Simultaneous Analysis of Amino Acids and Acylcarnitines in Dried Blood Spots

Application Note

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Abstract

A rapid method for the simultaneous detection and quantification of 37 acylcarnitines and 12 amino acids in dried blood spots was developed using the Agilent Triple Quadrupole LC/MS System in neutral loss scan, precursor ion scan and multiple reaction monitoring (MRM) data acquisition modes. With a run time of 1.6 minutes, the assay provides the ability to analyze 400–500 samples per day, and custom reports provide all the information needed to rapidly list the critical elements for the analysis.

Agilent Technologies

Introduction

The first analysis of dried blood spots (DBS) began in the 1960s with the introduction of a bacterial inhibition assay for phenylketonuria by Robert Guthrie. In the years since, DBS analysis has been expanded to include additional bacterial inhibition assays and other technologies (immunochemistry and electrophoresis) for amino acids and acylcarnitines, and MS/MS analysis was introduced in the 1990s. MS/MS is now the preferred technology for this analysis in dried blood spots, because of its multiplex capabilities to measure several analytes simultaneously.

This application note describes a unique method for the rapid determination of amino acids and acylcarnitines on DBS that is capable of processing 400 to 500 samples per day, using the Agilent Triple Quadrupole LC/MS System in neutral loss scan, precursor ion scan and multiple reaction monitoring data acquisition modes.[1, 2] The assay is capable of quantifying all acylcarnitines and most of the amino acids (Table 8), from both scan data and MRM data. As a result, the researcher has the option to use the scan data to quantify these analytes and compare it to the MRM quantification results, in order to eliminate the possibility of data misrepresentation due to chemical interferences.

Experimental

Reagents and standards

Reference standards on blood cards were obtained from the Centers for Disease Control (CDC), and isotopic internal standard mixes for amino acids and acylcarnitines were obtained from Cambridge Isotope Laboratories (Catalog Nos. NSK-A and NSK-B, respectively). Working solutions of the isotopic internal standards were prepared in methanol by diluting the contents of the vial with 200 mL of methanol. The concentrations of the working solutions of isotopic internal standards, which were stored at ≤ 20 °C, are given in **Table 1**. Methanol and isopropyl alcohol were obtained from Fisher Scientific, and butanol-HCl from Regis Chemicals.

Instruments

This method was developed on an Agilent 1200 Series LC system coupled with an Agilent 6460 Triple Quadrupole LC/MS and an Agilent 1200 Series G1367A Well-Plate Autosampler. The instrument conditions are listed in **Table 2**.

Sample preparation

Blood spot samples (3.2 mm) were extracted with 100 µL of methanol containing the appropriate isotopic internal standard at 30 °C for 30 minutes. Each extracted sample was then filtered using a 0.45 µm Low-Binding Hydrophilic PTFE filter (Millipore, Cat # MSRLN0410) and dried at 60 °C. Derivatization was then performed with butanol-HCl (100 µL) at 60 °C for 30 minutes, and the samples were dried at 40 °C for 20 minutes. Each sample was then reconstituted in 100 µL of methanol:water (80:20) prior to injection.

Internal Standard	Concentrations (nmol/mL)
Glycine-D3	12.5
Alanine-D4	2.5
Valine-D8	2.5
Leucine-D3	2.5
Methionine-D3	2.5
Phenylalanine-D6	2.5
Tyrosine-D6	2.5
Aspartate-D3	2.5
Glutamate-D3	2.5
Ornithine-D2	2.5
Citrulline-D2	2.5
Arginine-D5	2.5
C0-Carnitine	0.76
C2-Carnitine	0.19
C3-Carnitine	0.04
C4-Carnitine	0.04
C5-Carnitine	0.04
C8-Carnitine	0.04
C14-Carnitine	0.04
C16-Carnitine	0.08

Table 1. Daily Working Internal Standard Concentrations

LC Run Conditions	
Column	None
Injection volume	5 µL
Autosampler temperature	6 °C
Needle wash	Flush port (50:25:25 H_2O, IPA:MeOH:H_2O, 5 sec)
Mobile phase	A = H_2O + 0.1% formic acid B = methanol + 0.1% formic acid
Analysis time	1.8 min
Flow rate	0.5 mL/min
Isocratic Analysis	A = 20%, B = 80%
MS Conditions 6460	
Ion mode	Positive, ESI
Drying gas temperature	300 °C
Sheath Gas temperature	300 °C
Drying gas flow	5 L/min
Nebulizer pressure	60 psi
Capillary voltage	4000 V
Charging voltage	2000 V
MRM acquisition	Q1 peak and Q2 peak widths = 0.7 *m/z*
Delta EMV	200 V

Table 2. LC and Mass Spectrometer Conditions

Analysis parameters

The mass spectrometer was operated in 3 modes: neutral loss scan, precursor ion scan, and MRM. All three modes were performed sequentially on the Agilent 6460 Triple Quadrupole LC/MS using an injector program that injected each sample three times and automatically changed the acquisition parameters to suit each of the three acquisition modes. **Figure 1** shows representative reconstructed total ion current chromatograms (RTICCs) for the three consecutive scans performed on the same DBS sample.

