Technical note | 002655

Clinical research

# Enhanced disease detection in newborns

## Quantitation of amino acids and acylcarnitines in dried blood spots by FIA-Orbitrap mass spectrometry

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**Keywords**

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### Goal

Develop a flow injection analysis, high-resolution accurate-mass, $MS^2$ mass spectrometric method to quantitate 12 amino acids and 13 acylcarnitines in dried blood spots using a hybrid Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer, which improves detection confidence when implemented in public health laboratories performing primary screening for inherited metabolic disorders in newborns in clinical research.

### Introduction

The screening of newborns for inborn errors of metabolism (IEM) is a government-sponsored public health program aiming to identify asymptomatic newborns for rare and severe inherited genetic disorders to prevent unnecessary suffering for newborns and their families. This program is considered the largest and most successful disease prevention system in the United States and saves or improves the lives of over 12,000 babies a year.[1]

The quantitation of derivatized or non-derivatized amino acids and acylcarnitines from dried blood spots (DBS) via flow injection analysis – triple quadrupole tandem mass spectrometry (FIA-QqQ-MS/MS) serves as the main first-tier testing method.[2] Flow-injection mass spectrometry is fast and offers the lab analyst high sample throughput, as it is direct injection of samples into the mass spectrometer without requiring liquid chromatographic separation. The positive test results are usually followed by the second-tier tests with more sensitive methodologies of the same analytes or more specific biomarkers on the same DBS samples before reporting to the clinician to perform confirmative tests for the diseases. This method can detect all the metabolic disorders of organic acid, fatty acid oxidation, and amino acid listed on the Recommended Uniform Screening Panel (RUSP, as of Jan 2023) for which the US Department of Health and Human Services recommends local public health laboratories screen.[3]

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Although triple quadrupole (QqQ) MS provides robustness and sensitivity when quantitating analytes in selected reaction monitoring (SRM) mode, the assay is incapable of separating nominal mass isobaric compounds with similar SRM transitions in the absence of liquid chromatography separation, as in the cases of FIA-QqQ-MS/MS. For example, the nominal mass isobars leucine (Leu, $C_6H_{13}NO_2$, $[M+H]^+$ *m/z* 132.1019) and 4-hydroxyproline (4-OHPro, $C_5H_9NO_3$, $[M+H]^+$ *m/z* 132.0655) utilize the same SRM transition 132 → 86, where the fragment ions are also isobars: Leu fragment $C_5H_{12}N^+$ *m/z* 86.0964, and 4-OHPro, $C_4H_8NO^+$, *m/z* 86.0600.[2,4] Both the precursor and fragment ions are 0.03 Da apart, which cannot be distinguished by QqQ MS.

High-resolution accurate-mass (HRAM) mass spectrometers, such as Orbitrap™ MS, detect ions with low to sub-ppm mass accuracy, enabling the separation of isobaric analyte precursors or fragment ions otherwise indistinguishable by FIA-QqQ-MS/MS. Hybrid Orbitrap mass spectrometers, such as the Thermo Scientific™ Q Exactive™ mass spectrometers and Orbitrap Exploris mass spectrometers, also record the entire full scan $MS^2$ fragmentation spectra after high energy collision-induced dissociation. The $MS^2$ spectra are used for library matching to improve the analyte identification confidence. An Orbitrap Exploris mass spectrometer routinely operates at a sufficiently high resolution of 15,000 (*m/z* 200 at full-width at half maximum, FWHM), which can easily distinguish Leu and 4-OHPro at both the precursor and fragment ion levels. Figure 1 shows the isotope simulation of the precursor and fragment ions of Leu and 4-OHPro under nominal mass resolution (employed by a QqQ mass spectrometer), resolutions needed to achieve the 5% valley separation, and the typical resolutions utilized in the full-MS and $MS^2$ scan events in Orbitrap Exploris MS.

In this report, an FIA-HRAM-MS/MS method for clinical research was developed using a Thermo Scientific™ Vanquish™ Flex UHPLC and Orbitrap Exploris 120 mass spectrometer. The quality control dried blood spot samples, reagents, and consumables used were ClinSpot™ LC-MS/MS Complete Kits, Amino Acids and Acylcarnitines in Dried Blood Spots (DBS) – non-derivatized (Ref MS10200, RECIPE Chemicals + Instruments GmbH, Germany).

