Technical note | 002656

Clinical research

Method development to quantify amino acids, acylcarnitines, and succinylacetone in dried blood spots by FIA-MS/MS

Authors

Jitnapa Voranitikul,[1] Niramol Jitsommai,[1] Watcharapon Wongsupa,[1] Khunnalak Khitmoh,[2] Jingshu Guo,[3] Gina Tan,[3] Kerry Hassell[3]

[1]SciSpec Co., Ltd. Bangkok, Thailand

[2]Chromsystems Instruments & Chemicals GmbH. Munich, Germany

[3]Thermo Fisher Scientific, San Jose, CA, United States

Keywords

Dried blood spots, non-derivatized MassChrom Amino Acids and Acylcarnitines from Dried Blood kit, flow injection analysis-tandem MS, Vanquish Flex UHPLC, TSQ Quantis Plus mass spectrometer, TraceFinder software, relative response factor, inborn errors of metabolism, first-tier screening, newborn screening

Goal

Develop and verify a flow injection analysis-tandem MS (FIA-MS/MS) method to quantitate 13 amino acids, 13 acylcarnitines, and succinylacetone in dried blood spots for clinical reasearch of inherited metabolic disorders

Introduction

Newborn screening (NBS) programs are population-based public health services intended to identify newborns who may be at an increased risk of developing certain rare but treatable congenital disorders. Many disorders are not evident at birth. An early-stage diagnosis can lead to immediate medical intervention and treatment, therefore minimizing the unnecessary suffering of the newborn and the family. NBS 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]

In the U.S., the inherited metabolic disorders that the Department of Health and Human Services recommended states screen for in newborns are listed in the Recommended Uniform Screening Panel (RUSP). As of January 2023, RUSP consists of 42 metabolic disorders, two endocrine disorders, four hemoglobin disorders, and 15 other disorders.[2] These disorders were chosen based on evidence that supports the potential net benefit of screening, the ability of states to screen for the disorder, and the availability of effective treatments. Newer conditions are nominated to the Advisory Committee and can be adopted into future RUSP updates. All disorders are recommended to be screened for in newborns, but each state makes the final decisions based on its funding, internal capabilities, and expertise.

Among the disorders listed in the RUSP, all metabolic disorders are readily detected by flow-injection analysis tandem-MS (FIA-MS/MS), where abnormal ratios of some amino acids and acylcarnitines are indicative of many organic acid, fatty acid oxidation, and amino acid disorders.[3]

In this technical note, an FIA-MS/MS method was developed on a Thermo Scientific™ Vanquish™ Flex UHPLC coupled to a Thermo Scientific™ TSQ Quantis™ Plus mass spectrometer in selected reaction monitoring (SRM) mode. Quality control dried blood spot samples, reagents, and consumables were obtained from MassChrom™ Amino Acids and Acylcarnitines from Dried Blood (non-derivatized) for LC-MS/MS analysis (order no. 57000, Chromsystems Instruments & Chemicals GmbH, Gräfelfing, Germany). The developed method detects 13 amino acids (AA), 13 acylcarnitine (AC), and succinylacetone (SUAC) from DBS samples and verifies the semi-quantitative determination of those analytes in DBS.

Experimental

Sample preparation

The MassChrom™ Internal Standards (IS for AA and AC, order no. 57004, Lot 0321, and $^{13}C_5$-SUAC, order no. 57044, Lot 3522) were reconstituted using Extraction Buffer (order no. 57008 for AA and AC, and 57012 for SUAC) following the instruction of the MassChrom™ Amino Acids and Acylcarnitines from Dried Blood (non-derivatized) (order no. 57000). These solutions were used to extract AA, AC, and SUAC from the MassCheck Amino Acids, Acylcarnitines incl. Succinylacdetone Dried Blood Spot Control Bi-Level (I+II) (order no. 0191, Lot 2821).