Comprehensive DBS Analysis by Precursor Ion Scan, Neutral Loss Scan, and MRM

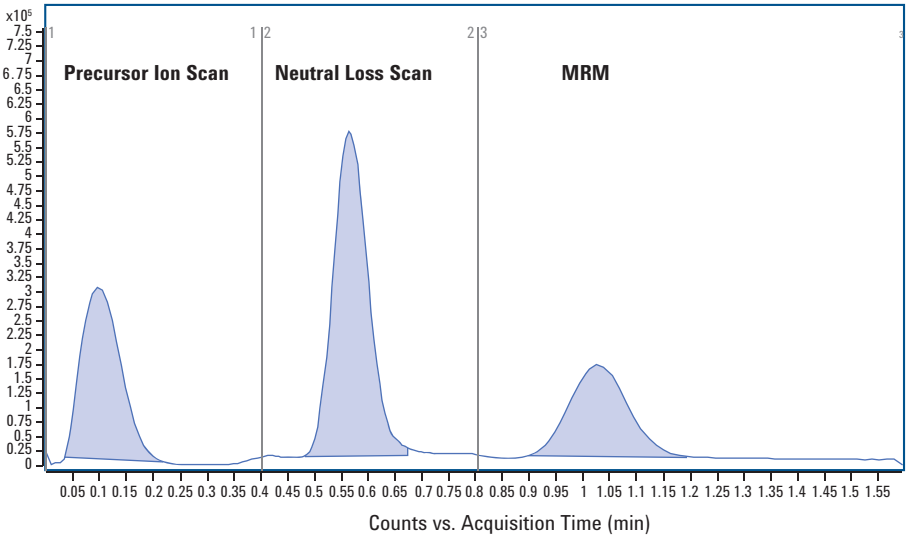


Figure 1. Reconstructed Total Ion Current Chromatograms (RTICC) showing the three sequential scans run on the same dried blood spot sample.

The neutral loss scan of 102 *m/z* was used to analyze the sample for the presence of 8 amino acids (**Figure 2**). In the first quadrupole, scanning is performed across an *m/z* range of 140 to 270. The third quadrupole (the second providing the molecule fragmentation) is set at a fixed difference in the *m/z* (102) from the first quadrupole, while scanning over an *m/z* range of 38 to 168. The combination of a specific precursor *m/z* and a known neutral loss in *m/z* in the product ion is characteristic for each amino acid. The analysis parameters used are shown in **Table 3**, and a representative scan is shown in **Figure 3**.

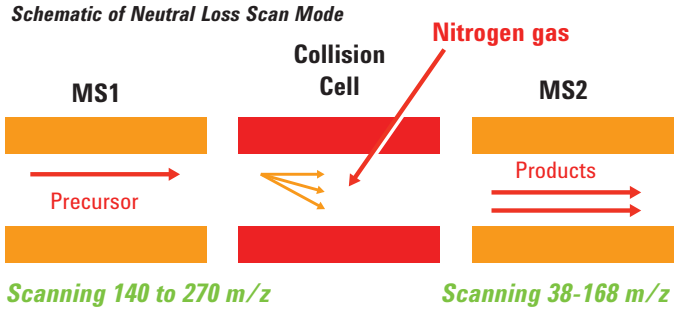


Figure 2. The first quadrupole mass analyzer (MS1) and third quadrupole (MS2) are set at a fixed difference (102 Da) in the mass-to-charge ratio (m/z) while both are scanning over a user-defined mass range (MS1: 140 to 270; MS2: 38 to 168).

Neutral Loss (*m/z*)	MS1 From (*m/z*)	MS1 To (*m/z*)	Scan Time (ms)	Frag Mode	Fragmentor Voltage	Collision Energy (EV)
102.1	140	270	300	Fixed	100	9

Table 3. Neutral Loss Scan Acquisition Parameters

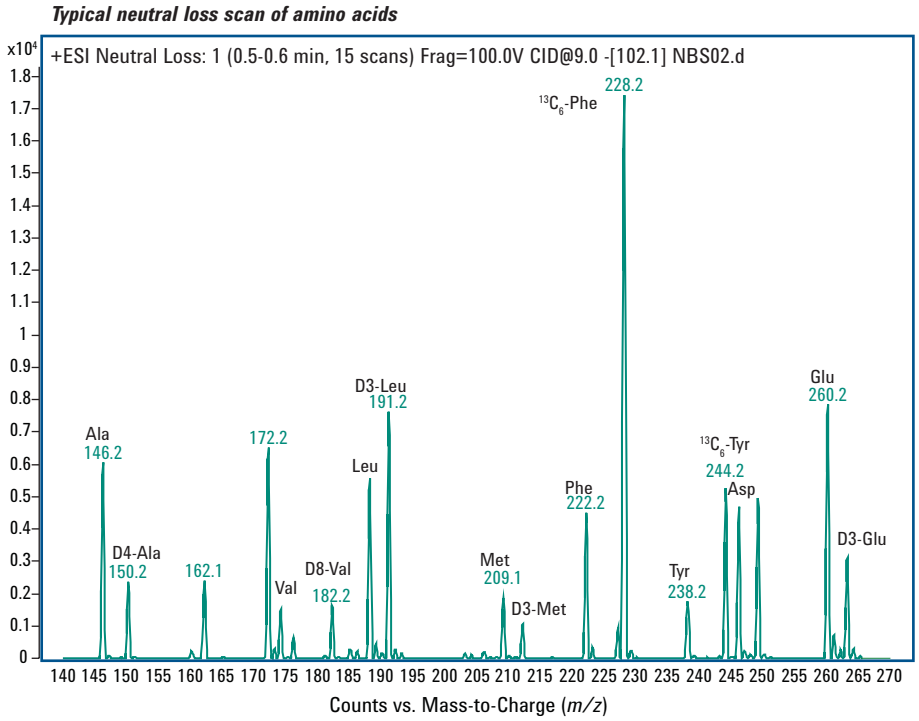


Figure 3. Neutral Loss Scan of 102 m/z for amino acids.

The precursor ion scan was used to analyze the sample for the presence of 37 acylcarnitines (**Figure 4**). The first quadrupole mass analyzer scans over a defined *m/z* of 200 to 510, while the third quadrupole is fixed to detect only product ions of *m/z* 85. The combination of a specific precursor *m/z* with the product ion *m/z* of 85 is characteristic for each acylcarnitine. The analysis parameters used are shown in **Table 4**, and a representative scan is shown in **Figure 5**.