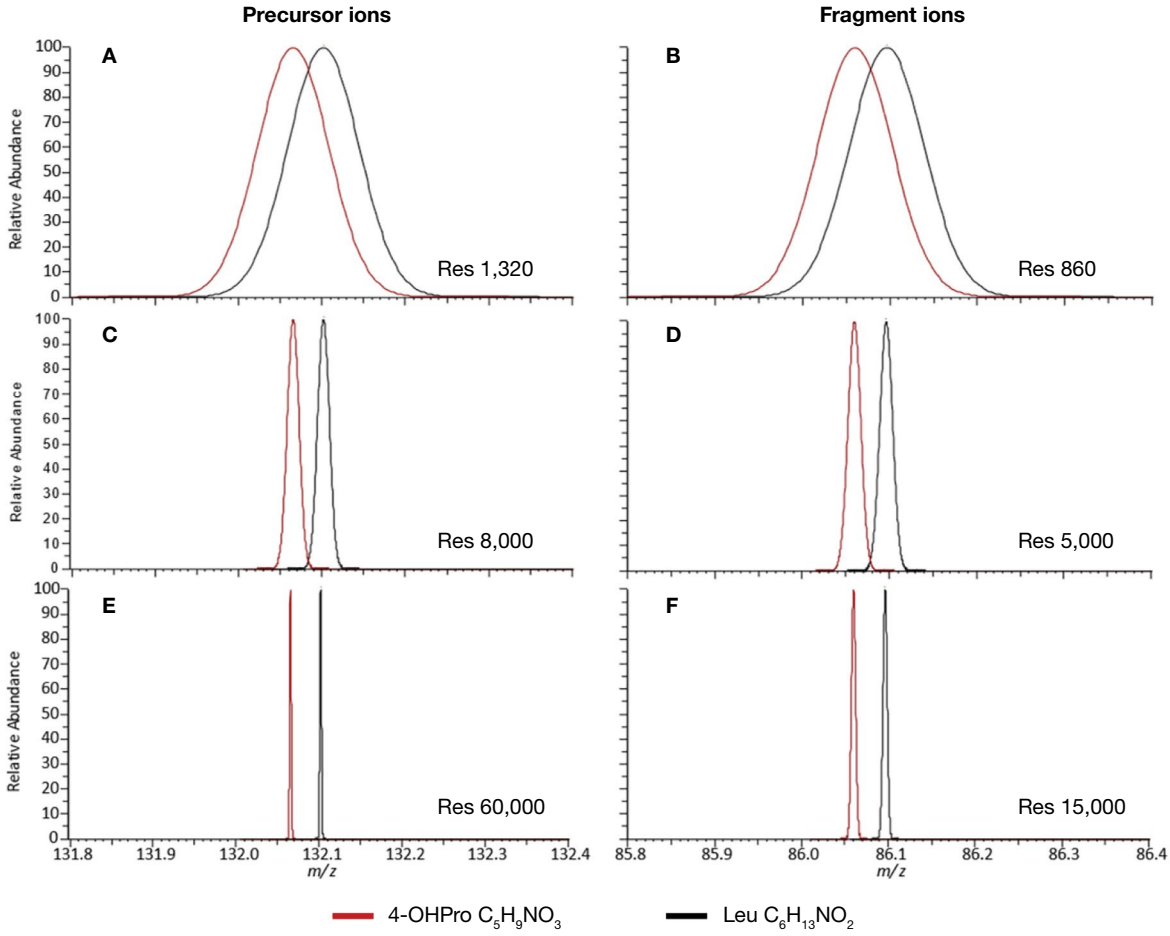


**Figure 1. Simulation of the $[M+H]^+$ monoisotopic peaks of Leu and 4-OHPro precursor and their fragment ions under (A, B) nominal mass resolution employed by a QqQ MS, (C, D) a resolution that achieves 5% valley separation, and (E, F) the resolution utilized by the Orbitrap Exploris 120 mass spectrometer for the full-MS and $MS^2$ scan events**

The measured levels of the 12 amino acids and 13 acylcarnitines from DBS samples were highly reproducible with good accuracy and precision. The results were comparable with those previously obtained using a Thermo Scientific™ Vanquish™ MD HPLC and Thermo Scientific™ TSQ Quantis™ MD mass spectrometer (TN001293)[5] but with higher levels of confidence due to the low ppm mass accuracy and good spectra library and fragment matching results. In addition, the full MS spectra over the *m/z* ranges of common metabolites were recorded for retrospective analysis for novel biomarker discovery. High-resolution mass spectrometry methods, such as those employing Orbitrap MS, have the potential to be implemented in routine IEM screening laboratories for newborns to improve the detection accuracy over FIA-QqQ-MS/MS and perform screening of metabolite profiles.

## Experimental

### Sample preparation

The ClinMass™ Internal Standard (IS, Ref MS10012A, Lot 2412) was reconstituted using Reagent A (Ref MS10021, Lot 2013) following the instruction of the ClinSpot LC-MS/MS Complete Kits, Amino Acids and Acylcarnitines in Dried Blood Spots (DBS) – non-derivatized (Ref MS10200). This solution was used to extract analytes from ClinChek™ – Control Dried Blood Spot (DBS) (Ref MS10182, Lot 2382), which came with two control levels (Levels I and II). A 3.2 mm disc was punched from the DBS control cards and placed in a 96/370 µL well plate (Ref MS10040). To extract the analytes, 100 µL of the reconstituted IS solution was added to the well plate, which was sealed by the PE/PP protective sheet (Ref MS10042) and left on an Eppendorf™ ThermoMixer™ mixing device with agitation at 700 rpm at room temperature. After 30 min, the extract was transferred to another 96/370 µL well plate and sealed by the PE/PP protective sheet for FIA-MS analysis. For the inter-day and intra-day precision measurements, each control level was prepared five times over three days.

### Liquid chromatography

A Vanquish Flex UHPLC system was conditioned with the provided mobile phase (Ref MS10010, Lot 2063) for at least 10 minutes prior to sample injection. Each sample was injected twice at an injection volume of 5 µL. The flow rate and the rest of the LC gradient is specified in Table 1. The sample needle was washed before and after each injection using the provided needle wash solution (Ref MS10005, Lot 2063).