A 3.2 mm disc from the DBS control cards was punctured and placed in a 96 well plate (plate 1, order no. 57010). To extract the analytes, 100 µL of the reconstituted AA and AC IS solution was added to the well plate, which was sealed with a protective sheet (order no. 55011) and left on a room-temperature thermomixer with agitation at 600 rpm. After 20 min, the extract was transferred to another 96 well plate (plate 2, order no. 57010) and sealed with a new protective seal. To extract SUAC, 75 µL of SUAC IS solution and 75 µL of Extraction Buffer for SUAC were added to the remaining DBS disk in plate 1 and sealed. The extraction took plate on an Eppendorf™ ThermoMixer™ mixing device set at at 600 rpm and 45 °C. After 30 min, the supernatant was added to plate 2 which was sealed with a pierceable adhesive seal (order no. 55013). The the mixture was agitated at 500 rpm for 1 min and left at room temperature for 20 min prior to the LC-MS/MS analysis. For the inter-day and intra-day precision measurements, each control level was prepared five times over five days.

Liquid chromatography

A Vanquish Flex UHPLC was used to deliver the extracted analyte to the mass spectrometer via isocratic flow injection analysis. PEEK tubing was used for all flow paths per the kit manufacturer's instruction. The LC-MS system was conditioned with the provided mobile phase (order no. 57001) for at least 10 minutes prior to sample injection. Each sample was injected three times at an injection volume of 10 µL. The LC conditions are specified in Table 1.

Table 1. Vanquish Flex UHPLC gradient

Time (min)	Flow rate (mL/min)	Mobile phase (included) (%)
0.00	0.60	100
0.12	0.60	100
0.13	0.35	100
1.00	0.35	100
1.01	0.80	100
1.10	0.80	100
1.11	0.80	100
1.50	0.80	100

Mass spectrometry

The analyte detection was performed using a TSQ Quantis Plus mass spectrometer equipped with a Thermo Scientific™ OptaMax™ NG ion source with a heated electrospray ionization (HESI) probe in the positive mode. The MS source parameters and SRM properties are listed in Table 2. Selected reaction monitoring (SRM) transitions of compounds, their IS, optimized collision energies, and RF lens settings are shown in Table 3.

Table 2. The HESI source and SRM parameters of TSQ Quantis Plus mass spectrometer

MS source parameters	
Ion source type	HESI (OptaMax NG ion source)
HESI probe position	Center - 1.0 - L (x - y - z)
Spray voltage	+4,500 V
Sheath gas (Arb)	45
Aux gas (Arb)	10
Sweep gas (Arb)	0
Ion transfer tube temp. (°C)	300
Vaporizer temp. (°C)	250
SRM Properties	
Chromatographic peak width (s)	30
Cycle time (s)	1.5
Points per peak	20
Q1 resolution (FWHM)	0.7
Q3 resolution (FWHM)	0.7
CID gas (mTorr)	1.5
Source fragmentation	0

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ software (ver 5.2 SP1 Clinical).

Table 3. SRM transitions, collision energies, and RF lens for the analytes and their internal standards

Analyte	Precursor (*m/z*)	Product (*m/z*)	IS	Precursor (*m/z*)	Product (m/z)	CE (V)	RF lens (V)
Alanine (Ala)	90	44	$^{2}H_{4}$-Ala	94	48	11	51
Arginine (Arg)	175	70	$^{2}H_{7}$-Arg	182	77	21	70
Aspartic acid (Asp)	134	116	$^{2}H_{3}$-Asp	137	119	7	50
Citrulline (Cit)	176	113	$^{2}H_{2}$-Cit	178	115	16	40
Glutamate (Glu)	148	130	$^{2}H_{5}$-Glu	153	135	7	50
Glycine (Gly)	76	30	$^{13}C_{2}$,^{15}N-Gly	79	32	18	40
Leucine (Leu)	132	86	$^{2}H_{3}$-Leu	135	89	10	59
Methionine (Met)	150	133	$^{2}H_{3}$-Met	153	136	10	70
Ornithine (Orn)	133	70	$^{2}H_{6}$-Orn	139	76	17	50
Phenylalanine (Phe)	166	120	$^{2}H_{5}$-Phe	171	125	13	75
Proline (Pro)	116	70	$^{2}H_{7}$-Pro	123	77	16	66
Succinylacetone (SUAC)	155	137	$^{13}C_{5}$-SUAC	160	142	11	70
Tyrosine (Tyr)	182	136	$^{2}H_{4}$-Tyr	186	140	12	74
Valine (Val)	118	72	$^{2}H_{8}$-Val	126	80	11	56
Carnitine (C0)	162	85	$^{2}H_{9}$-C0	171	85	20	100
Acetylcarnitine (C2)	204	85	$^{2}H_{3}$-C2	207	85	19	93
Propionylcarnitine (C3)	218	85	$^{2}H_{3}$-C3	221	85	20	87
Butyrylcarnintine (C4)	232	85	$^{2}H_{3}$-C4	235	85	20	100
Isovalerylcarnitine (C5)	246	85	$^{2}H_{9}$-C5	255	85	21	99
Glutarylcarnitine (C5DC)	276	85	$^{2}H_{6}$-C5DC	282	85	24	131
Hexanoylcarnitine (C6)	260	85	$^{2}H_{3}$-C6	263	85	22	107
Octanoylcarnitine (C8)	288	85	$^{2}H_{3}$-C8	291	85	23	116
Decanoylcarnitine (C10)	316	85	$^{2}H_{3}$-C10	319	85	24	135
Dodecanoylcarnitine (C12)	344	85	$^{2}H_{3}$-C12	347	85	25	164
Tetradecanoylcarnitine (C14)	372	85	$^{2}H_{3}$-C14	375	85	27	166
Hexadecanoylcarnitine (C16)	400	85	$^{2}H_{3}$-C16	403	85	28	152
Octadecanoylcarnitine (C18)	428	85	$^{2}H_{3}$-C18	431	85	30	208