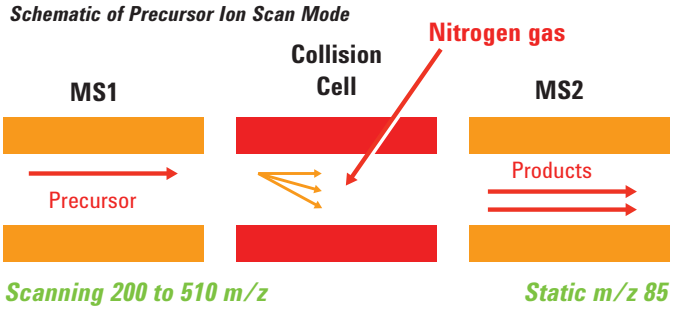


Figure 4. The first quadrupole mass analyzer (MS1) scans over a defined mass range of 85 to 200, while the second quadrupole (MS2) is fixed to detect only product ions of m/z 85. The combination of a specific precursor m/z with the product ion m/z of 85 is characteristic for each acylcarnitine.

Product Ion (*m/z*)	MS1 From (*m/z*)	MS1 To (*m/z*)	Scan Time (ms)	Frag Mode	Fragmentor Voltage	Collision Energy (EV)
85	85	200	510	Fixed	500	25

Table 4. Precursor Ion Scan Acquisition Parameters

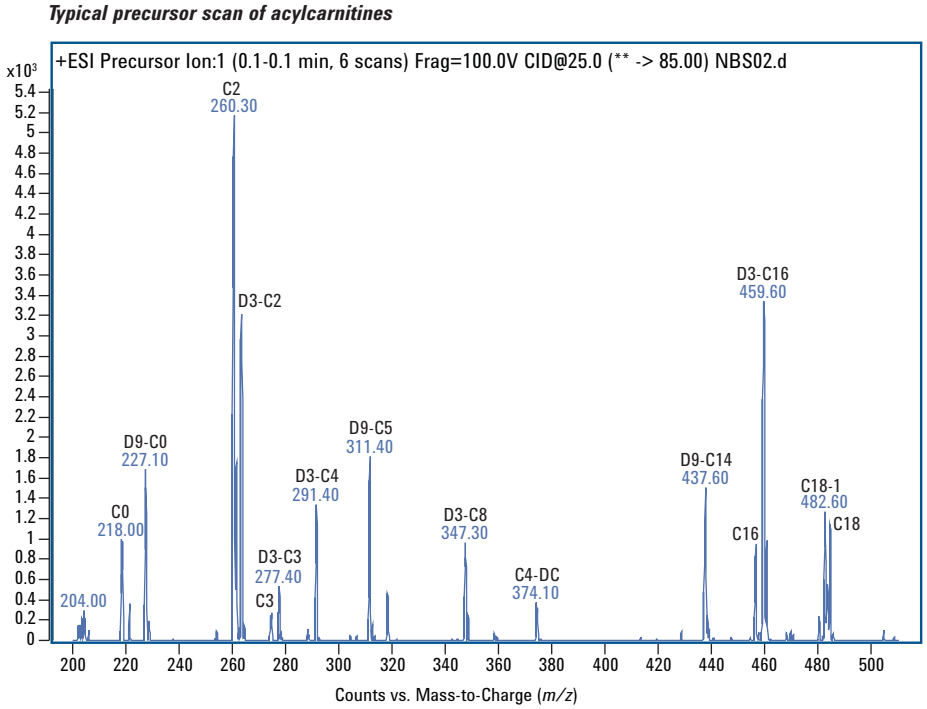


Figure 5. Spectrum profile of acylcarnitines.

Multiple Reaction Monitoring was used to quantify 12 amino acids and 37 acylcarnitines (**Figure 6**). Both the first and second quadrupole mass analyzers are held static at the mass-to-charge ratios (*m/z*) of the precursor ion and the most intense product ion, respectively, characteristic of a particular amino acid or acylcarnitine. The analysis parameters used are shown in **Table 5**, and a representative MRM transition is shown for leucine in **Figure 7**.

The MassHunter Universal integrator was used to perform the data analysis, and peak height was used for quantification.

Schematic of MRM Mode

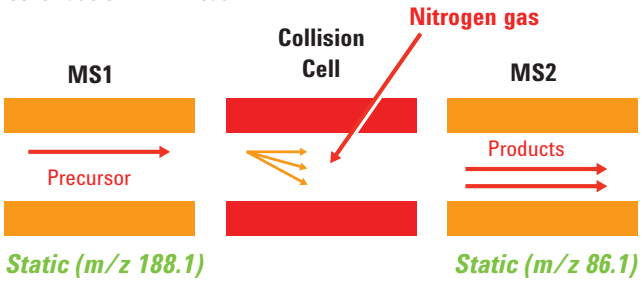


Figure 6. Both the first (MS1) and third quadrupole (MS2) mass analyzers are held static at the mass-to-charge ratios (m/z) of the precursor ion and the most intense product ion, respectively, characteristic of a particular amino acid or acylcarnitine, in this case.

Typical MRM analysis of an amino acid

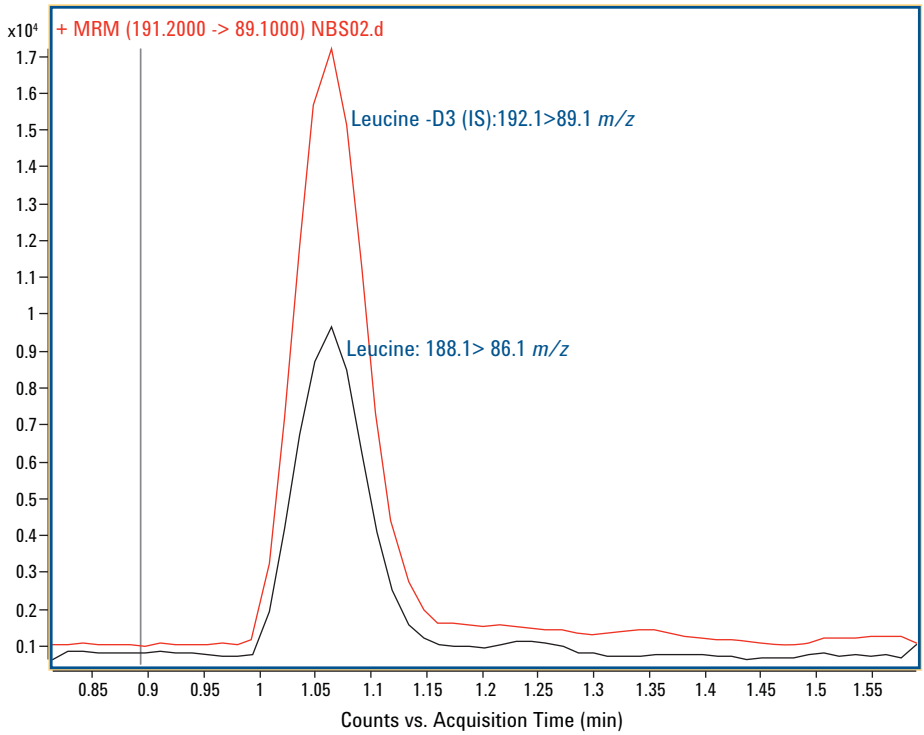


Figure 7. MRM for leucine and the leucine-d3 internal standard (IS).