**Table 1. Vanquish Flex UHPLC gradient**

| Time (min) | Flow rate (mL/min) | Mobile phase (Ref MS10010) (%) |
|---|---|---|
| 0.00 | 0.05 | 100 |
| 0.70 | 0.05 | 100 |
| 0.71 | 0.50 | 100 |
| 0.89 | 0.50 | 100 |
| 0.90 | 0.10 | 100 |
| 1.00 | 0.10 | 100 |

### Mass spectrometry

The analyte detection was achieved using the Orbitrap Exploris 120 mass spectrometer. $tMS^2$ scan events were used for all analytes, except for Gly and $^{13}C,^{15}N$-Gly, whose sole fragment ions had *m/z* values below the lowest *m/z* detection range (*m/z* 40) of the Orbitrap mass spectrometer (*m/z* 30.0338 for Gly and *m/z* 32.0342 for $^{13}C,^{15}N$-Gly). Thus, Gly was quantitated in the full-MS scan with the precursor ion. The mass spectrometer was equipped with a Thermo Scientific™ OptaMax™ NG ion source with a heated electrospray ionization probe in the positive mode. The MS source parameters and scan event properties are listed in Table 2 and Table 3. The precursor and fragment ions *m/z* values of the analytes and their internal standard (IS) are shown in Table 4.

### Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ software (ver 5.1 SP3 Clinical). The data processing parameters for the mzVault library matching, isotope pattern, and fragment matching are shown in Table 5. The mass tolerance for the analyte quantitation was set to 5 ppm.

**Table 2. Orbitrap Exploris 120 mass spectrometer global settings**

| MS source parameters | |
|---|---|
| Ion source type | HESI (OptaMax NG ion source) |
| HESI probe position | Center - 1.0 - L/M (x - y - z) |
| Spray voltage | +3,200 V |
| Sheath gas (Arb) | 50 |
| Aux gas (Arb) | 5 |
| Sweep gas (Arb) | 0 |
| Ion transfer tube temp. (°C) | 325 |
| Vaporizer temp. (°C) | 75 |
| **MS scan properties** | |
| Internal calibrant | EASY-IC, run - start |
| Mild trapping | On |

**Table 3. Orbitrap Exploris 120 mass spectrometer scan event properties**

| | |
|---|---|
| **Scan event 1 tMS²** | |
| Resolution | 15,000 |
| Isolation width (Da) | 0.8 |
| Targeted compound list | See Table 4 |
| RF lens | 60 (precursor *m/z* < 200); 120 (*m/z* > 200) |
| Normalized CE | 40 |
| AGC | 100% |
| Max injection time (ms) | Standard |
| **Scan event 2 full-MS** | |
| Resolution | 60,000 |
| *m/z* range | 70–200 |
| RF lens | 60 |
| AGC | 100% |
| Max injection time (ms) | Standard |
| **Scan event 3 full-MS** | |
| Resolution | 60,000 |
| *m/z* range | 200–450 |
| RF lens | 120 |
| AGC | 100% |
| Max injection time (ms) | Standard |

**Table 5. TraceFinder software data processing settings**

| | |
|---|---|
| **mzVault** | |
| Enabled | Checked |
| Search type | HighChem |
| Prefilter type | Precursor |
| Scan filter | MS² |
| Precursor tolerance | 5 ppm |
| Score threshold | 10 |
| Passing value | 60 |
| Reverse search | Checked |
| **Fragments** | |
| Enabled | Checked |
| Fragment tolerance | 5 ppm |
| Min # of fragments | 1 |
| **Isotopes (for Gly and Gly-IS)** | |
| Enabled | Checked |
| Fit threshold, % | 60 |
| Allowed mass deviation (ppm) | 5 |
| Allowed intensity deviation (%) | 20 |

**Table 4. The precursor and fragment ion *m/z* values of analytes and their internal standards.** The ions used for analyte quantification are indicated with "*", and the mass tolerance for the quantification was set to 5 ppm.

