuszewski et al., 2003). A syringe pump was used to continuously infuse (10 µL/min) of an external solution containing 100 nmol/mL of leucine, isoleucine and leucine-d3 in the flow path to the mass spectrometer post column. This provided a high baseline response for transitions associated with each compound so that when matrix is eluted pre-column, ion suppression would appear as a negative response shift in this high baseline, whereas ion enhancement would appear as a positive shift. The time duration of these shifts indicate elution times where ion suppression and enhancement occur. Blank matrix blood spots were eluted using the optimized chromatographic conditions while monitoring for shifts in the specific mass transitions for the analytes and internal standard. Figure 3.8, shows a representative chromatogram of a sodium heparin human blood blank eluted using this post column infusion technique. The initial negative shift seen at 1.07 minutes is attributed to a change in flow rate from 0.1 mL/min to 0.35 mL/min. This decrease in ionization is due to the increase in flow rate and should not impact the quantification of the analytes. Additional suppression shifts in the chromatogram were observed throughout the separation for both the analytes and internal standard channels.

As previously discussed it is difficult to assess matrix affects in samples that contain endogenous levels of the analytes of interest. In these dried blood spot samples, peaks for leucine and isoleucine were observed (Figure 3.8B) at the expected retentions for leucine and isoleucine, due to the endogenous levels in human whole blood. Therefor the internal standard transition was used to assess matrix affects. The greatest suppression is less than 20% of the total peak response for leucine and isoleucine.



Legend: A: Mass transition for leucine-d3, internal standard 134.6 > 88.8. B: Mass transition for leucine (3.30 min) and isoleucine (3.41 min) 131.6 > 85.7. Flow rate adjusted from 0.1 mL/min to 0.35 mL/min at 1.07 minutes resulting in a decrease in ionization.

Figure 3.8: Matrix suppression evaluation for analysis of a dried blood spot sample using the in-line desportion device and chromatographic separation on the HILIC Amide column, post-column infusion of leucine, isoleucine and internal standard at 100 nmol/mL

3.4 Conclusions

In conclusion, direct analysis of dried blood spots using a prototype device was applied to the quantification of biomarkers for MSUD. Work has been presented that shows that this device provides a leak free seal allowing for direct analysis of DBSs eliminating the need for any offline sample preparation. The method was validated and provides good precision and accuracy for leucine from 128 to 383 nmol/mL and isoleucine from 59.9 to 324 nmol/mL appropriate concentration ranges for identification of MSUD. Direct analysis provides several advantages over current second-tier tests for MSUD in that there is no need for derivatization, analysis can be done with the addition of the device to current instrumentation and direct analysis can be conducted with original dried blood spot. This prototype design could be easily automated using conventional robotics, which would allow for hundreds of samples to be processed with minimal human intervention, greatly improving the efficiency of newborn screening.

3.5 Acknowledgements

We would like to thank the Virginia Division of Consolidated Laboratory Services for supplying us with the internal standard reference material and Altria Client Services for providing additional supplies and LC/MS/MS instrumentation.

CHAPTER 4

A NOVEL ON-CARD APPROACH FOR DETERMINING HEMATOCRIT OF DRIED BLOOD SPOTS ALLOWING FOR CORRECTION OF SAMPLE VOLUME

4.1 Introduction

Analysis of dried blood spots has been used in newborn screening for decades and more recently it has become increasingly applied in bioanalytical analysis of pharmaceuticals for new drug development and for therapeutic drug monitoring (Denniff & Spooner, 2010; Edelbroek et al., 2009; O'Mara, Hudson-Curtis, Olson, Yueh, Dunn, Spooner, 2011). This sample collection technique offers many advantages including minimal sample collection volume, reduced costs associated with shipping and storage, it is less invasive than collecting venous samples and minimizes risks associated with transmission of some infectious diseases such as HIV (Li & Tse, 2010; Parker & Cubitt, 1999) Despite the numerous advantages to this sample collection technique, the most common concern is how to correct for sample volume variations. Hematocrit, which is a measure of concentration of red blood cells per unit volume of blood, has the largest influence on the sample volume for dried blood spot analysis. This is primarily due to the viscosity of blood being greater at high hematocrit and less at low hematocrit (Edelbroek et al., 2009). When blood is applied to filter paper, the blood will diffuse at different rates for blood samples with low and high hematocrits affecting the size of the blood spot

(Adam et al., 2000; Denniff & Spooner, 2010; Edelbroek et al., 2009; Mei et al., 2001). Since a fixed punch size is used for sample analysis, the sample amount is dependent on hematocrit and it can be significantly different in cases of abnormally low and high hematocrit (Denniff & Spooner, 2010; Mei et al., 2001). Typical hematocrit ranges from 28–67% in most healthy individuals and the normal range for newborns is from 42-64% (Denniff & Spooner, 2010). However, abnormal hematocrit can be characteristic of polycythemia or anemias, causing high and low hematocrit respectively. Several publications have reported the effects of hematocrit on concentration of pharmaceuticals (Denniff & Spooner, 2010; Wilhelm et al., 2009) and amino acids and acylcarnitines (Holub et al., 2006) in dried blood spots. In addition, some compounds show a chromatographic effect, where the concentration of the analyte is higher in a center punch relative to a peripheral punch (Holub et al., 2006; O'Mara, Hudson-Curtis, Olson,Yueh, Dunn, Spooner, 2011). Others have reported minimal differences in sample volume relative to normal hematocrit levels (Denniff & Spooner, 2010). It is difficult to accurately quantify sample volume unless a predefined volume of blood is spotted on filter paper and the whole spot is analyzed. Edelbroek has reviewed several publications using this technique which eliminates issues with hematocrit variations (Edelbroek et al., 2009). Although this approach may work for clinical analysis or therapeutic drug monitoring, it is not practical for newborn screening.

Newborn screening relies on a simplistic approach of spotting whole blood from a heal puncture on the filter paper and the sample volume is approximated at 3.1 µL from a 3 mm punch (Holub et al., 2006). In most cases the identification of a specific disorder is fairly distinguishable however; there are times when correction for volume could mean the

difference between a test positive and a test negative result (Holub et al., 2006; Orsini et al., 2010). Routine DBS analysis has been shown to be acceptable for newborn screening for decades but, there is still the potential to further improve the positive predictive value of this test. Since cut-off values are set independent of hematocrit the potential to misidentify samples is increased with high or low hematocrit samples. It has been reported that sample volume in DBS analysis can vary by as much as 47% depending on the hematocrit (Mei et al., 2001). A recent study evaluated the effect hematocrit has on the measured concentration of succinylacetone, which showed that samples with low hematocrit quantitated approximately 45% low and samples with high hematocrit quantitated 24% higher than expected (Peng, Liu, & Peng, 2012). Hematocrit effects on the analysis of amino acids and acylcarnitnes, markers for organic acid disorders, amino acid disorders and fatty acid oxidation disorders has been reported (Holub et al., 2006). Holub showed a positive correlation between analyte concentration and hematocrit. A chromatographic effect for some analytes showed significant differences in their concentration between peripheral and center punches (Holub et al., 2006). Attempts have been made to correct for differences in hematocrit using hemoglobin variant testing to approximate sample volume (Orsini et al., 2010) Orsini reported an average accuracy of 108% using this technique, however the results ranged from 73.5% to 142% (Orsini et al., 2010).

UV and NIR spectroscopy has been used for years to evaluate the composition and properties of blood in vitro and in vivo (Johns et al., 2001; Meinke et al., 2005; Schmitt et al., 1986; Serebrennikova et al., 2008; Steinke & Shepherd, 1986; Steinke & Shepherd, 1988; Zhang et al., 2000; Zijlstra et al., 1991; Zonios & Dimou, 2006). Several models and theories have been

used to explain the light scattering, absorption and reflectance properties of whole blood to determine hemoglobin concentration, hematocrit and blood oxygenation levels. Many of these theoretical models have resulted in medical devices used by health care providers including oximeters (Schmitt et al., 1986; Steinke & Shepherd, 1986) and hemoglobinometers (Zhang et al., 2000). These devices offer several major advantages over other sampling techniques including: nondestructive analysis, less invasive sampling, portable instrumentation and faster analysis time.

Analysis of whole blood in vivo and in vitro solutions has several complexities, due to the nature of the medium and other biological factors. Several different models and theories have been used to explain the properties of blood either in vitro or in vivo. Mie theory supports the light scattering properties of whole blood to evaluate red blood cell size and concentration in dilute solutions (Steinke & Shepherd, 1988). Although there has been some criticism to using Mie theory due to the shape of the red blood cells not being spherical, experimental results were comparable the theoretical predictions. Absorption measurements have been reported to be inversely proportional to reflectance of blood in thin films of non-hemolyzed blood and reflectance increases with increasing the thickness of the film (Anderson and Sekelj, 1967). The absorption properties of whole blood deviates from Beers law at high concentrations where the optical density does not appear to be related to hemoglobin concentration nor the path length (Lovell, Hebden, Goldstone, & Cope, 1999). More recently a paper by Serebrennikova et. al. proposed a new theoretical model that uses diffuse reflectance and light scattering to analyze whole blood (Serebrennikova et al., 2008). From their data it was apparent that reflectance is related to erythrocyte concentration. Although there are

many spectroscopic methods used for hematocrit determination for whole blood in vivo and in vitro, there have not been any published articles related to the spectroscopic analysis of dried blood spots to determine hematocrit.

We have developed a novel on card approach to evaluate hematocrit on dried blood spot using diffuse reflectance. Our method is the first reporting using a direct sampling approach for hematocrit that shows improved precision and accuracy by correcting for sample volume. Direct analysis by reflectance of can be used to relate sample hematocrit allowing for volume correction. This technique could be used in newborn screening or clinical analysis improving the quantification of dried blood spot analysis. Results are presented for quantitative analysis of acylcarnitines in dried blood spots from donor samples at various hematocrit levels. Correcting for sample volume is increasingly important to properly identify children with low hematocrit or high hematocrit.