Table 5. MRM Acquisition Parameters

Compounds	Segment	Precursor Ion	MS/MS	IS	IS Conc. (nmol/mL)	Collision Energy (EV)
Alanine	3	146.1 -> 44.0	MRM	Alanine-D4	2.5	9
Alanine-D4	3	150.1 -> 48.0	MRM	<None>	2.5	9
Arginine	3	231.2 -> 70.1	MRM	Arginine-D4-5C13	2.5	9
Arginine-D4-5C-13	3	236.2 -> 75.1	MRM	<None>	2.5	9
Aspartic Acid	3	246.2 -> 144.1	MRM	Aspartic Acid-D3	2.5	9
Aspartic Acid-D3	3	249.2 -> 147.1	MRM	<None>	2.5	9
C0	3	218.2 -> 103.0	MRM	C0-D9	0.76	16
C0-D9	3	227.2 -> 103.0	MRM	<None>	0.76	25
C10:1	3	370.3 -> 85.0	MRM	C8-D3	0.04	25
C10:2	3	368.3 -> 85.0	MRM	C8-D3	0.04	25
C10	3	372.3 -> 85.0	MRM	C8-D3	0.04	25
C12:1	3	398.3 -> 85.0	MRM	C14-D9	0.04	25
C12:1-OH	3	414.5 -> 85.0	MRM	C14-D9	0.04	25
C12	3	400.3 -> 85.0	MRM	C14-D9	0.04	25
C12-OH	3	416.5 -> 85.0	MRM	C14-D9	0.04	25
C14:1	3	426.4 -> 85.0	MRM	C14-D9	0.04	25
C14:1-OH	3	442.5 -> 85.0	MRM	C14-D9	0.04	25
C14:2	3	424.3 -> 85.0	MRM	C14-D9	0.04	25
C14	3	428.4 -> 85.0	MRM	C14-D9	0.04	25
C14-D9	3	437.4 -> 85.0	MRM	<None>	0.04	25
C14-OH	3	444.4 -> 85.0	MRM	C14-D9	0.04	25
C16:1	3	454.4 -> 85.0	MRM	C16-D3	0.08	25
C16:1-OH	3	470.4 -> 85.0	MRM	C16-D3	0.08	25
C16	3	456.4 -> 85.0	MRM	C16-D3	0.08	25
C16-D3	3	459.4 -> 85.0	MRM	<None>	0.08	25
C16-OH	3	472.4 -> 85.0	MRM	C16-D3	0.08	25
C18:1	3	482.4 -> 85.0	MRM	C16-D3	0.08	25
C18:1-OH	3	498.4 -> 85.0	MRM	C16-D3	0.08	25
C18:2	3	480.4 -> 85.0	MRM	C16-D3	0.08	25
C18:2-OH	3	496.4 -> 85.0	MRM	C16-D3	0.08	25
C18	3	484.4 -> 85.0	MRM	C16-D3	0.08	25
C18-OH	3	500.4 -> 85.0	MRM	C16-D3	0.08	25
C2	3	260.2 -> 85.0	MRM	C2-D3	0.19	25
C2-D3	3	263.2 -> 85.0	MRM	<None>	0.19	25
C3	3	274.2 -> 85.0	MRM	C3-D3	0.04	25
C3-D3	3	277.2 -> 85.0	MRM	<None>	0.04	25
C3-DC	3	360.4 -> 85.0	MRM	C3-D3	0.04	25
C4	3	288.2 -> 85.0	MRM	C4-D3	0.04	25
C4-D3	3	291.2 -> 85.0	MRM	<None>	0.04	25
C4-DC	3	374.3 -> 85.0	MRM	C8-D3	0.04	25
C4-OH	3	304.4 -> 85.0	MRM	C4-D3	0.04	25
C5:1	3	300.3 -> 85.0	MRM	C5-D9	0.04	25
C5	3	302.2 -> 85.0	MRM	C5-D9	0.04	25
C5-D9	3	311.3 -> 85.0	MRM	<None>	0.04	25
C5-DC	3	388.3 -> 85.0	MRM	C8-D3	0.04	25
C5-OH	3	318.4 -> 85.0	MRM	C5-D9	0.04	25
C6	3	316.3 -> 85.0	MRM	C8-D3	0.04	25
C6-DC	3	402.3 -> 85.0	MRM	C14-D9	0.04	25
C8:1	3	342.3 -> 85.0	MRM	C8-D3	0.04	25
C8	3	344.3 -> 85.0	MRM	C8-D3	0.04	25
C8-D3	3	347.3 -> 85.0	MRM	<None>	0.19	25
Citrulline	3	232.2 -> 113.1	MRM	Citrulline-D2	2.5	9
Citrulline-D2	3	234.2 -> 115.1	MRM	<None>	2.5	9
Glutamic Acid	3	260.2 -> 158.1	MRM	Glutamic Acid-D3	2.5	9
Glutamic Acid-D3	3	263.2 -> 161.2	MRM	<None>	2.5	9
Glycine	3	132.1 -> 76.1	MRM	Glycine-N15-2C13	12.5	4
Glycine-N-15-2C-13	3	134.1 -> 78.1	MRM	<None>	12.5	4
Leucine	3	188.1 -> 86.0	MRM	Leucine-D3	2.5	9
Leucine-D3	3	191.2 -> 89.1	MRM	<None>	2.5	9
Methionine	3	206.2 -> 104.1	MRM	Methionine-D3	2.5	9
Methionine-D3	3	209.2 -> 107.1	MRM	<None>	2.5	9
Ornithine	3	189.2 -> 70.1	MRM	Ornithine-D2	2.5	14
Ornithine-D2	3	191.2 -> 72.1	MRM	<None>	2.5	14
Phenylalanine	3	222.2 -> 120.1	MRM	Phenylalanine-6C13	2.5	9
Phenylalanine-6C-13	3	228.2 -> 126.1	MRM	<None>	2.5	9
Tyrosine	3	238.2 -> 136.1	MRM	Tyrosine-6C13	2.5	9
Tyrosine-6C-13	3	244.2 -> 142.1	MRM	<None>	2.5	9
Valine	3	174.2 -> 72.1	MRM	Valine-D8	2.5	9
Valine-D8	3	182.2 -> 80.1	MRM	<None>	2.5	9