| Analyte | Precursor (*m/z*) | Fragment ions (*m/z*) | IS | Precursor (*m/z*) | Fragment ions (*m/z*) |
|---|---|---|---|---|---|
| Alanine (Ala) | 90.0550 | 44.0495* | $^{13}C_3$,$^{15}$N-Ala | 94.0621 | 47.0532* |
| Arginine (Arg) | 175.1190 | 77.0651*, 60.0556, 116.0706, 130.0975 | $^{13}C_6$-Arg | 181.1391 | 74.0786*, 61.0590, 121.0873, 135.1143 |
| Aspartate (Asp) | 134.0448 | 88.0393*, 74.0237 | $^{13}C_4$-Asp | 138.0582 | 76.0304, 91.0494* |
| Citrulline (Cit) | 176.1035 | 159.0764*, 113.0709, 70.0651 | $^{2}H_7$-Cit | 183.1469 | 166.1204*, 120.1149, 77.1091 |
| Glutamate (Glu) | 148.0604 | 102.055*, 84.0444, 130.0499 | $^{13}C_5$-Glu | 153.0772 | 106.0684*, 88.05781, 135.0666 |
| Glycine (Gly) | 76.0393* | | $^{13}$C,$^{15}$N-Gly | 78.0397* | |
| Leucine (Leu) | 132.1019 | 86.0964* | $^{2}H_3$-Leu | 135.1207 | 89.1153* |
| Methionine (Met) | 150.0583 | 104.0528*, 61.0106, 56.0495, 133.0318 | $^{2}H_3$-Met | 153.0772 | 107.0717*, 64.0295, 56.0495, 136.0506 |
| Ornithine (Orn) | 133.0972 | 116.0706*, 70.0651 | $^{2}H_6$-Orn | 139.1348 | 122.1083*, 76.1028 |
| Phenylalanine (Phe) | 166.0863 | 120.0808* | $^{13}C_6$-Phe | 172.1064 | 126.1009* |
| Proline (Pro) | 116.0706 | 70.0651* | $^{13}C_5$-Pro | 121.0874 | 74.0785* |
| Tyrosine (Tyr) | 182.0812 | 136.0757*, 91.0542, 123.0441, 165.0546 | $^{13}C_6$-Tyr | 188.1013 | 142.0958*, 97.0744, 129.0642, 171.0748 |
| Valine (Val) | 118.0863 | 72.0808* | $^{2}H_8$-Val | 126.1365 | 80.1310* |
| Carnitine (C0) | 162.1125 | 85.0284*, 60.0808, 103.0390 | $^{2}H_9$-C0 | 171.1690 | 85.0284*, 69.1373, 103.0390 |
| Acetylcarnitine (C2) | 204.1230 | 85.0284*, 60.0808, 145.0495 | $^{2}H_3$-C2 | 207.1419 | 85.0284*, 63.0996, 145.0495 |
| Propionylcarnitine (C3) | 218.1387 | 85.0284*, 60.0808, 159.0652 | $^{2}H_3$-C3 | 221.1575 | 85.0284*, 63.0996, 159.0652 |
| Butyrylcarnintine (C4) | 232.1543 | 85.0284*, 60.0808, 173.0808 | $^{2}H_3$-C4 | 235.1732 | 85.0284*, 63.0996, 173.0808 |
| Isovalerylcarnitine (C5) | 246.1700 | 85.0284*, 60.0808, 187.0965 | $^{2}H_9$-C5 | 255.2265 | 85.0284*, 69.1373, 187.0965 |
| Glutarylcarnitine (C5DC) | 276.1442 | 85.0284*, 60.0808 | $^{2}H_9$-C5DC | 285.2007 | 85.0284*, 69.1373 |
| Hexanoylcarnitine (C6) | 260.1856 | 85.0284*, 60.0808, 201.1121 | $^{2}H_3$-C6 | 263.2045 | 85.0284*, 63.0996, 201.1121 |
| Octanoylcarnitine (C8) | 288.2169 | 85.0284*, 60.0808, 229.1434 | $^{2}H_3$-C8 | 291.2358 | 85.0284*, 63.0996, 229.1434 |
| Decanoylcarnitine (C10) | 316.2482 | 85.0284*, 60.0808, 257.1747 | $^{2}H_3$-C10 | 319.2671 | 85.0284*, 63.0996, 257.1747 |
| Dodecanoylcarnitine (C12) | 344.2795 | 85.0284*, 60.0808, 285.2060 | $^{2}H_3$-C12 | 347.2984 | 85.0284*, 63.0996, 285.2060 |
| Tetradecanoylcarnitine (C14) | 372.3108 | 85.0284*, 60.0808, 313.2373 | $^{2}H_3$-C14 | 375.3297 | 85.0284*, 63.0996, 313.2373 |
| Hexadecanoylcarnitine (C16) | 400.3421 | 85.0284*, 60.0808, 341.2686 | $^{2}H_3$-C16 | 403.3610 | 85.0284*, 63.0996, 341.2686 |
| Octadecanoylcarnitine (C18) | 428.3734 | 85.0284*, 60.0808, 369.3000 | $^{2}H_3$-C18 | 431.3923 | 85.0284*, 63.0996, 369.3000 |

## Results and discussion

The data shown was developed from a FIA-HRAM-MS/MS method with a Vanquish Flex UHPLC and an Orbitrap Exploris 120 mass spectrometer to quantitate 12 amino acids and 13 acylcarnitines using the ClinSpot LC-MS/MS Complete Kits, Amino Acids and Acylcarnitines in Dried Blood Spots (DBS) (non-derivatized). Using data generated by an Orbitrap Exploris 120 mass spectrometer, quantitation can be performed at either the precursor ion level in full-MS scan events or at the fragment ion level in the targeted $MS^2$ ($tMS^2$) scan event. For the purpose of easy method setup, we decided to perform the analyte quantitation in one scan event and use default parameters whenever possible. The selection of the optimum RF lens setting is crucial for efficient ion transmission from ion source to mass analyzers. We chose an RF lens setting of 60 for precursors within *m/z* 70 to 200, and 120 for those *m/z* 200 to 450; these two values were optimum for transferring different *m/z* ranges of analytes. When attempting to quantitate analytes in the precursor ion level, we noticed that glutarylcarnitine (C5DC) has an unknown isobar that could not be separated at 60,000 resolution. The presence of the isobar increased the measured level of C5DC by 5-fold compared to performing the quantitation at the $tMS^2$ scan event (data not shown). In addition, the IS of Met and Glu are isobars ($^2H_3$-Met, $C_5[^2H]_3H_8NO_2S$, $[M+H]^+$ *m/z* 153.07716; $^{13}C_6$-Gly, $[^{13}C]^5H_9NO_4$, $[M+H]^+$ *m/z* 153.07721) that require a minimum 3,000,000 resolution to be separated. However, these two compounds can be distinguished from characteristic fragment ions in the $MS^2$ spectra (Table 4). Thus, we chose to perform the analyte quantitation in $tMS^2$ mode for all analytes except Gly, which fragmented into only one ion with a *m/z* value below the Orbitrap Exploris 120 mass spectrometer mass range limit. Two full-MS scans with *m/z* range 70–200 and 200–450 were also recorded for retrospective analysis for novel biomarker discovery.