4.2 Materials and methods

4.2.1 Materials and reagents

Acylcarnitine reference standards were purchased from VU Medical Center Metabolic Laboratory, (Amsterdam, The Netherlands). Stable isotope labeled acylcarnitines reference materials were obtained from Cambridge Isotopes (Andover, MA, USA). HPLC grade acetonitrile, formic acid and ammonium formate were from Fisher Scientific (Pittsburgh, PA, USA). Control human potassium EDTA whole blood was obtained from Biochemed (Winchester, VA, USA). Newborn screening cards, 903 Protein Saver and DMPK filter paper cards were obtained from Whatman (Piscataway, NJ, USA).

4.2.2 Chromatographic instrumentation

Chromatographic separations were conducted with a Waters Acquity HILIC Amide column (2.1 x 50 mm, 1.7 µm) Waters Corp., (Milford, MA, USA). A Waters Acquity UPLC pump and sample manager were used for analysis and gradient separation. A Waters Premier triple quadrupole mass spectrometer (Milford, MA, USA) equipped with electrospray ionization (ESI) source in positive ion mode was used. Instrument parameters were optimized for analysis of acylcarnitines and their labeled internal standards by direct infusion of an external solution into the mass spectrometer. Optimal sensitivity was achieved using 3.8 kV for the capillary, 390 ^{o}C desolvation temperature with nitrogen as the desolvation gas at a flow of 800 L/hr. Cone gas was nitrogen at a flow of 20 L/hr with a source temperature of 110 ^{o}C. Argon was used for the collision gas at a flow of 0.4 L/hr. Multiple reaction monitoring (MRM) was used for all analytes and internal standard. Individual mass transitions for all analytes is shown in Table 4.1. Optimized MRMs were used based on the retention time of analytes to achieve a minimum 0.4 minute window around each peak with a 0.02 second dwell time and a 0.02 second inter-scan delay.

Table 4.1: Acylcarnitines and amino acids analyzed and the mass spectral settings for MRM mode

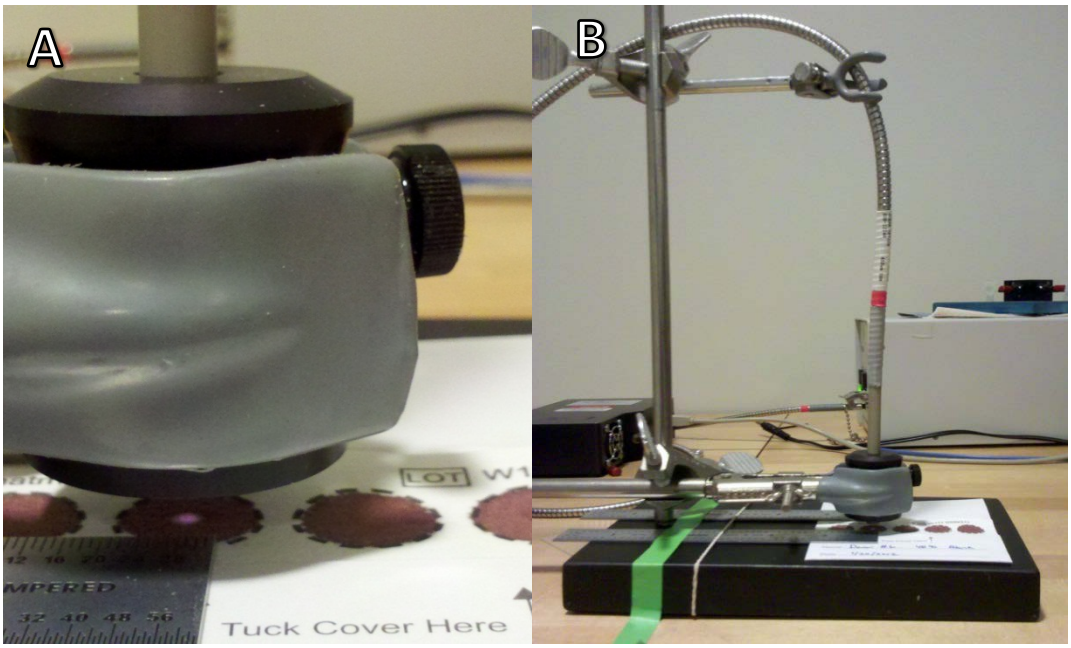
Analyte	ID	CV (V)	CE (eV)	MRM Transition	I.S.	I.S. MRM Transition	Time segment (min)
Carnitine	C0	22	20	161.9→84.6	C0-D9	170.9→84.6	1.73 to 2.03
Acetylcarnitine	C2	22	20	203.9→84.6	C2-D3	206.9→84.6	1.57 to 1.87
Propionylcarnitine	C3	22	20	217.9→84.6	C3-D3	220.9→84.6	1.43 to 1.73
Butyrylcarnitine	C4	22	21	231.9→84.6	C4-D3	235.0→84.6	1.31 to 1.61
Valerylcarnitine	C5	22	23	246.0→84.6	C5-D9	255.1→84.6	1.21 to 1.51
Hexanoylcarnitine	C6	22	23	260.0→84.6	C8-D3	291.1→84.6	1.10 to 1.40
Octanoylcarnitine	C8	22	23	288.1→84.6	C8-D3	291.1→84.6	0.97 to 1.27
Decanoylcarnitine	C10	22	28	316.1→84.6	C8-D3	291.1→84.6	0.80 to 1.10
Lauroylcarnitine	C12	22	28	344.1→84.6	C14-D9	381.2→84.6	0.63 to 1.01
Myristoylcarnitine	C14	22	28	372.1→84.6	C14-D9	381.2→84.6	0.63 to 1.01
Palmitoylcarnitine	C16	22	28	400.2→84.6	C16-D3	403.2→84.6	0.00 to 0.95
Stearoylcarnitine	C18	22	28	428.2→84.6	C16-D3	403.2→84.6	0.00 to 0.95

4.2.3 UV/NIR-instrumentation

Dried blood spot samples were analyzed with a QE65000 Scientific-Grade Spectrometer and deuterium tungsten halogen light source from Ocean Optics (Dunedin, FL). Reflectance measurements were made with a R400-7-VIS/NIR reflectance probe, Ocean Optics. Data was collected from 200-1000 nm using a 6 msec integration time and a boxcar smooth setting of 2. The reflectance probe was held in position using a gripper mounted to a ring stand. A picture of

the reflectance probe apparatus is shown in Figure 4.1. In order to analyze the samples in reflectance mode the background of the detector and the sample must be analyzed. First the background from the detector is accounted for by taking a reference "dark" sample in which the detector was blocked from all light. Next a reference "light" sample is collected, for this we used a blank Whatman 903 filter paper card. This was done in order to account for the inherent background peaks present in the Whatman 903 filter paper that absorb UV/NIR light. Using the paper as a reference allows for these peaks to be subtracted from the data collected for reflectance, absorbance and light scattering measurements. Also the ambient light was reduced by turning off all overhead lights to eliminate background light from interfering with the analysis. An alternative approach to block out all light would be to move the black collar around the end of the probe to be flush with the dried blood spot. However, using the collar to block all light also makes it difficut to see where you are taking measurements, so in order to make sure we sampled appropiately we elected not to use the collar.

For probe height optimization an adjustable stage was placed below the probe to adjust the height of the probe above the sample. Following the optimization experiment the adjustable stage was removed and the probe was fixed at the optimized distance from the base in order to make optical measurements more consistent.



Legend: A: Reflectance probe positioned above dried blood spot, **B:** Expanded view of reflectance probe set-up showing ring stand holder.

Figure 4.1: UV-reflectance fiber optic probe setup, showing how the probe is held in position above dried blood spot

4.2.4 Preparation of dried blood spots

Potassium EDTA human whole blood was used to prepare samples at 25%, 35%, 45%, 55%, 65% and 75% hematocrit by first measuring the hematocrit by centrifuging the samples at 3500 rpm for 20 minutes and measuring the volume of red blood cells. The hematocrit was adjusted by adding or removing serum. The red blood cells were re-suspended by gentle mixing. For quantitative purposes, individually prepared reference standards containing acylcarnitines (Carnitine, C2, C3, C4, C5, C6, C8, C10, C12, C14, C16 and C18) were fortified into the whole blood at approximately ten times the endogenous level. After thoroughly mixing the samples, 50 µL of unfortified and fortified whole blood samples at the various levels of hematocrit were spotted on three different lots of Whatman 903 Protein Saver newborn

screening cards. For DMPK filter paper cards only 15 µL of blood was spotted on filter paper due to cards are for smaller sample volumes. In addition, 10 µL, 25 µL and 100 µL of the same samples were spotted on separate Whatman 903 filter paper cards. All samples were allowed to thoroughly dry at room temperature and then stored at 0-5 ^{o}C until analyzed. Figure 4.2 is a picture of the dried blood spots from 25% hematocrit to 75% hematocrit after drying. There are some distinct differences observed in the dried blood spots at the various hematocrit levels. Samples prepared at 25% and 35% hematocrit appear to have a second lighter ring that diffuses out farther from the center. As the hematocrit level increases to about 45% this ring becomes less apparent. Samples prepared at 65 to 75% hematocrit appear much darker in color and do not fill the entire spot with 50 µL of blood.