Results and Discussion

Quantification and qualitative confirmation

This method provided quantitative and qualitative data within a 2 minute analysis time. The concentration of each analyte was determined from the MRM data using the known concentration of the internal isotopic standard and the calculation shown in **Figure 8**. In order to determine the accuracy of quantification, CDC reference standards for six amino acids and eleven acylcarnitines were quantified using MRM and the determined concentrations were plotted against the actual concentrations of the standards. **Tables 6** and **7**, as well as **Figure 9**, show that the R^2 values for these plots were all very close to 1, as were their slopes, indicating very high accuracy for the MRM quantification method.

Acylcarnitine	Y-Intercept (nmol/mL)	Slope	R^2
C0-Carnitine	2.9040	0.848	0.976
C2-Carnitine	0.7100	1.090	0.981
C3-Carnitine	0.2200	0.910	0.994
C4-Carnitine	-0.0099	0.903	0.998
C5-Carnitine	0.0032	1.053	0.988
C6-Carnitine	0.0019	0.997	0.988
C8-Carnitine	0.0406	0.919	0.978
C10-Carnitine	0.0352	0.970	0.977
C14-Carnitine	0.0517	0.813	0.990
C16-Carnitine	0.0254	0.941	0.986
C18-Carninite	0.0598	0.812	0.995

Table 6. Data from the Statistical Plot of Actual Concentrations of Acylcarnitines vs. Calculated Concentrations

Quantification of target analytes

$C_{analyte} = \frac{I_{analyte} \times V_{ex} \times C_{IS}}{V_{BS} \times I_{IS} \times RRF}$	
Where:	
$C_{analyte} =$	Target analyte concentration (mM)
$I_{analyte} =$	Target analyte intensity (counts per second)
$V_{ex} =$	Specimen extraction volume (mL)
$C_{IS} =$	Internal standard concentration (mM)
$V_{BS} =$	Volume of blood contained in a punch (3.1 µL for a 3.2 mm punch size at 50% hematocrit)
$I_{IS} =$	Internal standard intensity (counts per second)
RRF =	Extraction efficiency

Figure 8. Calculation of target analyte concentration.

Amino Acid	Y-Intercept (nmol/mL)	Slope	R^2
Phenylalanine	13.44	0.9917	0.987
Tyrosine	25.99	0.9266	0.992
Leucine	23.74	0.8950	0.989
Methionine	5.80	0.9770	0.990
Valine	15.90	0.8550	0.970
Citrulline	4.30	0.9700	0.992

Table 7. Data from the Statistical Plot of Actual Concentrations of Amino Acids vs. Calculated Concentrations

Excellent correlation between actual and MRM determined acylcarnitine and amino acid concentrations