Figure 2 shows the quantitation results for one analyte, Met, to demonstrate TraceFinder software data processing results. The measured analyte amount ("Calculated Amt"), %RSD (of the calculated amount), %CV (of the peak area), etc. are shown in the Sample Results table window in the TraceFinder view. The extracted ion chromatograms (EIC) of the Met and its IS, $^2H_3$-Met, displayed as "stick," the library matching results, and the fragment matching results are also shown to highlight the confidence for the analyte detection. TraceFinder software uses caution flags to facilitate the data review process. Samples outside the predefined acceptance criteria, such as %RSD, calculated amount ranges, and library matching scores, are flagged. For Gly, which was quantified in the full-MS scan event, a similar plot was shown in Figure 3 to highlight the isotope pattern matching compared to the theoretical calculation, which is calculated by the empirical formula.

The inter- and intra-day precision measurements from three different days with sample preparation performed five times are specified in Table 6 and Table 7, respectively. All the measured values were within the target range listed by the ClinChek – Control Dried Blood Spot (DBS) (Ref MS10182, Lot 2382) specification sheet, except Orn, which was slightly lower. This observation was consistent with the previous work on the verification of the same kit using a Vanquish MD HPLC and TSQ Quantis MD mass spectrometer, where a slight difference in ionization efficiency of the same analyte across MS platforms from different vendors was acknowledged. For all analyte quantitations, the %RSD was below 12%, and the %CV of the IS was below 9%, which supports the fact that the developed method was highly robust and reproducible.

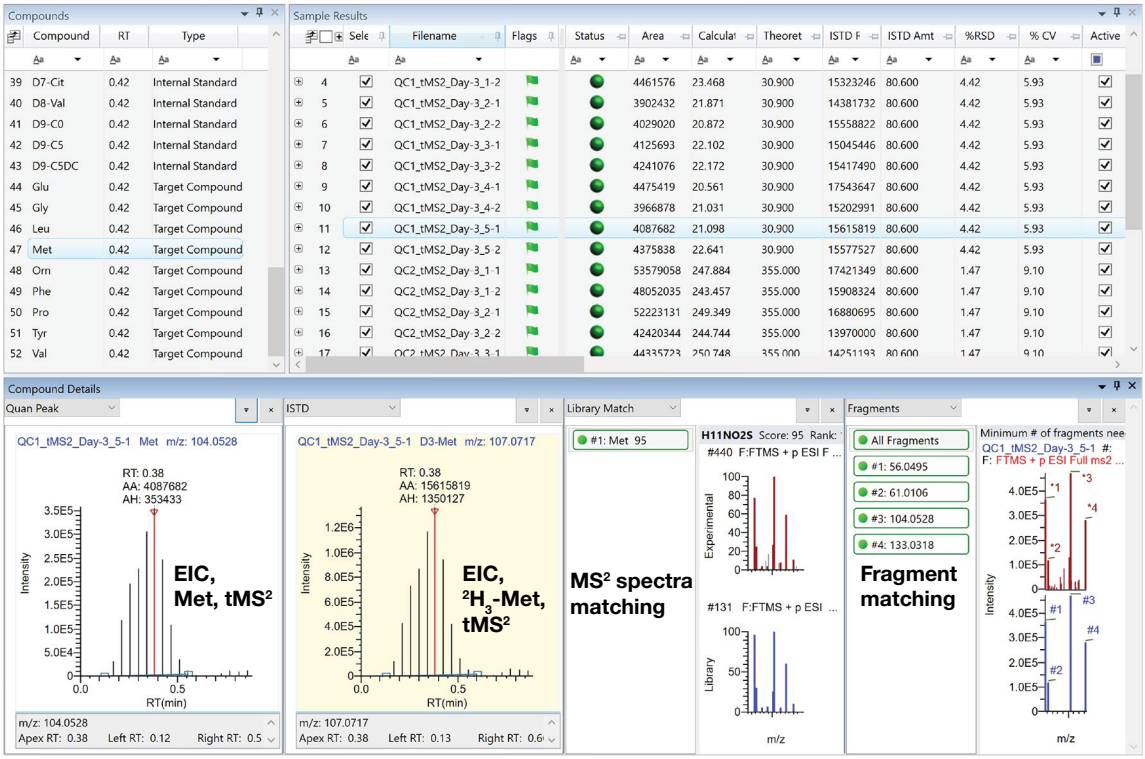


**Figure 2. TraceFinder software interface showing the quantification results of Met in a tMS² scan event with Calculated Amt, %RSD (of the Calculated Amt), and %CV (of the peak area) in the Sample Results table.** The EIC of Met and its IS, $^{2}H_{3}$-Met, used for quantification are shown in the lower left panel, while the matching results of the MS² fragment spectra and fragment ions in red against those from the library or database in blue are shown to the right.