Results and discussion

The preferred first-tier testing method for the metabolic disorder screening in the NBS program is flow-injection analysis tandem MS (FIA-MS/MS), which is easy to set up and has high throughput. Utilizing the Vanquish Flex UHPLC and TSQ Quantis Plus mass spectrometer, we verified the reliable detection of 13 AA, 13 AC, and SUAC using the MassChrom Amino Acids and Acylcarnitines from Dried Blood (non-derivatized) for LC-MS/MS analysis kit. The quantification was achieved by comparing the SRM peak area ratio of the analyte with the corresponding internal standards.

Figure 1 shows alanine as a representative analyte to demonstrate TraceFinder software data processing results. The measured amount, %Diff (comparing to the theoretical amount), %RSD (of the calculated amount), %CV (of the peak area), etc. are shown in the Sample Results table. TraceFinder software uses caution flags to expedite the data review process to highlight samples outside the predefined acceptance criteria. The analyte and IS chromatograms from one raw file are shown in the Compound Details Plot, and the chromatograms of the same analyte from different raw files are shown in the Compound-Centric Plot.

To compensate for the slight ionization efficiency differences of triple quadrupole MS among vendors, the relative-response factors (RRF) for each analyte can be determined during the system installation and verification using the control DBS samples of the kit. RRF is calculated based on the ratio between the targeted analyte value provided by the kit manufacturer and the measured average analyte amount.

RRF = (target value)/(measured value)

As an example, RRF calculated from 75 continuous analyses of samples are reported in Table 4. The RRF values are then applied to samples to correct the ionization efficiency difference (“RRF-corrected amount” in Table 4). In the TraceFinder software data processing method, the RRF values are inserted into the “Min RF” column in the “QAQC” tab, which automatically updates the “calculated amount” of the analytes into “RRF-corrected amount” in the report template. Figure 2 gives an example of RRF conversion from the calculated amount of analytes for one of the raw files.