Figure 4.2: Dried blood spots prepared at various levels of hematocrit, 50 µL of blood was spotted on Whatman 903 filter paper

4.3 Results and Discussion

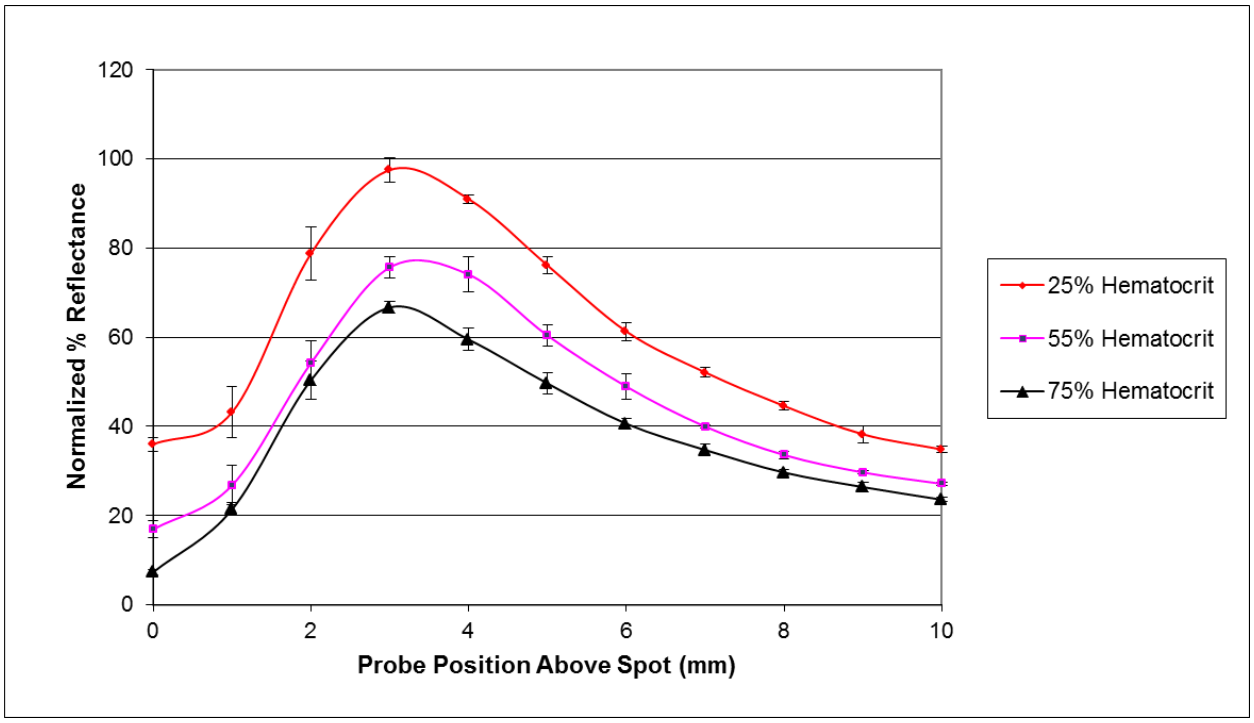
4.3.1 UV reflectance of dried blood spots

For our initial investigations we collected absorbance, reflectance and light scattering data when the probe was positioned perpendicular to the dried blood spot samples. There did appear to be some similarities between reflectance and absorbance spectra, however light scattering alone did not provide identifiable differences between samples at different levels of hematocrit. The absorbance spectra appears to be the inverse of the reflectance spectra. Since we are measuring diffuse reflectance which is a measure of both light scattering and absorbance, we decided to collect the data in reflectance mode; however absorbance could provide similar results.

4.3.2 Probe height optimization

Since reflectance is related to the intensity of the light we first optimized the probe position above the sample. The probe height was optimized by measuring the reflectance from 210 to 1000 nm for 25%, 55% and 75% hematocrit dried blood spot samples. The position of the probe was adjusted from 0 mm to 10 mm from the surface of the spot in 1 mm increments by raising and lowering an adjustable stage. Triplicate analysis of each sample was performed at different locations for each hematocrit at 25%, 55% and 75%. Reflectance measurements were evaluated for several different wavelengths from 200 to 1000 nm. Wavelengths below 800 nm did show provide a relationship between % hematocrit and reflectance, however wavelengths above 800 nm all had a similar correlation but the slope was larger. A larger slope provides improved sensitivity for this analysis allowing for better discrimination between

samples. For this experiment the responses were plotted for 980 nm to show the effect of probe position above the spot related to reflectance for several hematocrit levels, Figure 4.3.

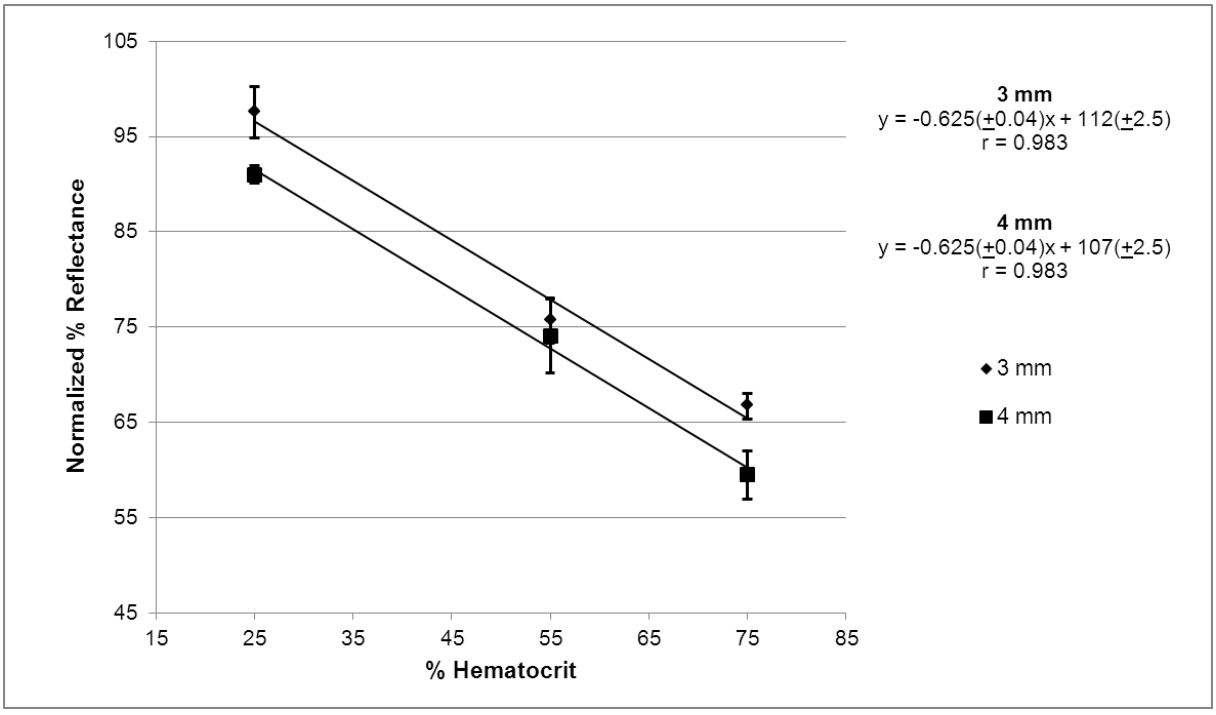


Legend: Error bars represent 1 standard deviation

Figure 4.3: Optimization of probe height above dried blood spot for reflectance measurements at 980 nm, evaluated with samples prepared at 25%, 55% and 75% hematocrit, samples analyzed in triplicate

From our data it was apparent that 3-4 mm above the surface of the probe provided the highest relative intensity for each hematocrit level. At positions lower than 3 mm the mean reflectance values for 55% and 75% hematocrit were not significantly different using a t-test, $p = 0.396$. Measurements above 4 mm had less intensity which was probably due to the diffusion of light created by moving the probe farther away from the samples. For this reason we choose 3-4 mm to evaluate the relationship between percent reflectance and hematocrit. Figure 4.4 shows a plot for the percent reflectance versus hematocrit plotted for 3 mm and 4

mm. The correlation coefficient was calculated both 3 mm and 4 mm using linear regression in Excel and determined to be 0.983 and the slope of both lines were -0.625 ±0.04. Although there was no apparent difference between the two heights, either position could be used for this analysis. We decided to use a probe height of 4 mm for all additional experiments.