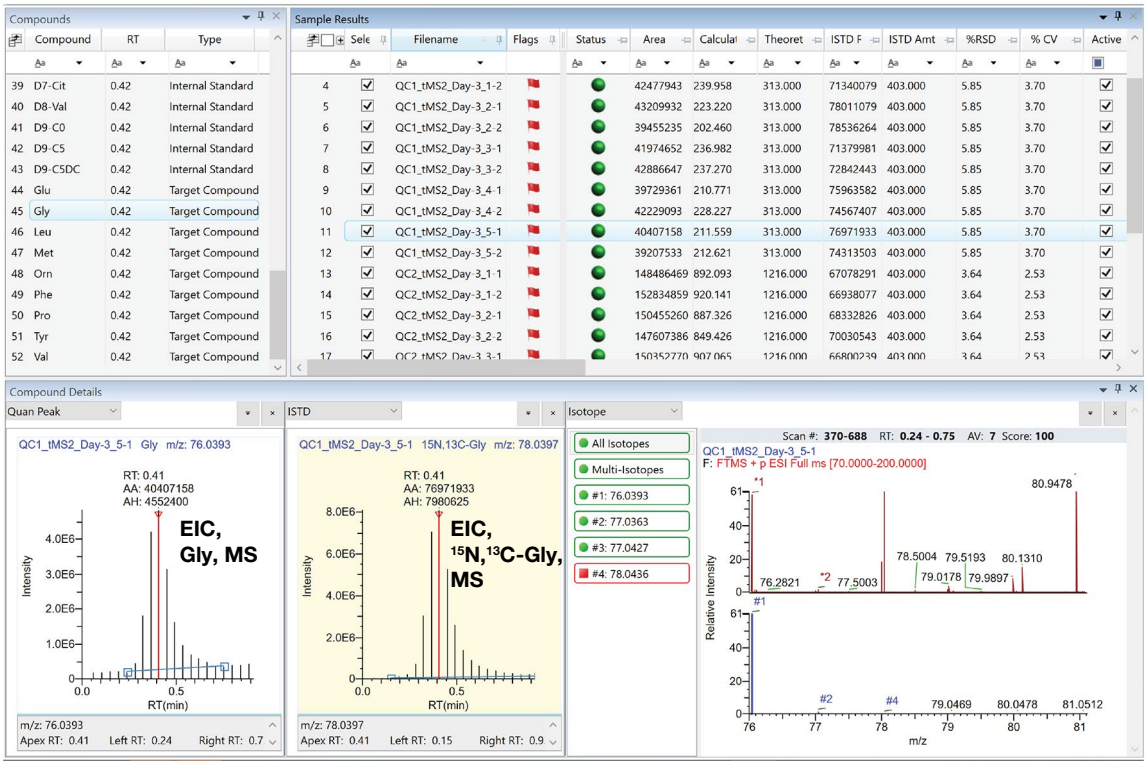


**Figure 3. TraceFinder software interface showing the quantification results of Gly in a full-MS scan event with Calculated Amt,** %RSD (of the Calculated Amt), and %CV (of the peak area) in the Sample Results table. The EIC of Gly and its IS, $^{13}C,^{15}N$-Met, used for quantification are shown in the lower left panel, while the match result of the isotope pattern from the raw file (in red) against the theoretical calculation (in blue) is shown to the right.

Table 6. Inter-day and intra-day precision of the control level – I (acceptance criteria: %CV < 25), and the reproducibility of the IS (N = 30)