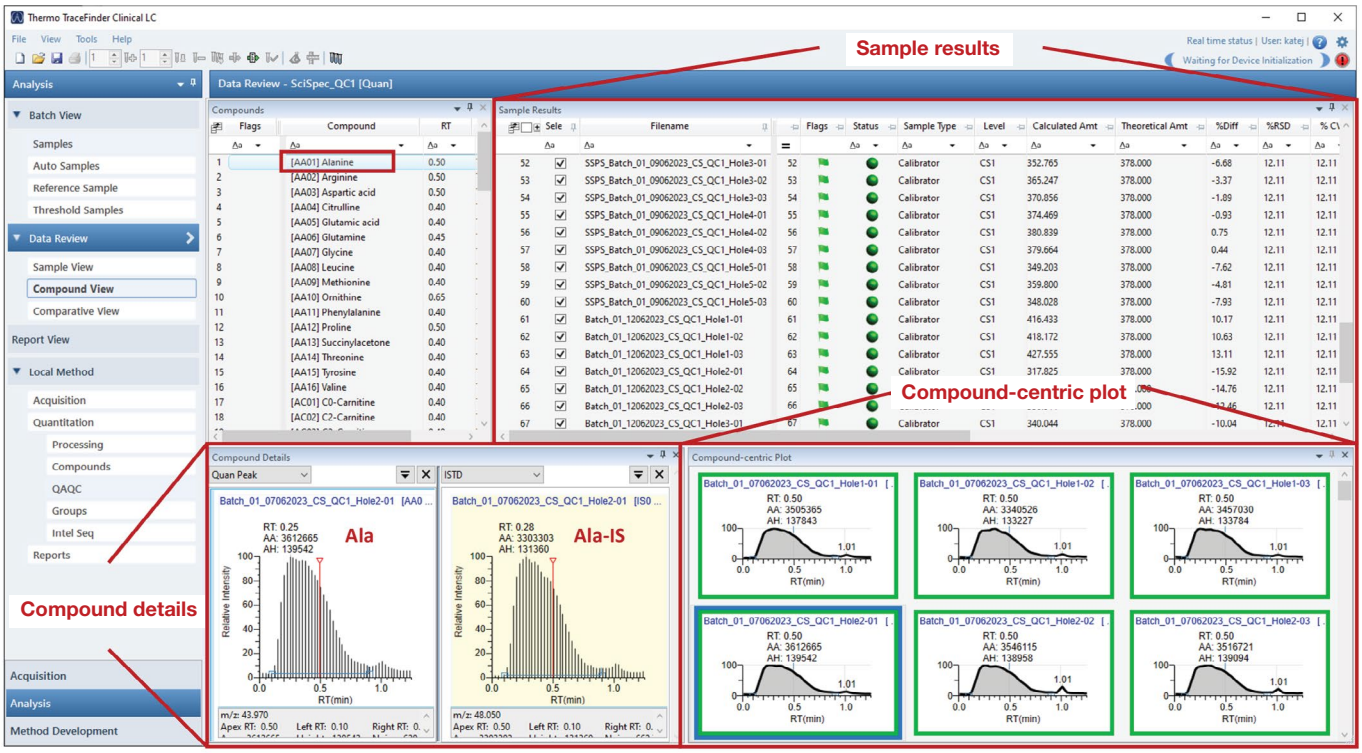

Figure 1. The TraceFinder software processing results of Ala are shown in the Compound View tab under Data Review. The chromatograms of Ala and Ala-IS from one raw file are shown in the Compound Details Plot, and the chromatograms of Ala from different raw files are shown in the Compound-Centric Plot.