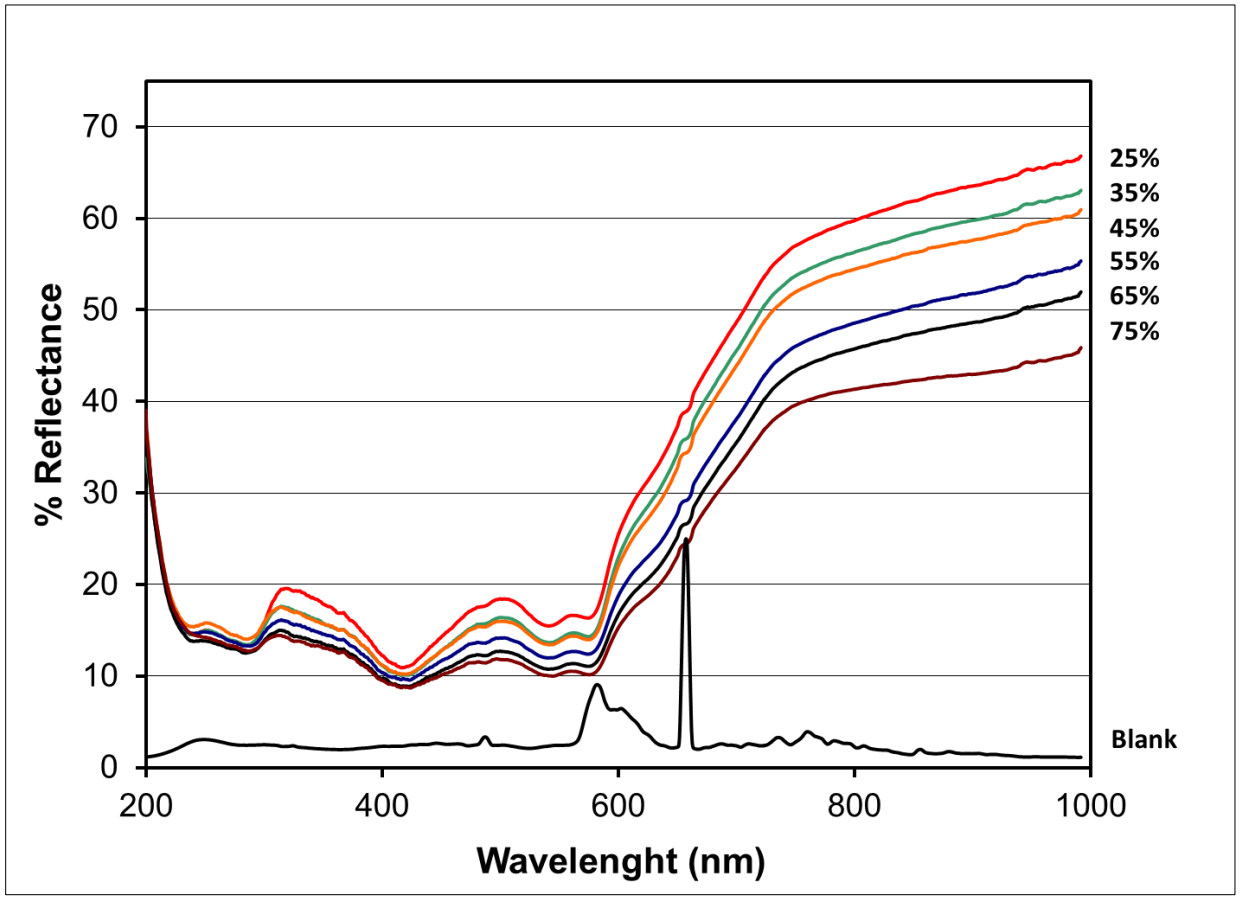
Legend: Error bars represent 1 standard deviation

Figure 4.4: Percent reflectance at 980 nm plotted for probe positioned at 3 mm and 4 mm above the dried blood spot for samples prepared at 25%, 55% and 75% hematocrit, samples analyzed in triplicate

4.3.3 UV reflectance analysis of control samples

Reflectance measurements were made for control samples prepared at 25%, 35%, 45%, 55%, 65% and 75% hematocrit. Samples were analyzed in triplicate at different positions on a dried blood spot. In addition three different lots of Whatman 903 filter paper and one lot of DMPK filter paper were analyzed. UV spectra from 200 nm to 1000 nm was collected for each sample to generate UV reflectance spectra, Figure 4.5. The reflectance measurements for samples with

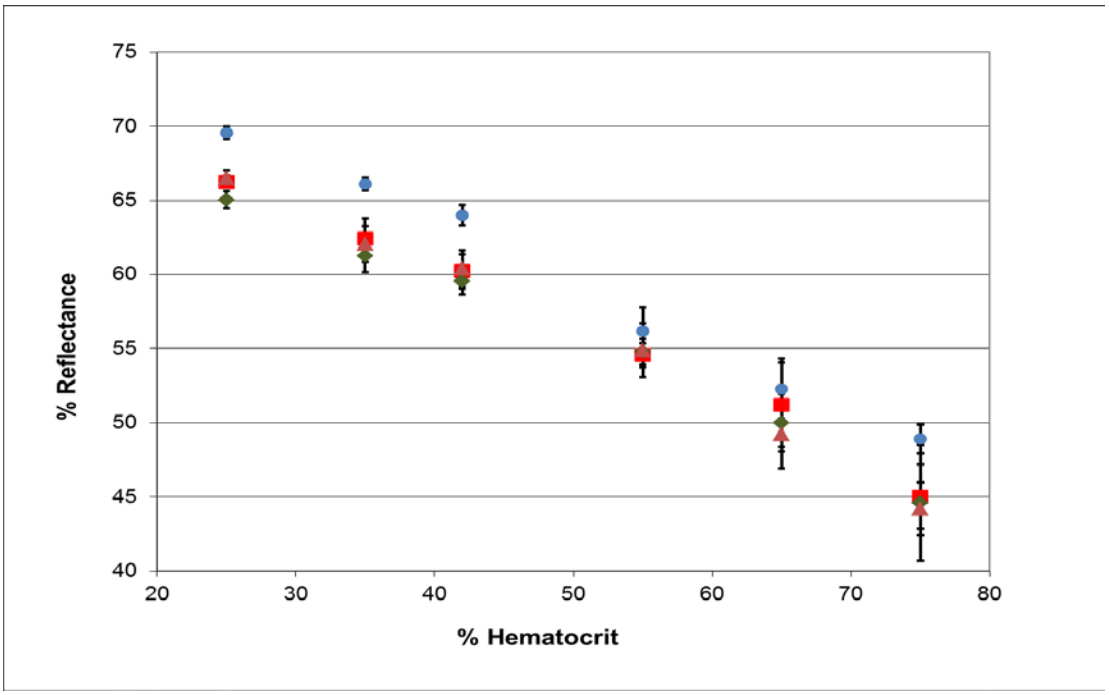
different hematocrits do have some distinct features as referenced by Serebrennikova (Serebrennikova, Smith, Huffman, Leparc, & García-Rubio, 2008). Also, there were two distinct bands at 540 nm and 570 nm which have been previously reported as characteristic hemoglobin bands (Yu et al., 2008; Zijlstra et al., 1991). The region above 800 nm has few distinct features and provides a relationship between hematocrit and reflectance.



Legend: UV reflectance measurements for dried blood spot samples at various hematocrit levels as indicated. Blank is a blank newborn screening card. Distinct hemoglobin bands can be seen at 540 nm and 570 nm.

Figure 4.5: UV/NIR reflectance spectra for dried blood spots prepared at various levels of hematocrit and an absorbance spectra for a blank Whatman 903 filter paper sample

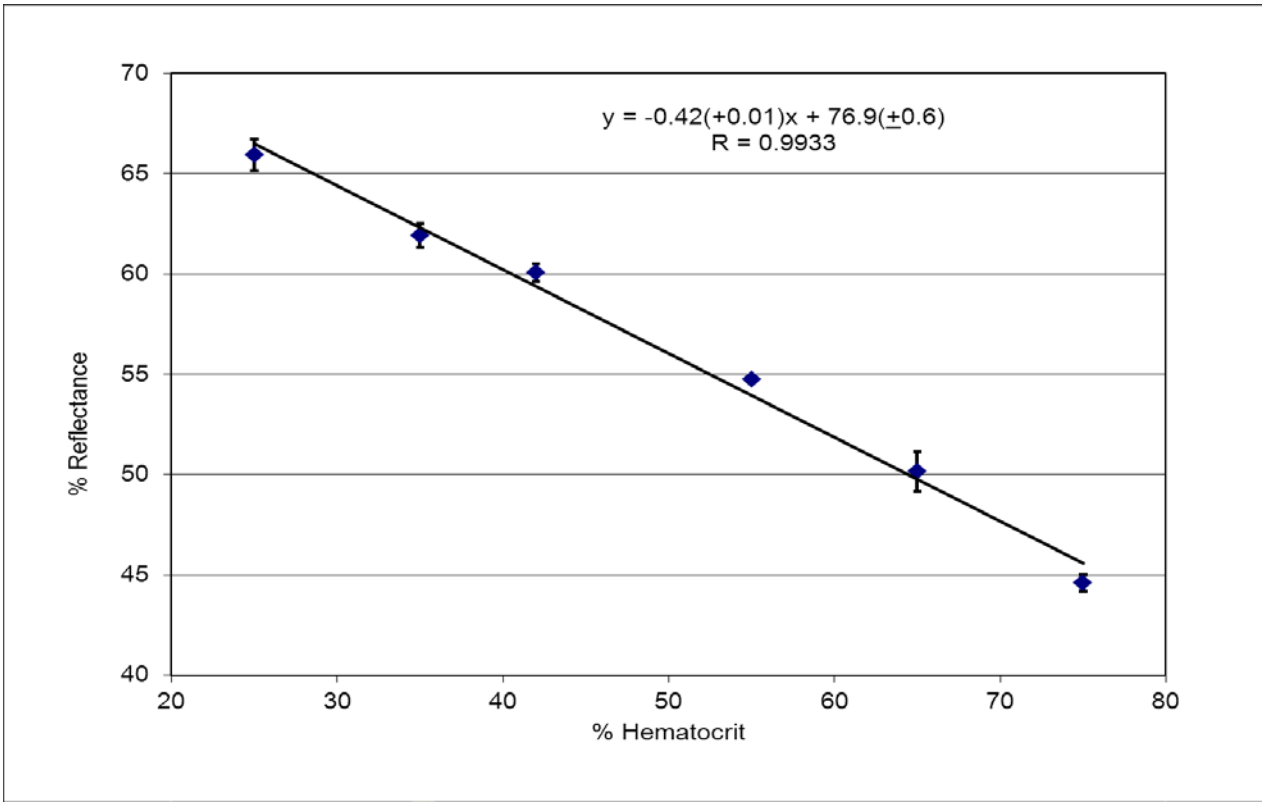
Data analysis of the UV reflectance spectra was performed by extracting percent reflectance at different wavelengths and plotting the signal response versus percent hematocrit. Several wavelengths show a linear correlation between percent hematocrit and hemoglobin; however 980 nm showed the best correlation and had the largest slope. Since the slope is the sensitivity of the analysis the linear relationship with the largest slope was chosen. Dried blood spots prepared at various levels of hematocrit were evaluated on two additional lots of Whatman 903 filter paper and on lot of DMPK filter paper cards. Acceptable, linear relationships with correlation coefficients greater than 0.9939 were observed for each set of samples, however there did appear to be slight differences between the reflectance response for samples prepare with DMPK filter paper and the Whatman 903 filter paper (Figure 4.6). This difference may be related to the volume of blood spotted on the DMPK filter paper cards was only 15 µL , where the volume of blood spotted on the Whatman 903 filter paper cards was 50 µL. It has been previously reported that volume of blood spotted on filter paper affects the volume of sample analyzed from a 6 mm punch (Mei et al., 2001).