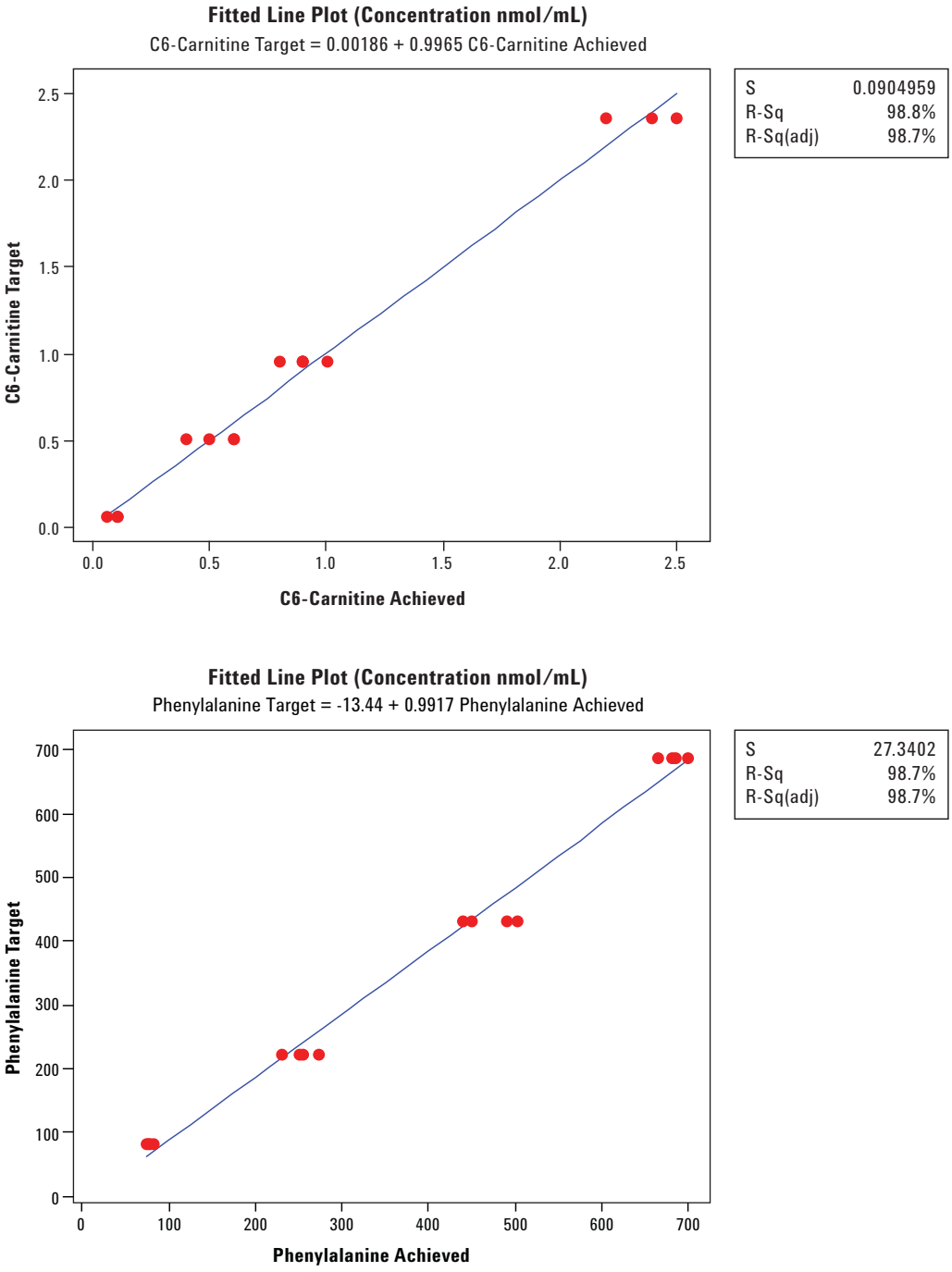


Figure 9. Statistical plots of C6-carnitine assay performance (upper panel) and phenylalanine assay performance (lower panel).

In addition to quantification, there can be a need for qualitative information on the relative levels of amino acids and acylcarnitines. This information is provided by precursor ion and neutral loss scans, such as that shown in Figures 3 and 5. The data is acquired in scan mode for all the acylcarnitines and eight of the amino acids. As a routine procedure, acquired MRM data is used for the quantification and custom reporting, but precursor ion scan and neutral loss scan data can also be used to quantify the acylcarnitines and amino acids. This is provided as an option to the researcher, in order to eliminate the possibility of misinterpreting the data due to chemical interferences (**Table 8**).