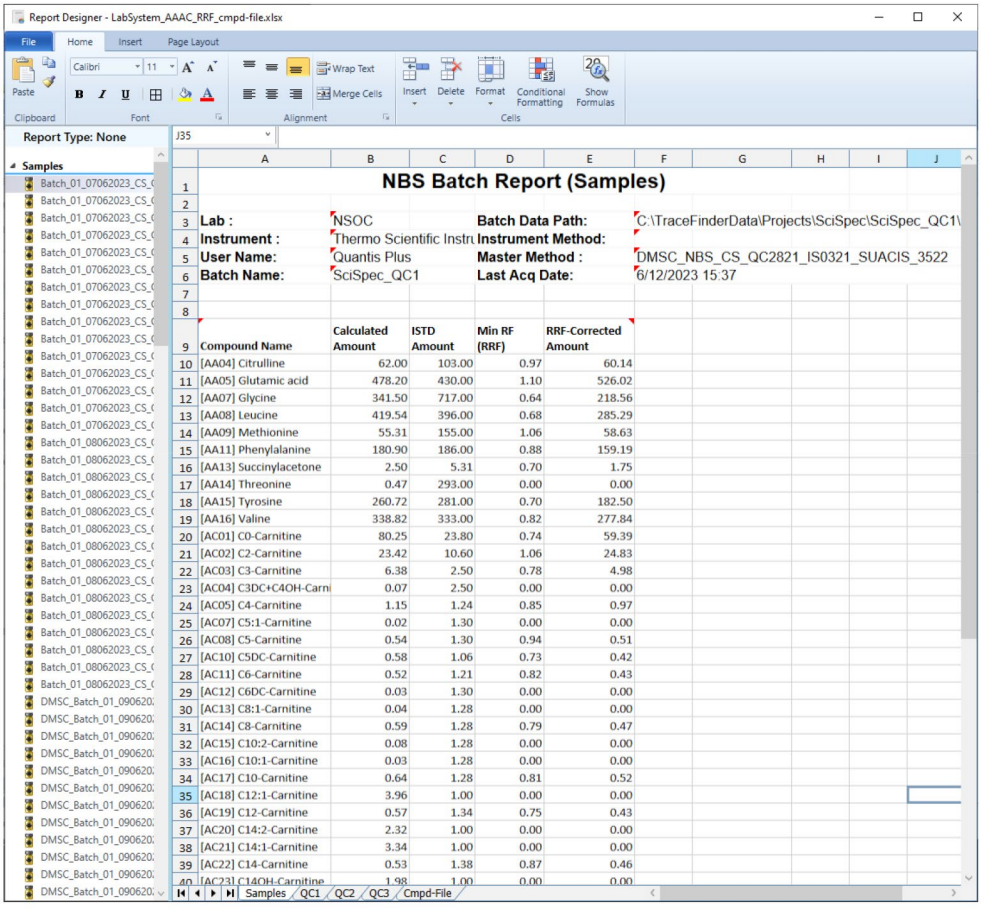

Figure 2. The TraceFinder software report template for calculating the RRF-corrected amount of analytes using the RRF values

The trueness of measurement (accuracy) results after RRF correction are shown in Table 4. All the measured values were within the target range listed by MassCheck Amino Acids, Acylcarnitines incl. Succinylacetone Dried Blood Spot Control Bi-Level (I+II) (lot no. 2821) with %Bias below 30.

The inter- and intra-day precision measurements results (N = 15) are listed in Table 5. The % RSD of the inter- and intra-day precision were under the acceptance criteria of 20% for AA and AC, and 30% for SUAC. The reproducibility of the method was represented by the %CV of the IS peak areas of each analyte in Table 5. For the two control levels, %CV of all IS (N = 75) was below 16%.

Table 4. Trueness of measurement (N = 75, acceptance criteria: %Bias <30)