Legend: Three different lots of Whatman 903 filter paper (Squares, Diamonds and Triangles) One lot of DMPK filter paper (Circles). Error bars represent 1 standard deviation

Figure 4.6: Plot of reflectance measurement at 980 nm versus hematocrit for control samples prepared at different levels of hematocrit on three different lots of Whatman 903 filter paper and one lot of DMPK filter paper, samples analyzed in triplicate each measurement made at a different location on the dried blood spot

The average response for each hematocrit level on the three different lots of Whatman 903 filter paper was used for our model. A linear model was achieved between percent reflectance and hematocrit with an overall correlation coefficient 0.9933. This model was used to calculate the percent hematocrit for the donor samples at low, normal and high hematocrit levels.



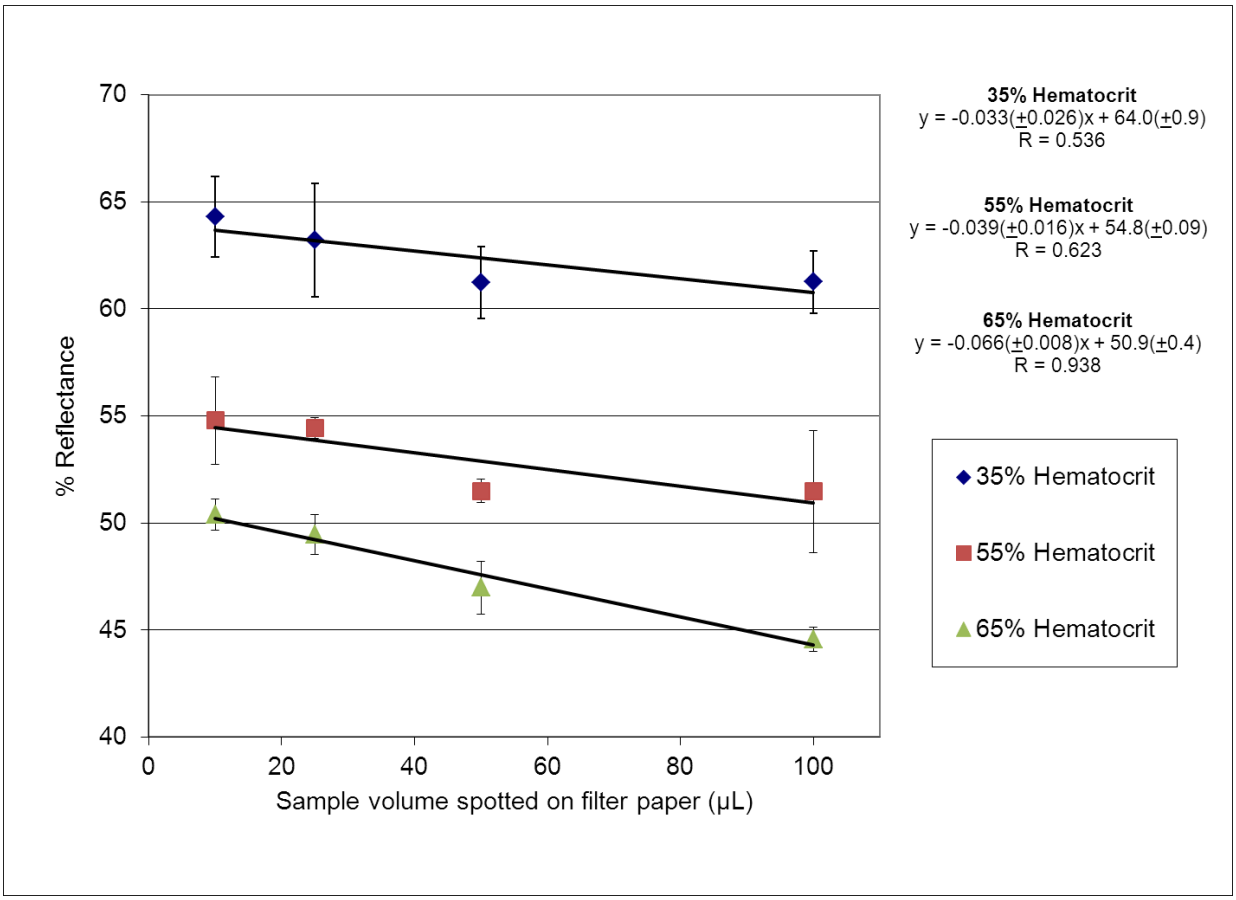
Legend: Error bars represent 1 standard deviation from the average of three different lots of Whatman 903 filter papers.

Figure 4.7: Plot of the average reflectance at 980 nm versus hematocrit for control samples prepared at different levels of hematocrit on three different lots of Whatman 903 filter paper, each filter paper lot was analyzed in triplicate

4.3.4 Evaluation of sample volume on reflectance

The volume of blood spotted has also been shown to affect the sample size (Mei et al., 2001). When spotting small volumes (15 µL) of blood the percent hematocrit has been shown to have less effect on sample size (Denniff & Spooner, 2010). However the sample volume can increase by as much as 15% between 25 µL of blood spotted and 125 µL of blood spotted on filter paper (Mei et al., 2001). Therefore we decided to investigate the reflectance measurement for samples that were prepared with different volumes of blood. Samples prepared with 10 µL, 25 µL 50 µL and 100 µL at 35%, 55% and 65% hematocrit were analyzed

using UV reflectance at 980 nm. Samples were analyzed in triplicate for each volume and hematocrit level at various positions on dried blood spots, results are shown in Figure 4.8.



Legend: Error bars represent 1 standard deviation.

Figure 4.8 Plot of blood volume spotted on Whatman 903 filter paper cards versus reflectance measurements at 980 nm for blood containing 35%, 55% and 65% hematocrit, dried blood spots were prepared by spotting 10 µL, 25 µL, 50 µL and 100 µL of blood on filter paper, samples were analyzed in triplicate

It is apparent from Figure 4.8 that as the volume of sample spotted on the filter paper increases there is a decrease in the percent reflectance. Also the decrease in reflectance is not consistent from 35% hematocrit to 65% hematocrit, as shown by the slope of the lines -0.033 and –0.066 respectively. Samples prepared with 65% hematocrit showed the largest decrease

in reflectance as the volume of blood spotted increased from 10 µL to 100 µL. Using just reflectance alone, it would not be possible to distinguish between a 10 µL sample with a 65% hematocrit spotted and 100 µL sample with a 55% hematocrit. However, 50-100 µL of blood spotted have differences between 35%, 55% and 65%. The manufacture indicates that the amount of blood that will fill the circular region on a dried blood spot is between 75 to 100 µL of blood.

4.3.5 Evaluation of hematocrit in donor samples

In order to evaluate the accuracy of our model donor samples prepared at low, normal and high hematocrits were evaluated with UV reflectance. Each dried blood spot was analyzed in triplicate at different positions to test the intra spot variability. Reflectance data was collected for each sample from 200 nm to 1000 nm for each sample. The response at 980 nm was used to back calculate the hematocrit level using our model, shown in Figure 4.7. Results from this experiment are presented in Table 4.2, along with the predetermined hematocrit levels and the percent difference from our measured value. The overall percent hematocrit determined using UV reflectance was within $\pm$ 11% of the measured value. In addition, the overall percent relative standard deviations for three replicate measurements at three hematocrit levels were within 11%.

Table 4.2: Determination of hematocrit for donor samples using reflectance at 980 nm.

Low Level	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
Rep 1	32	35	39	24	30	24
Rep 2	28	33	39	25	32	24
Rep 3	32	34	42	23	34	24
Mean	31	34	40	24	32	24
S.D.	2	1	2	1	2	0
%RSD	7%	4%	5%	5%	6%	1%
Measured Hematocrit	29	34	36	25	32	27
% Difference from Measured	6%	0%	11%	-6%	0%	-11%

Normal Level	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
Rep 1	49	52	50	39	47	37
Rep 2	49	49	48	38	45	38
Rep 3	48	54	52	41	50	35
Mean	49	51	50	39	47	36
S.D.	0	2	2	2	2	1
%RSD	1%	5%	4%	4%	5%	4%
Measured % Hematocrit	46	51	50	38	48	41
% Difference from Measured	6%	1%	0%	4%	-2%	-11%

High Level	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
Rep 1	76	64	71	65	58	69
Rep 2	69	73	73	70	68	67
Rep 3	64	79	68	64	59	61
Mean	69	72	70	66	62	66
S.D.	6	8	3	3	6	4
%RSD	9%	11%	4%	5%	9%	6%
Measured % Hematocrit	66	71	71	68	67	68
% Difference from Measured	5%	0%	-1%	-3%	-8%	-4%

4.4 Quantitative analysis

4.4.1 Quantitative analysis of control samples to determine sample volume

Quantitative analysis of the dried blood spots was used to evaluate the relationship between sample volume and hematocrit. There have been others that have evaluated this relationship for selected pharmaceutical compounds and for amino acids and acylcarnitines for

dried blood spots at various hematocrit levels. We are reporting data for a wider range of hematocrit than was previously reported. And it was necessary to establish measurements based on our samples to show the relationship between UV reflectance and sample volume.