Compound	Segment	Transition	Scan	Analyte	Precurosor	Product
Alanine	2	146.1 -[102.1] -> 44.0	Neutral Loss	Target	146.1	44
Alanine-D4	2	150.1 -[102.1] -> 48.0	Neutral Loss	ISTD	150.1	48
Valine (nl)	2	174.1 -[102.1] -> 72.0	Neutral Loss	Target	174.1	72
Valine-D8 (nl)	2	182.1 -[102.1] -> 80.0	Neutral Loss	ISTD	182.1	80
Leucine (nl)	2	188.1 -[102.1] -> 86.0	Neutral Loss	Target	188.1	86
Leucine-D3 (nl)	2	191.1 -[102.1] -> 89.0	Neutral Loss	ISTD	191.1	89
Methionine (nl)	2	206.2 -[102.1] -> 104.1	Neutral Loss	Target	206.2	104.1
Methionine-D3 (nl)	2	209.2 -[102.1] -> 107.1	Neutral Loss	ISTD	209.2	107.1
Phenylalanine (nl)	2	222.2 -[102.1] -> 120.1	Neutral Loss	Target	222.2	120.1
Phenylalanine-6C-13 (nl)	2	228.2 -[102.1] -> 126.1	Neutral Loss	ISTD	228.2	126.1
Glycine (nl)	2	132.1 -[56.0] -> 76.1	Neutral Loss	Target	132.1	76.1
Tyrosine (nl)	2	238.2 -[102.1] -> 136.1	Neutral Loss	Target	238.2	136.1
Tyrosine-6C-13 (nl)	2	244.2 -[102.1] -> 142.1	Neutral Loss	ISTD	244.2	142.1
Aspartic Acid (nl)	2	246.2 -[102.1] -> 144.1	Neutral Loss	Target	246.2	144.1
Aspartic Acid-D3 (nl)	2	249.2 -[102.1] -> 147.1	Neutral Loss	ISTD	249.2	147.1
Glutamic Acid (nl)	2	260.2 -[102.1] -> 158.1	Neutral Loss	Target	260.2	158.1
Glutamic Acid-D3 (nl)	2	263.2 -[102.0] -> 161.2	Neutral Loss	ISTD	263.2	161.2
Phenylalanine/Tyrosine (nl)	2	222.2 -[102.1] -> 120.1	Neutral Loss	Target	222.2	120.1
Methionine/Phenylalanine (nl)	2	206.2 -[102.1] -> 104.1	Neutral Loss	Target	206.2	104.1
Glycine-N-15-2C-13 (nl)	2	134.1 -[56.0] -> 78.1	Neutral Loss	ISTD	134.1	78.1
Leucine/Phenylalanine (nl)	2	188.1 -[102.1] -> 86.0	Neutral Loss	Target	188.1	86
Citrulline (nl)	2	232.2 -[119.1] -> 113.1	Neutral Loss	Target	232.2	113.1
Aspartic Acid/Arginine (nl)	2	246.2 -[102.1] -> 144.1	Neutral Loss	Target	246.2	144.1
Citrulline-ISTD (nl)	2	234.2 -[119.1] -> 115.1	Neutral Loss	ISTD	234.2	115.1
Ornithine (nl)	2	189.2 -[119.1] -> 70.1	Neutral Loss	Target	189.2	70.1
Ornithine-ISTD (nl)	2	191.2 -[119.1] -> 72.1	Neutral Loss	ISTD	191.2	72.1
Arginine (nl)	2	231.2 -[161.1] -> 70.1	Neutral Loss	Target	231.2	70.1
Arginine-ISTD (nl)	2	236.2 -[161.1] -> 75.1	Neutral Loss	ISTD	236.2	75.1
C0 (prec)	1	218.3 -> 85.0	Precursor Ion	Target	218.3	85
C2 (prec)	1	260.3 -> 85.0	Precursor Ion	Target	260.3	85
C3 (prec)	1	274.3 -> 85.0	Precursor Ion	Target	274.3	85
C4 (prec)	1	288.3 -> 85.0	Precursor Ion	Target	288.3	85
C5:1 (prec)	1	300.3 -> 85.0	Precursor Ion	Target	300.3	85
C5 (prec)	1	302.3 -> 85.0	Precursor Ion	Target	302.3	85
C4-OH (prec)	1	304.4 -> 85.0	Precursor Ion	Target	304.4	85
C6 (prec)	1	316.4 -> 85.0	Precursor Ion	Target	316.4	85
C5-OH (prec)	1	318.4 -> 85.0	Precursor Ion	Target	318.4	85
C8:1 (prec)	1	342.4 -> 85.0	Precursor Ion	Target	342.4	85
C8 (prec)	1	344.4 -> 85.0	Precursor Ion	Target	344.4	85
C3-DC (prec)	1	360.4 -> 85.0	Precursor Ion	Target	360.4	85
C10:2 (prec)	1	368.4 -> 85.0	Precursor Ion	Target	368.4	85
C10:1 (prec)	1	370.4 -> 85.0	Precursor Ion	Target	370.4	85
C10 (prec)	1	372.4 -> 85.0	Precursor Ion	Target	372.4	85
C4-DC (prec)	1	374.4 -> 85.0	Precursor Ion	Target	374.4	85
C5-DC (prec)	1	388.5 -> 85.0	Precursor Ion	Target	388.5	85
C12:1 (prec)	1	398.5 -> 85.0	Precursor Ion	Target	398.5	85
C12 (prec)	1	400.5 -> 85.0	Precursor Ion	Target	400.5	85
C6-DC (prec)	1	402.5 -> 85.0	Precursor Ion	Target	402.5	85
C12:1-OH (prec)	1	414.5 -> 85.0	Precursor Ion	Target	414.5	85
C12-OH (prec)	1	416.5 -> 85.0	Precursor Ion	Target	416.5	85
C14:2 (prec)	1	424.5 -> 85.0	Precursor Ion	Target	424.5	85
C14:1 (prec)	1	426.5 -> 85.0	Precursor Ion	Target	426.5	85
C14 (prec)	1	428.4 -> 85.0	Precursor Ion	Target	428.4	85
C14:1-OH (prec)	1	442.5 -> 85.0	Precursor Ion	Target	442.5	85
C14-OH (prec)	1	444.5 -> 85.0	Precursor Ion	Target	444.5	85
C16:1 (prec)	1	454.6 -> 85.0	Precursor Ion	Target	454.6	85
C16 (prec)	1	456.6 -> 85.0	Precursor Ion	Target	456.6	85
C16:1-OH (prec)	1	470.6 -> 85.0	Precursor Ion	Target	470.6	85
C16-OH (prec)	1	472.6 -> 85.0	Precursor Ion	Target	472.6	85
C18:2 (prec)	1	480.6 -> 85.0	Precursor Ion	Target	480.6	85
C18:1 (prec)	1	482.6 -> 85.0	Precursor Ion	Target	482.6	85
C18 (prec)	1	484.6 -> 85.0	Precursor Ion	Target	484.6	85
C18:2-OH (prec)	1	496.6 -> 85.0	Precursor Ion	Target	496.6	85
C18:1-OH (prec)	1	498.6 -> 85.0	Precursor Ion	Target	498.6	85
C18-OH (prec)	1	502.6 -> 85.0	Precursor Ion	Target	502.6	85
C0-D9 (prec)	1	227.3 -> 85.0	Precursor Ion	ISTD	227.3	85
C2-D3 (prec)	1	263.2 -> 85.0	Precursor Ion	ISTD	263.2	85
C3-D3 (prec)	1	277.3 -> 85.0	Precursor Ion	ISTD	277.3	85
C4-D3 (prec)	1	291.3 -> 85.0	Precursor Ion	ISTD	291.3	85
C5-D9 (prec)	1	311.3 -> 85.0	Precursor Ion	ISTD	311.3	85
C8-D3 (prec)	1	347.3 -> 85.0	Precursor Ion	ISTD	347.3	85
C14-D9 (prec)	1	437.4 -> 85.0	Precursor Ion	ISTD	437.4	85
C16-D3 (prec)	1	459.6 -> 85.0	Precursor Ion	ISTD	459.6	85
C14:1/C12:1 (prec)	1	426.5 -> 85.0	Precursor Ion	Target	426.5	85
C14:1/C16 (prec)	1	426.5 -> 85.0	Precursor Ion	Target	426.5	85
C14:1/C2 (prec)	1	426.5 -> 85.0	Precursor Ion	Target	426.5	85
C16/C2 (prec)	1	456.6 -> 85.0	Precursor Ion	Target	456.6	85
C3/C0 (prec)	1	274.3 -> 85.0	Precursor Ion	Target	274.3	85
C3/C2 (prec)	1	274.3 -> 85.0	Precursor Ion	Target	274.3	85
C3-DC/C10 (prec)	1	360.4 -> 85.0	Precursor Ion	Target	360.4	85
C4/C2 (prec)	1	288.3 -> 85.0	Precursor Ion	Target	288.3	85
C4/C3 (prec)	1	288.3 -> 85.0	Precursor Ion	Target	288.3	85
C5/C3 (prec)	1	302.3 -> 85.0	Precursor Ion	Target	302.3	85
C5-DC/C8 (prec)	1	388.5 -> 85.0	Precursor Ion	Target	388.5	85
C5-OH/C8 (prec)	1	318.4 -> 85.0	Precursor Ion	Target	318.4	85
C8/C10 (prec)	1	344.4 -> 85.0	Precursor Ion	Target	344.4	85
C8/C2 (prec)	1	344.4 -> 85.0	Precursor Ion	Target	344.4	85
Ornithine-ISTD (nl)	2	191.2 -[119.1] -> 72.1	Neutral Loss	ISTD	191.2	72.1
Arginine (nl)	2	231.2 -[161.1] -> 70.1	Neutral Loss	Target	231.2	70.1