| | Control level I | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Day 1 | | Day 2 | | Day-3 | | Inter-day | | IS |
| Analytes | Target | Range | Conc. | %RSD | Conc. | %RSD | Conc. | %RSD | Conc. | %RSD | %CV |
| Ala | 356.00 | 231.00 – 481.00 | 232.55 | 5.50 | 232.93 | 5.25 | 228.64 | 4.35 | 231.37 | 5.03 | 7.08 |
| Arg | 11.60 | 4.05 – 19.10 | 5.94 | 6.50 | 6.03 | 5.23 | 6.18 | 7.13 | 6.05 | 6.28 | 2.94 |
| Asp** | NA | NA | 17.69 | 2.67 | 17.79 | 5.10 | 19.37 | 4.46 | 18.28 | 4.08 | 3.58 |
| Cit | 19.70 | 9.85 – 29.50 | 11.50 | 10.46 | 11.19 | 4.23 | 11.39 | 5.02 | 11.36 | 6.57 | 3.84 |
| Glu | 274.00 | 178.00 – 370.00 | 332.24 | 4.72 | 327.35 | 4.29 | 336.59 | 5.60 | 332.06 | 4.87 | 4.10 |
| Gly | 313.00 | 188.00 – 439.00 | 222.47 | 4.43 | 224.98 | 4.83 | 222.88 | 5.85 | 223.44 | 5.04 | 3.37 |
| Leu | 225.00 | 146.00 – 304.00 | 176.74 | 3.17 | 173.69 | 3.57 | 173.95 | 4.25 | 174.79 | 3.66 | 6.95 |
| Met | 30.90 | 18.50 – 43.20 | 22.10 | 4.49 | 21.88 | 4.26 | 21.87 | 4.42 | 21.95 | 4.39 | 6.41 |
| Orn | 144.00 | 50.40 – 238.00 | 41.65* | 2.45 | 39.11* | 4.48 | 37.94* | 3.87 | 39.57* | 3.60 | 4.00 |
| Phe | 81.80 | 53.20 – 110.00 | 67.32 | 3.72 | 67.03 | 3.91 | 67.48 | 4.24 | 67.28 | 3.96 | 8.49 |
| Pro | 274.00 | 192.00 – 356.00 | 207.72 | 4.10 | 206.03 | 3.54 | 207.28 | 3.42 | 207.01 | 3.68 | 4.51 |
| Tyr | 61.10 | 36.70 – 85.50 | 53.88 | 3.24 | 54.58 | 2.91 | 54.41 | 3.16 | 54.29 | 3.10 | 7.64 |
| Val | 176.00 | 96.90 – 255.00 | 112.41 | 3.34 | 111.49 | 3.70 | 111.45 | 4.26 | 111.78 | 3.77 | 6.05 |
| C0 | 24.10 | 10.80 – 37.30 | 26.85 | 5.44 | 26.43 | 4.24 | 26.47 | 4.67 | 26.58 | 4.78 | 4.18 |
| C2 | 13.40 | 4.71 – 22.20 | 8.37 | 3.02 | 8.34 | 1.93 | 8.43 | 5.23 | 8.38 | 3.39 | 4.27 |
| C3 | 2.38 | 1.31 – 3.46 | 1.64 | 4.33 | 1.61 | 7.53 | 1.64 | 5.26 | 1.63 | 5.71 | 6.74 |
| C4 | 0.80 | 0.52 – 1.08 | 0.57 | 6.40 | 0.60 | 5.48 | 0.60 | 6.74 | 0.59 | 6.21 | 6.65 |
| C5 | 0.45 | 0.25 – 0.65 | 0.29 | 4.73 | 0.28 | 5.59 | 0.28 | 4.97 | 0.28 | 5.10 | 7.35 |
| C5DC | 1.01 | 0.35 – 1.66 | 0.90 | 5.76 | 0.88 | 8.27 | 0.89 | 5.34 | 0.89 | 6.46 | 6.84 |
| C6 | 0.44 | 0.24 – 0.64 | 0.29 | 4.25 | 0.30 | 3.36 | 0.29 | 3.02 | 0.29 | 3.54 | 7.46 |
| C8 | 0.47 | 0.26 – 0.67 | 0.35 | 9.54 | 0.35 | 8.23 | 0.35 | 5.31 | 0.35 | 7.69 | 9.79 |
| C10 | 0.24 | 0.13 – 0.35 | 0.16 | 7.90 | 0.16 | 3.80 | 0.16 | 11.94 | 0.16 | 7.88 | 9.33 |
| C12 | 0.40 | 0.22 – 0.58 | 0.28 | 7.99 | 0.28 | 2.68 | 0.28 | 6.68 | 0.28 | 5.78 | 7.73 |
| C14 | 0.41 | 0.23 – 0.60 | 0.26 | 4.05 | 0.27 | 8.31 | 0.26 | 5.95 | 0.26 | 6.10 | 6.82 |
| C16 | 1.57 | 0.86 – 2.27 | 1.12 | 5.21 | 1.11 | 3.81 | 1.10 | 8.94 | 1.11 | 5.99 | 6.39 |
| C8 | 0.57 | 0.29 – 0.86 | 0.41 | 13.03 | 0.41 | 3.57 | 0.41 | 5.76 | 0.41 | 7.46 | 7.71 |

* The measured Orn level was slightly lower than the target range, which was consistent with the previous measurement of the same kit on a TSQ Quantis MD mass spectrometer.

** Asp target value and ranges were not provided by the kit. NA, not available

Table 7. Inter-day and intra-day precision of the control level – II (acceptance criteria: %CV < 25), and the reproducibility of the IS (N = 30)