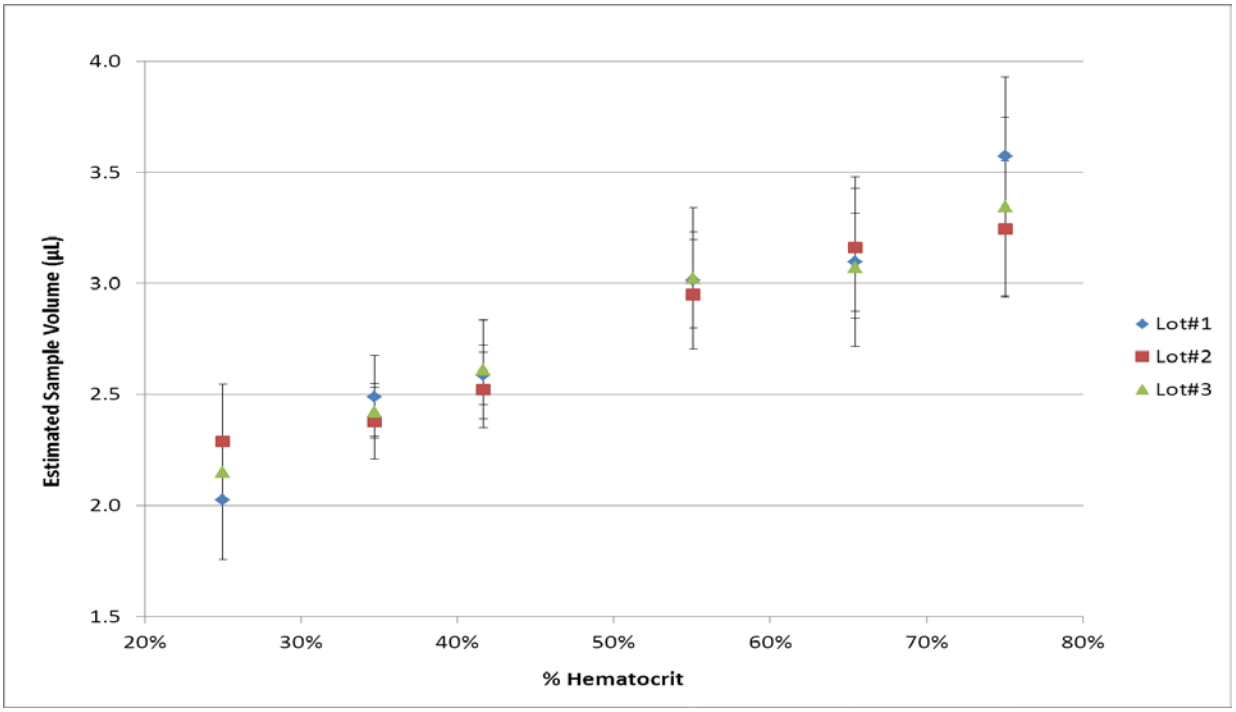
For this experiment, a 3.0 mm punch from dried blood spots using samples prepared with 50 µL of whole blood spotted on Whatman 903 filter paper. Samples were extracted in 0.100 mL of a mixture of 90:10 acetonitrile/water with 10 mM ammonium formate and analyzed by LC/MS/MS for acylcarnitines using a HILIC Amide (2.1 x 50 mm, 1.7 µm) column at a flow rate of 0.3 mL/minute. Compounds were eluted using a gradient elution form 95% B to 55% B over 3 minutes with 90:10 acetonitrile/10 mM ammonium acetate for mobile phase B and 0.1% formic acid in water for mobile phase A. Multiple reaction monitoring (MRM) of 12 acylcarnitines was performed on a Waters Premier mass spectrometer with positive electrospray ionization, with optimized MRMs shown in Table 4.1. Samples were quantified using an external calibration curve prepared in 90:10 Acetonitrile/ 10mM ammonium formate containing Carnitine, C2, C3, C4, C5, C6, C8, C10, C12, C14, C16, C18.

Dried blood spot samples from each of the three lots of Whatman 903 filter paper were analyzed in triplicate at each hematocrit level from 25% to 75%. Back calculated values of each analyte were determined using the external calibration curve. The volume of the sample was determined based on the fortified level and back calculated concentration using Equation 4.1:

Equation 4.1: Calculation for sample volume from an extracted dried blood spot sample

$$\text{Sample Volume} = \frac{\text{Calculated Concentration} * \text{Extraction Volume}}{\text{Fortified Concentration}}$$

Sample volume was calculated for each analyte Carnitine, C2, C3, C4, C5, C6, C8, C10, C12, C14, C16 and C18, since it was previously reported that some analytes appear to have a chromatographic effect on the filter paper cards. Although there were some differences observed in the sample volume when calculated using different analytes the overall differences were less than 10%. The volume for each hematocrit level was calculated by averaging the results obtained from all the analytes. The overall % relative standard deviations were less than 10%. Results for the average volume verses hematocrit determined on the three lots of Whatman 903 filter paper are shown in Figure 4.9.



Legend: Error bars represent 1 standard deviation.

Figure 4.9: Plot of sample volume versus hematocrit for dried blood spots analyzed from a 3.0 mm punch, sample volume calculated using quantitative analysis of Carnitine, C2, C3, C4, C5, C6, C8, C10, C14, C16, C18 for 3 different lots of Whatman 903 filter paper, each data point represents the average volume calculated using the all the analytes and each sample was analyzed in triplicate

It was apparent from our results that as the percent hematocrit increases there is an increase in sample volume. There is approximately a 55% increase in sample volume from 25% hematocrit to 75% hematocrit. The three different lots of Whatman 903 filter paper cards showed consistent results for each of the different hematocrit levels. The overall average from the three different lots of Whatman 903 filter paper were used to determine the average volume for each hematocrit level. A linear correlation with a slope of 2.42(± 0.14), a y-intercept of 1.58 (±0.07) and a coefficient of determination (R^2) of 0.9860 was obtained, Figure 4.10. This curve was used to determine sample volume of the donor samples based on the response from the reflectance measurements.

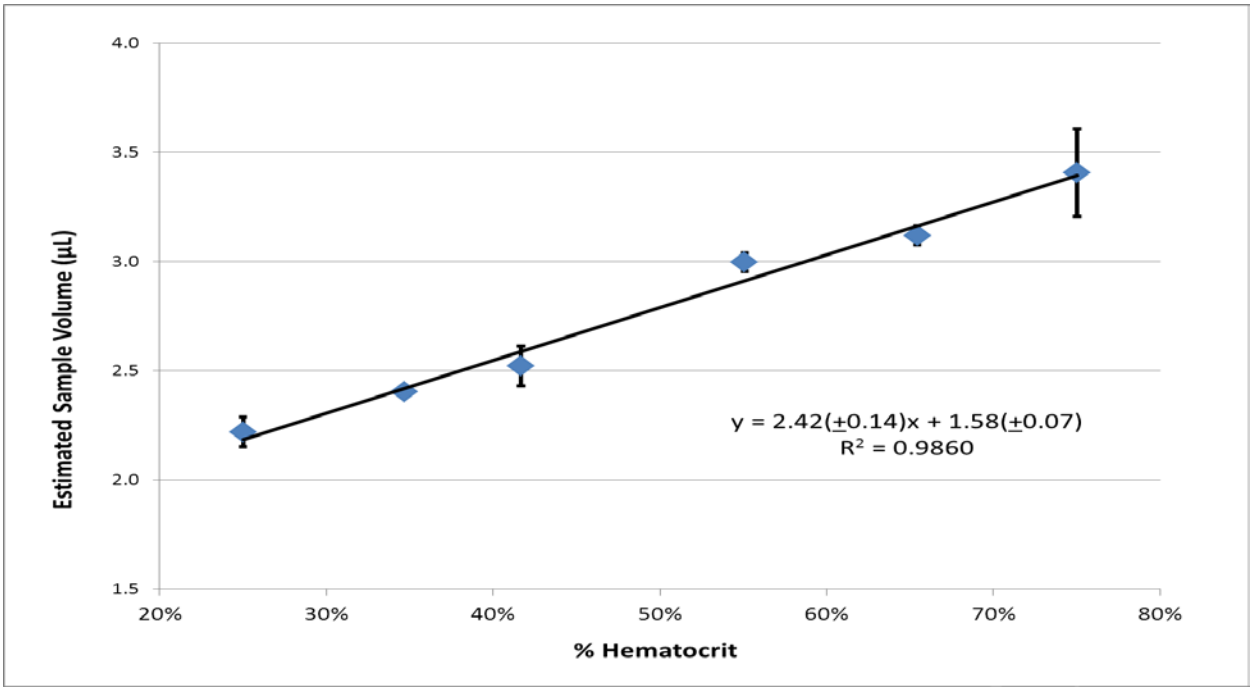


Figure 4.10: Plot of the average volume from a dried blood spot calculated from quantitative analysis of Carnitine, C2, C3, C4, C5, C6, C8, C10, C14, C16, C18 from a 3 mm punch using 3 different lots Whatman 903 filter paper versus hematocrit from 25% to 75%.

4.4.2 Quantitative analysis of donor samples

Eighteen donor samples at high, normal and low hematocrit levels were extracted using the same conditions as described earlier. Back calculated concentrations were determined for each acylcarnitne C0, C2, C3, C4, C5, C6, C8, C10, C12, C14, C16 and C18 using response ratios with labeled internal standards. Analyte concentrations were first calculated using assuming the sample volume extracted was 3.1 µL from a 3 mm punch. Table 4.3 shows the calculated accuracy for each analyte based on the known fortified concentration. The average accuracy for individual acylcarnitines in the low hematocrit samples ranged from 67% to 82% for an overall accuracy of 76% ± 4%. The average accuracy for individual acylcarnitines in the high hematocrit samples ranged from 94% to 136% for an overall accuracy of 110% ± 10%.