Analyte	RRF	Control I (µmol/L)	Control I range (µmol/L)	RRF-corrected amt. ave. Control I	Bias (%)	Outcome	Control II (µmol/L)	Control II range (µmol/L)	RRF-corrected amt. ave. Control II	Bias (%)	Outcome
Ala	1.01	378.00	173.00 – 582.00	379.00	-0.28	Normal	557.00	245.00 – 869.00	569.00	-2.22	Normal
Arg	1.10	78.30	28.80 – 128.00	83.30	-6.37	Normal	221.00	115.00 – 328.00	213.00	3.70	Normal
Asp	1.03	169.00	108.00 – 229.00	173.00	-2.32	Normal	423.00	280.00 – 566.00	431.00	-1.89	Normal
Cit	1.16	62.20	43.90 – 80.50	63.20	-1.56	Normal	240.00	176.00 – 303.00	243.00	-1.09	Normal
Glu	1.22	517.0	332.00 – 701.00	522.00	-0.91	Normal	781.00	537.00 – 1025.00	788.00	-0.95	Normal
Gly	0.75	211.00	153.00 – 268.00	229.00	-8.42	Normal	488.00	343.00 – 632.00	459.00	5.85	Normal
Leu	0.83	305.00	168.00 – 442.00	314.00	-2.87	Normal	543.00	360.00 – 725.00	544.00	-0.16	Normal
Met	1.14	60.20	18.80 – 102.00	59.70	0.86	Normal	222.00	88.60 – 354.00	228.00	-2.86	Normal
Orn	1.30	286.00	169.00 – 403.00	290.00	-1.37	Normal	557.00	350.00 – 764.00	572.00	-2.63	Normal
Phe	1.01	167.00	107.00 – 227.00	163.00	2.33	Normal	536.00	330.00 – 741.00	566.00	-5.52	Normal
Pro	1.25	291.00	214.00 – 368.00	293.00	-0.82	Normal	692.00	424.00 – 959.00	704.00	-1.79	Normal
SUAC	0.88	1.98	1.20 – 2.76	1.97	0.69	Normal	7.16	4.54 – 9.78	7.97	-11.34	Normal
Tyr	0.83	185.00	125.00 – 244.00	192.00	-4.02	Normal	507.00	347.00 – 666.00	497.00	1.93	Normal
Val	0.96	289.00	181.00 – 396.00	286.00	0.93	Normal	505.00	331.00 – 679.00	525.00	-3.98	Normal
C0	0.87	60.70	28.90 – 74.70	61.50	-1.30	Normal	122.00	75.90 – 168.00	124.00	-1.79	Normal
C2	1.27	25.00	15.90 – 30.50	25.00	-0.01	Normal	59.60	36.90 – 82.30	61.60	-3.38	Normal
C3	0.92	5.08	2.91 – 7.15	5.07	0.10	Normal	12.50	8.11 – 16.90	12.90	-3.45	Normal
C4	0.99	0.94	0.44 – 1.44	0.94	0.94	Normal	4.12	2.40 – 5.83	4.27	-3.55	Normal
C5	1.03	0.54	0.28 – 0.80	0.52	0.50	Normal	2.17	1.23 – 3.11	2.25	-3.52	Normal
C5DC	0.86	0.48	0.13 – 0.97	0.62	-12.24	Normal	1.91	0.88 – 2.93	1.76	7.64	Normal
C6	0.93	0.45	0.27 – 0.65	0.47	1.06	Normal	2.00	1.27 – 2.73	2.08	-4.11	Normal
C8	0.95	0.48	0.27 – 0.75	0.51	-1.86	Normal	2.13	1.30 – 2.95	2.18	-2.30	Normal
C10	0.98	0.53	0.26 – 0.63	0.54	-0.44	Normal	2.18	1.20 – 3.16	2.30	-5.57	Normal
C12	0.93	0.46	0.20 – 0.70	0.50	-2.18	Normal	2.11	1.38 – 2.83	2.18	-3.14	Normal
C14	0.99	0.50	0.24 – 0.68	0.50	-0.29	Normal	2.01	1.20 – 2.82	2.10	-4.35	Normal
C16	0.96	4.65	2.79 – 6.41	4.74	-1.85	Normal	11.80	7.25 – 16.4	12.10	-2.19	Normal
C18	0.84	3.00	1.45 – 3.77	3.01	-0.46	Normal	9.50	5.14 – 13.9	9.80	-2.63	Normal

Table 5. Inter-day and intra-day precision (N = 15, acceptance criteria: %RSD <20, and <30 for SUAC)