| | Control level II | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Day 1 | | Day 2 | | Day-3 | | Inter-day | | IS |
| Analytes | Target | Range | Conc. | %RSD | Conc. | %RSD | Conc. | %RSD | Conc. | %RSD | %CV |
| Ala | 1065.00 | 745.00 – 1384.00 | 678.81 | 3.97 | 659.91 | 5.51 | 690.84 | 5.43 | 676.52 | 4.97 | 6.62 |
| Arg | 102.00 | 40.80 – 163.00 | 53.24 | 4.67 | 53.46 | 5.30 | 54.77 | 3.47 | 53.82 | 4.48 | 2.50 |
| Asp** | NA | NA | 185.97 | 3.31 | 186.42 | 3.40 | 190.37 | 2.48 | 187.59 | 3.06 | 2.62 |
| Cit | 172.00 | 86.00 – 258.00 | 192.63 | 4.33 | 196.38 | 4.30 | 198.02 | 2.72 | 195.68 | 3.78 | 3.27 |
| Glu | 516.00 | 361.00 – 671.00 | 633.93 | 2.28 | 640.91 | 3.91 | 656.76 | 2.68 | 643.87 | 2.95 | 3.08 |
| Gly | 1216.00 | 730.00 – 1703.00 | 890.76 | 4.54 | 869.58 | 6.04 | 892.23 | 3.64 | 884.19 | 4.74 | 3.46 |
| Leu | 626.00 | 407.00 – 845.00 | 498.50 | 3.34 | 506.39 | 4.11 | 500.10 | 1.17 | 501.66 | 2.87 | 6.69 |
| Met | 355.00 | 231.00 – 480.00 | 250.59 | 3.90 | 249.85 | 4.91 | 247.24 | 1.47 | 249.23 | 3.42 | 6.13 |
| Orn | 441.00 | 242.00 – 639.00 | 179.11* | 4.36 | 171.99* | 5.42 | 171.07* | 4.19 | 174.06* | 4.66 | 2.63 |
| Phe | 664.00 | 431.00 – 896.00 | 606.61 | 3.40 | 611.44 | 3.56 | 615.30 | 1.54 | 611.12 | 2.83 | 8.56 |
| Pro | 497.00 | 323.00 – 671.00 | 389.99 | 3.59 | 390.16 | 3.41 | 394.21 | 0.90 | 391.45 | 2.64 | 4.80 |
| Tyr | 491.00 | 319.00 – 663.00 | 441.54 | 3.03 | 448.01 | 2.33 | 441.64 | 1.02 | 443.73 | 2.13 | 7.40 |
| Val | 550.00 | 330.00 – 770.00 | 354.23 | 4.00 | 351.05 | 4.52 | 356.26 | 2.37 | 353.85 | 3.63 | 5.56 |
| C0 | 97.80 | 48.90 – 147.00 | 102.23 | 5.74 | 102.33 | 2.78 | 102.38 | 2.78 | 102.31 | 3.77 | 4.68 |
| C2 | 102.00 | 41.00 – 164.00 | 65.30 | 3.76 | 65.55 | 3.32 | 65.50 | 2.82 | 65.45 | 3.30 | 4.69 |
| C3 | 15.20 | 9.86 – 20.50 | 10.35 | 2.40 | 10.16 | 4.27 | 10.54 | 2.07 | 10.35 | 2.91 | 5.66 |
| C4 | 8.52 | 5.96 – 11.10 | 6.10 | 3.33 | 6.09 | 6.33 | 6.22 | 3.56 | 6.14 | 4.41 | 6.14 |
| C5 | 1.97 | 1.18 – 2.76 | 1.44 | 6.93 | 1.44 | 3.51 | 1.48 | 4.64 | 1.45 | 5.02 | 7.68 |
| C5DC | 2.79 | 1.12 – 4.46 | 1.68 | 6.19 | 1.61 | 7.23 | 1.63 | 3.68 | 1.64 | 5.70 | 9.06 |
| C6 | 1.12 | 0.73 – 1.51 | 0.81 | 3.31 | 0.81 | 3.70 | 0.80 | 4.04 | 0.81 | 3.68 | 8.65 |
| C8 | 2.30 | 1.27 – 3.34 | 1.83 | 2.95 | 1.78 | 8.45 | 1.86 | 5.35 | 1.83 | 5.58 | 9.63 |
| C10 | 1.01 | 0.56 – 1.47 | 0.73 | 6.55 | 0.73 | 8.75 | 0.76 | 8.29 | 0.74 | 7.86 | 8.16 |
| C12 | 5.40 | 3.78 – 7.02 | 4.27 | 10.57 | 4.33 | 8.58 | 4.38 | 6.11 | 4.33 | 8.42 | 6.94 |
| C14 | 2.99 | 1.79 – 4.18 | 2.13 | 4.32 | 2.24 | 4.88 | 2.12 | 5.86 | 2.16 | 5.02 | 7.00 |
| C16 | 10.80 | 5.96 – 15.70 | 8.46 | 11.73 | 8.42 | 4.44 | 8.46 | 4.36 | 8.45 | 6.84 | 9.47 |
| C8 | 4.64 | 2.33 – 6.73 | 3.90 | 4.32 | 3.88 | 4.72 | 3.86 | 3.03 | 3.88 | 4.02 | 4.99 |

* The measured Orn level was slightly lower than the target range, which was consistent with the previous measurement of the same kit on a TSQ Quantis MD mass spectrometer.

** Asp target value and ranges were not provided by the kit. NA, not available

## Conclusions

We developed an FIA-HRAM-MS/MS method to quantify 12 amino acids and 13 acylcarnitines from the ClinSpot LC-MS/MS Complete Kits, Amino Acids and Acylcarnitines in Dried Blood Spots (DBS) (non-derivatized) using a Vanquish Flex UHPLC and Orbitrap Exploris 120 mass spectrometer. The method showed good accuracy and precision measurements that are comparable to those values quantified from the TSQ Quantis MD mass spectrometer (TN001293). Orbitrap MS also provided high mass accuracy detection, full-MS spectra over the *m/z* ranges of common metabolites, and complete $MS^2$ spectra to increase the analyte identification confidence, which alternatively has the potential to improve the detection accuracy and enable novel biomarker discovery for clinical research.

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