Next the same data was recalculated with the appropriate volume using the hematocrit determined from the reflectance measurements reported in Table 4.2. Sample volumes were calculated for the hematocrit level in each sample using the equation in Figure 4.10. Using the volume correction the accuracy was determined Table 4.4 shows the calculated accuracy for each analyte based on the known fortified concentration. The average accuracy for individual acylcarnitines in the low hematocrit samples ranged from 97% to 114% for an overall accuracy of 103% ± 5%. The average accuracy for individual acylcarnitines in the high hematocrit samples ranged from 81% to 115% for an overall accuracy of 98% ± 8%.

Overall there was a negative bias of 24% for low hematocrit samples when volume of the excised samples was not corrected for volume based on hematocrit. There was a slight positive bias of 10% observed for samples at high hematocrit when the excised sample was not corrected for volume based on hematocrit. However when sample volumes were corrected for

hematocrit that was determined by using our reflectance technique the overall bias was reduced to less than 3%. In addition correcting for sample volume improved the precision of the analysis by reducing the percent relative standard deviations from 18.7% to 11.7%.

Table 4.3: Calculation of percent accuracy for acylcarnitines in 6 donor samples at low, normal and high hematocrit. Samples not corrected for volume.

Donor Samples at Low Level Hematocrit

Donor	C0	C2	C3	C4	C5	C6	C8	C10	C12	C14	C16	C18
1	78%	65%	69%	79%	77%	75%	78%	75%	74%	83%	76%	77%
2	81%	74%	75%	80%	78%	76%	79%	76%	76%	80%	79%	79%
3	89%	81%	83%	86%	85%	84%	89%	82%	90%	91%	89%	86%
4	66%	65%	67%	70%	68%	64%	68%	70%	73%	75%	70%	73%
5	81%	72%	79%	81%	80%	73%	81%	76%	79%	85%	82%	83%
6	67%	44%	64%	74%	72%	69%	73%	72%	73%	78%	72%	72%
Mean	77%	67%	73%	78%	77%	73%	78%	75%	78%	82%	78%	78%
S.D.	8.8%	12.5%	7.5%	5.6%	5.9%	6.6%	7.2%	4.2%	6.6%	5.6%	6.8%	5.5%
%RSD	11.5%	18.7%	10.3%	7.1%	7.7%	9.0%	9.2%	5.5%	8.6%	6.8%	8.7%	7.0%
Overall	76%											

Donor Samples at Normal Level Hematocrit

	C0	C2	C3	C4	C5	C6	C8	C10	C12	C14	C16	C18
1	88%	95%	93%	95%	90%	87%	90%	83%	87%	94%	86%	86%
2	84%	98%	89%	85%	84%	81%	84%	74%	82%	83%	84%	85%
3	93%	103%	99%	93%	92%	92%	97%	87%	98%	96%	98%	95%
4	75%	87%	83%	82%	76%	76%	83%	79%	83%	85%	82%	81%
5	87%	98%	95%	96%	91%	90%	92%	85%	99%	96%	95%	93%
6	68%	68%	74%	81%	78%	73%	77%	76%	80%	82%	76%	76%
Mean	82%	91%	89%	89%	85%	83%	87%	81%	88%	89%	87%	86%
S.D.	9.4%	12.7%	8.9%	6.9%	6.9%	8.0%	7.2%	5.2%	8.3%	6.7%	8.1%	7.1%
%RSD	11.4%	13.9%	10.1%	7.7%	8.1%	9.6%	8.3%	6.4%	9.4%	7.5%	9.3%	8.2%
Overall	87%											

Donor Samples at High Level Hematocrit

	C0	C2	C3	C4	C5	C6	C8	C10	C12	C14	C16	C18
1	94%	142%	115%	109%	108%	110%	113%	102%	118%	114%	108%	106%
2	96%	138%	114%	108%	107%	100%	106%	95%	108%	101%	101%	98%
3	101%	141%	121%	114%	110%	107%	115%	108%	118%	113%	110%	103%
4	95%	143%	119%	113%	112%	105%	114%	100%	117%	111%	116%	111%
5	92%	128%	120%	122%	112%	105%	112%	104%	109%	110%	102%	97%
6	86%	125%	113%	120%	115%	111%	110%	98%	106%	99%	99%	95%
Mean	94%	136%	117%	114%	111%	107%	112%	101%	113%	108%	106%	102%
S.D.	4.8%	7.8%	3.5%	5.5%	2.8%	3.8%	3.3%	4.4%	5.5%	6.2%	6.8%	6.4%
%RSD	5.2%	5.7%	3.0%	4.8%	2.5%	3.6%	2.9%	4.3%	4.9%	5.7%	6.4%	6.3%
Overall	110%											

Table 4.4: Calculation of percent accuracy for acylcarnitines in 6 donor samples at low, normal and high hematocrit. Samples corrected for volume using reflectance measurements

Donor Samples at Low Level Hematocrit

Donor	C0	C2	C3	C4	C5	C6	C8	C10	C12	C14	C16	C18
1	111%	97%	92%	104%	101%	98%	103%	98%	97%	108%	102%	104%
2	111%	107%	96%	102%	98%	96%	100%	96%	97%	101%	102%	104%
3	113%	106%	99%	102%	101%	99%	105%	97%	106%	107%	106%	103%
4	112%	117%	100%	102%	98%	93%	99%	101%	105%	108%	108%	111%
5	119%	111%	105%	106%	104%	95%	105%	98%	103%	110%	110%	111%
6	118%	83%	94%	107%	103%	99%	105%	104%	104%	112%	109%	109%
Mean	114%	104%	98%	104%	101%	97%	103%	99%	102%	108%	106%	107%
S.D.	3.6%	11.7%	4.7%	2.2%	2.4%	2.4%	2.9%	2.8%	4.1%	3.7%	3.4%	3.9%
%RSD	3.1%	11.3%	4.8%	2.1%	2.3%	2.5%	2.8%	2.9%	4.0%	3.4%	3.2%	3.6%
Overall	103%											

Donor Samples at Normal Level Hematocrit

Donor	C0	C2	C3	C4	C5	C6	C8	C10	C12	C14	C16	C18
1	94%	102%	101%	102%	96%	94%	97%	89%	94%	101%	93%	93%
2	89%	105%	93%	90%	89%	84%	88%	78%	86%	87%	88%	89%
3	102%	114%	105%	99%	98%	98%	103%	93%	104%	102%	105%	102%
4	115%	97%	99%	95%	94%	97%	121%	105%	108%	106%	108%	102%
5	100%	115%	105%	106%	100%	99%	101%	94%	108%	105%	105%	104%
6	94%	98%	93%	100%	96%	90%	94%	94%	98%	101%	97%	98%
Mean	99%	105%	99%	99%	96%	94%	101%	92%	100%	100%	99%	98%
S.D.	8.8%	7.7%	5.3%	5.7%	3.9%	5.7%	11.1%	8.8%	8.8%	6.9%	7.8%	5.6%
%RSD	8.9%	7.3%	5.4%	5.8%	4.1%	6.0%	11.0%	9.5%	8.8%	6.8%	7.9%	5.7%
Overall	98%											

Donor Samples at High Level Hematocrit

Donor	C0	C2	C3	C4	C5	C6	C8	C10	C12	C14	C16	C18
1	77%	111%	101%	97%	97%	98%	101%	91%	105%	102%	95%	92%
2	79%	112%	99%	94%	94%	88%	93%	83%	94%	89%	87%	84%
3	84%	115%	106%	101%	97%	95%	102%	95%	105%	100%	96%	90%
4	83%	123%	108%	104%	102%	96%	104%	91%	107%	102%	105%	100%
5	85%	118%	114%	116%	107%	101%	107%	99%	104%	105%	97%	92%
6	76%	108%	103%	110%	106%	103%	102%	91%	98%	91%	90%	86%
Mean	81%	115%	105%	104%	100%	97%	102%	92%	102%	98%	95%	91%
S.D.	4.0%	5.5%	5.5%	8.3%	5.4%	5.1%	4.9%	5.2%	4.8%	6.4%	6.0%	5.8%
%RSD	4.9%	4.8%	5.3%	8.0%	5.4%	5.3%	4.8%	5.7%	4.7%	6.6%	6.4%	6.3%
Overall	98%											

4.5 Conclusions

We have developed a novel approach that allows for the determination of hematocrit directly on dried blood spots using reflectance. In addition, we have shown that the sample volume is dependent on the percent hematocrit and can be used to correct for sample volume. Donor samples at various hematocrits were used to test both models and demonstrated acceptable precision and improved accuracy over the standard technique which assumes that all samples are 3 µL. The ability to predetermine the hematocrit directly on dried blood spots using UV reflectance can be used to correct for sample volume, improving the accuracy of this sample collection technique. Correcting for volume greatly improves the accuracy of analysis and the reflectance format used allows for direct automatable volume corrections on the card without the need for off line procedures to test whole blood.

4.6 Acknowledgements

We would like to thank the Virginia Division of Consolidated Laboratory Services for supplying us with the internal standard reference material and Altria Client Services for providing additional supplies and LC/MS/MS instrumentation.

CHAPTER 5

SUMMARY AND OVERALL CONCLUSIONS

According to the CDC there are over 4 million children born every year in the United States. It is a state mandated requirement that all of these children be tested for inheritable metabolism disorders, endocrinopathies, hemoglobinopathies and genetic disorders. Dried blood spot analysis has been pivotal to newborn screening, since it is an extremely cost effective way of collecting, shipping and storing samples. It has been more than 50 years since dried blood spots were developed for the analysis of PKU by Robert Guthrie and newborn screening has grown to test for more than 40 metabolism disorders. Although this testing program has been claimed to be one of the most successful public health programs, there is an increasing need to further improve this testing. Newborn screening allows for the quick identification of disorders; however the current methods are not fully quantitative and often require second-tier testing or follow-up testing by an independent laboratory to confirm a disorder. This adds additional time to diagnosis and an increase cost to state newborn screening laboratories and an increase cost to health care. By providing newborn screening laboratories with new techniques and new technologies we can further improve the accuracy of analysis, reduce costs and shorten the time to diagnosis. This is why we decided to investigate

new approaches to dried blood spot analysis using automation and high resolution chromatography.