	ChromSystems Control I							ChromSystems Control II						
Analyte	Day 1 RSD (%)	Day 2 RSD (%)	Day 3 RSD (%)	Day 4 RSD (%)	Day 5 RSD (%)	Inter-day RSD (%)	IS (N = 75) CV (%)	Day 1 RSD (%)	Day 2 RSD (%)	Day 3 RSD (%)	Day 4 RSD (%)	Day 5 RSD (%)	Inter-day RSD (%)	IS (N = 75) CV (%)
Ala	5.47	12.86	6.34	9.49	12.07	12.11	9.77	7.00	10.91	5.66	8.01	9.81	10.21	7.74
Arg	7.30	9.36	5.19	5.49	14.23	11.97	14.64	4.74	7.70	3.28	7.06	8.12	9.48	6.89
Asp	7.98	13.01	7.90	10.94	10.18	16.90	12.09	9.74	12.13	5.15	8.33	10.52	12.97	9.65
Cit	10.53	10.62	7.56	10.36	12.31	13.56	12.23	6.65	10.26	5.07	6.58	9.48	9.57	8.80
Glu	5.04	8.19	5.76	6.14	9.43	9.09	12.46	6.79	10.58	5.49	8.72	9.81	10.22	9.58
Gly	4.95	9.31	6.09	7.45	11.81	9.37	11.02	6.69	10.14	4.46	6.69	8.78	8.66	8.27
Leu	8.05	12.67	6.12	10.20	16.41	13.99	9.27	9.08	10.90	4.95	7.88	10.25	10.70	6.79
Met	5.51	9.74	6.21	7.58	13.99	9.80	10.16	4.88	9.49	4.37	7.71	7.89	9.83	7.39
Orn	8.17	10.77	18.25	8.32	10.76	16.38	8.48	8.33	9.26	4.34	4.98	9.27	13.12	5.11
Phe	6.62	12.29	6.13	10.21	15.30	13.37	10.21	5.74	10.42	4.95	7.29	10.00	10.30	7.26
Pro	5.50	12.20	5.40	9.85	13.02	12.81	10.22	5.65	10.72	5.35	7.58	9.86	10.21	6.97
SUAC	9.43	11.74	10.41	13.39	15.05	25.82	11.47	7.64	16.03	8.41	6.88	18.24	17.42	8.35
Tyr	4.26	8.78	5.65	7.89	10.57	10.62	10.95	4.62	8.55	4.67	6.20	8.20	8.88	7.47
Val	6.09	12.83	6.15	9.96	15.46	13.67	9.24	7.00	10.72	4.97	7.73	9.93	10.38	6.86
C0	5.47	12.56	6.43	10.75	12.84	13.84	15.86	4.54	11.45	5.30	7.80	11.21	10.57	11.11
C2	5.21	12.22	5.94	9.63	13.53	14.58	16.03	4.38	10.94	5.10	7.42	10.35	10.62	10.56
C3	4.30	11.54	6.56	10.66	14.12	14.11	15.29	4.80	11.39	5.18	7.39	11.05	10.87	10.06
C4	6.42	11.85	5.24	9.24	12.37	12.52	14.64	4.43	9.97	5.39	8.03	9.71	9.59	9.83
C5	7.76	12.00	5.66	11.08	11.04	13.14	12.57	4.67	12.15	5.66	7.88	11.41	10.58	8.72
C5DC	10.63	14.14	7.64	8.80	13.12	13.45	14.76	5.89	9.35	5.31	6.52	12.85	9.68	11.01
C6	4.64	12.16	7.19	8.66	15.08	13.60	13.05	4.64	10.65	5.30	7.25	10.93	10.07	8.72
C8	6.31	14.66	5.78	13.14	14.80	16.00	12.75	4.46	13.30	5.84	7.84	13.05	12.45	8.99
C10	7.95	18.55	6.79	15.92	18.72	19.20	11.87	7.26	15.01	7.81	8.45	16.14	14.05	8.44
C12	7.97	18.12	7.53	16.71	19.67	18.29	12.03	8.04	14.03	7.08	8.94	16.12	13.34	9.09
C14	8.13	15.41	7.82	15.04	19.72	16.76	11.14	8.04	13.75	6.66	9.01	13.84	12.84	8.80
C16	8.21	15.85	6.60	13.46	18.20	16.53	11.61	7.30	12.36	6.54	8.36	11.78	11.39	9.37
C18	7.97	12.65	6.27	12.00	15.88	14.36	11.67	6.25	10.97	6.17	8.06	10.37	10.14	9.34

Conclusions

We developed a Vanquish Flex UHPLC and TSQ Quantis Plus mass spectrometer-based FIA-MS/MS method to verify the quantitation of 13 amino acids, 13 acylcarnitine, and succinylacetone from the MassChrom Amino Acids and Acylcarnitines from Dried Blood (non-derivatized) kit. The method showed good accuracy, precision, and reproducibility, meeting the needs of clinical research laboratories.

References

1. Association of Public Health Laboratories. APHL Newborn Screening Resources. https://www.aphl.org/programs/newborn_screening/Documents/nbs_resource_brochure_educationaltool_april2019_se.pdf
2. Health Resources & Services Administration. Recommended Uniform Screening Panel. Aug **2023.** https://www.hrsa.gov/advisory-committees/heritable-disorders/rusp/index.html
3. Clinical and Laboratory Standards Institute (CLSI). Newborn Screening by Tandem Mass Spectrometry. 2ns ed. CLSI guideline NBS04 (ISBN 1-56238-818-5). Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087 USA, **2017.**

Acknowledgments

We wish to acknowledge the National Neonatal Screening Operation Centre, MoPH Thailand, for support in this technical note.

Learn more at **thermofisher.com/ClinicalResearchApps**