Table 8. Neutral Loss Scan and Precursor Ion Scan Acquisition Parameters

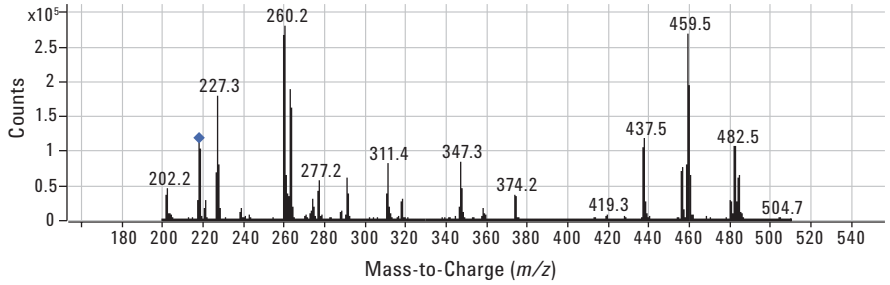
Custom reporting of results

The Agilent MassHunter software can be customized to generate reports that display the concentrations and scan spectra for the amino acids and acylcarnitines in the DBS sample, providing the researcher with the information required to determine the presence of inborn errors of metabolism (**Figure 10**). The scan spectra provide characteristic patterns for the amino acids and acylcarnitines that can be visually observed for interferences and abnormal relative levels of any of the analytes. In addition, the custom report provides flags for abnormal analyte levels and calculates the concentration ratios for the analytes required by the researcher.

Custom reports provide quantitative and confirmatory qualitative information

Instrument	Instrument 1	**Sample Name**	NBS-1-96-AC
Operator		**Data File**	NBS-1-961-AC.d
Inj. Volume		**Acq Method**	100119-NBS-SCN-MRM-ALL-CB2.m
Position	P1-A1	**Acq Time**	1/20/2010 16:08
Dilution	1		

+ Precursor Ion (0.045-0.414 min, 44 scans) (** -> 85.0) NBS-1-961-AC.d



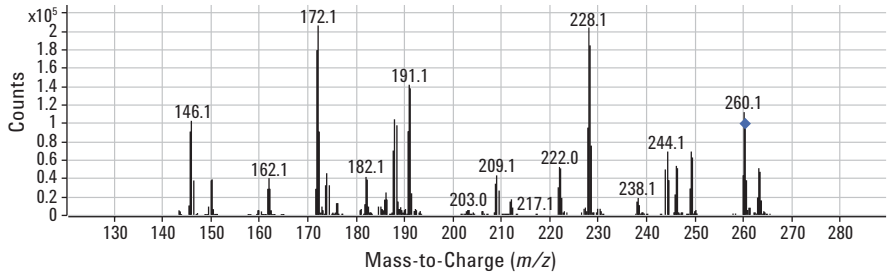
Compound	Result	Normal Range	Flag	Response	ISTD	ISTD Response
C3	0.0 nmol/mL	0-0.98	Low	277341	C3-D3	387052
C4	0.0 nmol/mL	0-0.16	Low	119872	C4-D3	635182
C5:1	0.0 nmol/mL	0-1.3	Low	2810	C5-D9	868554
C4-OH	0.0 nmol/mL	0.0-1.0	Low	23665	C4-D3	635182
C6	1.0 nmol/mL	0-0.08	High	50651	C8-D3	990593
C5-OH	0.0 nmol/mL	0-0.7	Low	285403	C5-D9	868554

Figure 10. Custom reports for DBS acylcarnitines (upper panel), and amino acids (lower panel). These reports display the spectrum profiles and quantification for 12 amino acids and 37 acylcarnitines. The researcher establishes the normal ranges and enters the normal values in the MassHunter Quantification Method. The custom report then displays each result as normal or flags it as low or high. The quantification number for each peak is displayed on the spectrum profile.

Batch Data Path
C:\MassHunter\data\QQQ\Mt Sinai\100120\QuantResults\NBS003 One Sample.batch.bin

Instrument	Instrument 1	**Sample Name**	NBS-1-96-AC
Operator		**Data File**	NBS-1-961-AC.d
Inj. Volume		**Acq Method**	100119-NBS-SCN-MRM-ALL-CB2.m
Position	P1-A1	**Acq Time**	1/20/2010 16:08
Dilution	1		

+ Neutral Loss (0.547-0.826 min, 33 scans) NL NBS-1-961-AC.d



Compound	Result	Normal Range	Flag	Response	ISTD	ISTD Response
Methionine	0.3 nmol/mL	0.3-15.0	Low	135296	Methionine-D3	1218973
Arginine	18.0 nmol/mL	0.3-15.0	High	50716	Arginine-D4-5C13	1752101

Compound	Result	Normal Range	Flag	Response	ISTD	ISTD Response
Leucine/ Phenylalanine	2.4	0.3-1.5	High			
Citrulline/ Arginine	4.7	0.3-1.5	High			

Conclusions

A method has been demonstrated for the accurate detection and quantification of the amino acids and acylcarnitines that are analyzed in dried blood spots. The assay provides a run time of 1.6 minutes, and the ability to analyze 400–500 samples per day. Custom reports provide all the information needed to rapidly and simply show all relevant data. The assay has the option to verify the MRM quantification of the selected samples with the acquired scan data for all the acylcarnitines and eight of the twelve amino acids determined by MRM (only eight exhibit a neutral loss of 102 *m/z*). The Universal Integrator of the MassHunter software is capable of detecting any concentrations of amino acids and acylcarnitines, so that no false negative results are reported. In fact, no false negative results were observed in over one thousand DBS samples analyzed using this method.

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2. D. H. Chase, J. C. DiPerna, B. L. Michell, B. Sgroi, L. F. Hoffman, E. W. Naylor, "Electrospray Tandem Mass Spectrometry for Analysis of Acylcarnitines in Unexplained Cause of Death," *Clin. Chem.* 47:1166-1182, **2001**.

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Published in the U.S.A. July 20, 2010
5990-6036EN

Agilent Technologies