The first chapter of this dissertation covers the background to newborn screening dried blood spot analysis for the identification of metabolism disorders. Although current flow injection approach used by newborn screening laboratories does provide a rapid screening technique there are several potential issues with this method. In order to address some of these issues we developed a high resolution liquid chromatographic separation that could be conducted in approximately the same time as the current screening method. Chapter 1, also provides a review of current techniques available for direct analysis of dried blood spots which have been developed since the start of our research. Several of these devices have been automated to increase efficiencies in dried blood spot analysis and minimize sample handling requirements ultimately reducing costs and improving laboratory efficiencies. We were the first to publish a device that allowed for direct sampling of newborn screening cards without the need for punching disks from dried blood spots. Chapter 1, also reviews recent literature that has investigated the affect hematocrit has on sample volume in dried blood spot analysis. It has been reported that hematocrit has the largest effect of sample size for dried blood spot analysis compared to volume spotted on filter paper and punch location (Mei et al., 2001). Although, there is some controversy as to how dramatic this effect is for normal hematocrit levels there does appear to be a significant bias of analyte concentration in samples with low and high hematocrit.

In chapter 2, we provide a full description of our high resolution chromatographic separation along with data supporting the method validation. We showed the ability to

separate key isobars that are diagnostic for different metabolism disorders, which is not possible with the current flow injection approach. We also address several key issues not previously addressed in newborn screening such as matrix effects and quantification errors associated with response factors. A comparison between the current newborn screening method and our modified screening method using high resolution chromatography is outlined in table 5.1.

Table 5.1: Comparison between the current newborn screening method used by most state laboratories and our modified method using a high resolution chromatographic separation

Current newborn screening method	Modified screening method with chromatographic separation
Labor intensive extraction: 1. Samples must be transferred twice 2. Requires hazardous derivatization 3. Evaporation	**Fewer extraction steps:** 1. Samples transferred once 2. No derivatization 3. No evaporation
Not fully quantitative: 1. Samples quantified based on single point response factors with labeled internal standards 2. Matrix effects could cause errors in quantification	**Improved quantification:** 1. Samples quantified using internal calibration curve encompassing a range above and below cut-off value 2. Minimizes matrix effects by chromatographically resolving interferences
Poor selectivity and sensitivity: 1. No Chromatographic separation of isobars, requires additional testing for differential diagnosis of certain disorders 2. Precursor ion scan and neutral loss scan used for analysis reduces sensitivity	**Improved selectivity and sensitivity:** 1. Chromatographically separates key isomers allowing for differential diagnosis of multiple disorders 2. Scheduled MRMs are used for analysis improving the analyte sensitivity

Chapter 3, provides a practical application using our in-line desorption device with a chromatographic separation. A method was developed and validated for quantification of

leucine and isoleucine which are markers for MSUD. Our approach allowed for the direct analysis of dried blood spots without the need for any off-line sample processing. Our chromatographic separation is capable of resolving leucine, isoleucine and hydroxylproline, and could be used as a rapid second tier test for MSUD. This was the first published paper that incorporated a flow through device with a high resolution chromatographic separation.

Finally we addressed the major concern in dried blood spot analysis which is the influence of hematocrit on sample volume. In the fourth chapter we evaluated the ability to correct for sample volume using a hematocrit determined by direct analysis of the dried blood spot with UV reflectance at 980nm. Our technique provides a relationship between reflectance and hematocrit that can be used prior to sample analysis to correct sample volume. We validated our reflectance model to determine hematocrit through the analysis of donor samples prepared at various hematocrit levels. We also showed that sample volume is dependent on hematocrit and is consistent from lot to lot of Whatman 903 filter paper cards. Using both the reflectance measurement and the quantitative analysis of samples we could relate the reflectance measurement to sample volume. To validate our technique donor samples were analyzed at various levels of hematocrit and quantified with and without correcting for sample volume. Correcting for sample volume showed a dramatic improvement in accuracy at low and high hematocrit levels.

Overall we addressed several of the current issues associated with newborn screening dried blood spot analysis. We addressed the lack of specificity in newborn screening by adding a high resolution chromatographic separation that could be conducted in approximately the same time as the flow injection analysis. We provided a direct analysis technique that could be

automated increasing laboratory efficiencies which minimizes sample handling requirements. Finally, we addressed a major concern which was how to correct for differences in hematocrit by applying UV-NIR reflectance as a means to directly analyze dried blood spots to determine hematocrit and relating this to sample volume. The device that we evaluated was a fiber optic probe that allowed for direct measurements to be made to the dried blood spot. The simplicity of this device and the use of fiber optics would allow for it to be easily integrated by newborn screening laboratories. These improvements that we have investigated could dramatically improve the efficiencies and accuracy of newborn screening dried blood spot analysis.

Although our research does provide significant improvements to the newborn screening methodology we were not able to test our methodologies with incurred samples from real newborns. This is due to the limited availability of newborn screening samples and potential controversies associated with analysis of samples from other laboratories. We believe the next step to this research would be to apply our methods in parallel with the current methods using incurred samples from newborns. Future work could also investigate the ability to add additional markers to the high resolution chromatographic separation. This would allow for more accurate diagnosis of disorders that have more selective markers available for identification such as methylmalonic acid for methylmalonic acidemia (MMA). Also, several in-line desorption devices are now commercially available and could be investigated for newborn screening dried blood spot analysis with or without chromatographic separation for identification of metabolism disorders. This would dramatically decrease the workloads of newborn screening reducing costs and improving efficiencies. Finally the use of NIR reflectance analysis should be evaluated with incurred samples from newborns to evaluate the potential to

evaluate hematocrit on dried blood spots. Our approach was acceptable under controlled conditions; however it should be further evaluated with real samples spotted as they would be by health care providers.

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CURRICULUM VITAE

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Education

B.S. Chemistry, 1992, Virginia Military Institute, Lexington VA
Ph.D. Pharmaceutics, 2012, VCU, Richmond VA

Work Experience

Job Title: Research Scientist, Sensory and Analytical Sciences 2010-2012
Location: Center for Research and Technology, Altria, Richmond VA
Duties: Responsible for developing and validating methods related to sensory and analytical chemistry to support new product development and manufacturing. LC/MS/MS technical leader, responsible for supervising and training other scientist.

Job Title: Associate Research Scientist, Analytical Sciences 2008-2010
Location: RD&E, Philip Morris U.S.A. Richmond VA
Duties: Responsible for developing and validating methods related to process analytical chemistry to support manufacturing. Test method project leader for implementation of methods to Factor QA.

Job Title: Scientist, Analytical Sciences, 2006-2008
Location: RD&E, Philip Morris U.S.A. Richmond VA
Duties: Develop and validate methods for the analysis of constituents in new products including tobacco and smoke. Transferred methods from HPLC platforms to UHPLC Platforms reducing analysis times and costs.

Job Title: Scientist, Analytical Sciences, 2004-2006
Location: RD&E, Philip Morris U.S.A. Richmond VA
Duties: Develop and validate methods for the analysis of constituents in tobacco and smoke. Conduct method validations on a variety of instrumentation platforms including Micromass Ultima and Premier equipped with UPLC, Shimadzu GCMS, HP1100 HPLC and HP GC with Antek nitrogen detector.

Job Title: Method Validation Group Leader, 2001-2004
Location: PPD DEVELOPMENT, Richmond, VA
Duties: Manage multiple validation projects simultaneously, checking methods, notebooks and data packets for accuracy, completeness and GLP compliance. Schedule the activities of the validation group to maximize the usage of instruments and coordinate with production and development groups to meet deadlines. Oversee the daily activities of the group, helping setup systems and troubleshoot any problems. Responsible for teaching inexperienced scientists how to operate LCMS systems and evaluate extractions. Provide technical support to the production group to ensure methods will perform properly.

Job Title: Research Scientist R&D, 2000-2001
Location: PPD DEVELOPMENT, Richmond, VA
Duties: Responsible for developing and validating LCMS bioanalytical methods. Worked independently to optimize extractions and establish chromatography. Supported production group and supervised other R&D scientists in the development of methods.

Job Title: Senior Scientist R&D, 1997-2000
Location: PPD DEVELOPMENT, Richmond, VA
Duties: Responsible for developing and validating HPLC and LCMS bioanalytical methods. Reviewed project data, notebooks and prepared validation reports and methods. Communicated with Department Manager concerning assigned projects and provide updates on progression or problems

Job Title: Scientist, 1992-1997
Location: PPD DEVELOPMENT, Richmond, VA
Duties: Responsible for developing and validating HPLC bioanalytical methods. Established proficiency in both solid phase and liquid/liquid extractions to isolate analytes from plasma or urine samples. Prepared calibration standards, quality control samples and working standards for analysis of biological samples. Reviewed and compiled data in regression programs and made judgments on data acceptability. Wrote validation and method reports.

Technical Publications and Posters

1. Miller IV, J.H., Poston P.A., Rutan, S.C., Karnes H. T. (2012) Diffuse Reflectance for Relating Percent Hematocrit to Correct for Sample Volume on Dried Blood Spots, American Association of Pharmaceutical Scientists Convention (AAPS), Poster Session, October 2012.

2. Miller, J.H., Poston, P.A., Karnes, H.T. (2012) A quantitative method for acylcarnitines and amino acids using high resolution chromatography and tandem mass spectrometry in newborn screening dried blood spot analysis, Journal of Chromatography B, 903(1) 142.

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Professional Memberships and Activities

President of The Richmond Chromatography Discussion Group 2011-2012
American Association of Pharmaceutical Scientists (AAPS)
American Society of Mass Spectrometry (ASMS)
American Chemical Society (ACS)