A Fast & Reliable Liquid Chromatography Mass Spectrometry Method for Determination of Amino acids & Acylcarnitines including Succinyl acetone in dried blood spots for newborn screening.

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Abstract
Introduction: We developed & validate first time TMS based non derivatized 1.5 min protocol to quantify 25 amino acids & 30 acylcarnitines along with Succinyl acetone.
Method: 3.2mm DBS disk was extracted with a mixture of methanol: Hydrate hydrazine: oxalic acid: Acetonitrile: Water. Analysis was performed on LCMSMS with direct injection method. Water: formic acid & Methanol was used in gradient elution. Optimized multiple reaction monitoring (MRM) was used for detection of amino acids and acylcarnitines on a Shimatzu mass spectrometer. The method was validated with respect to linearity, precision, recovery, accuracy.
Result: Precision, accuracy and analytical measurement range considering overall precision within 10% and accuracy within 15% of for all amino acids for Acylcarntine at one fortified level, which quantitated 21.8% lower than the expected value. Inter method comparison shows only 8.5% & 10.5 % error bias for AA & AC respectively. Analytes were stable for 1 month at room temperature & 6 months at -20^{0}C storage condition.
Conclusion: The present design protocol is sensitive with significant LOD and cost effective which can be effectively applied as fast primary & second-tier test for newborn screening of metabolic disorders associated with abnormal levels of acylcarnitines and amino acids, potentially reducing false positive cases and shortening the time to diagnosis.

Keywords — *Amino acids & Acylcarnitines, Dried blood spot, ESI-MS, newborn screening,*

I. INTRODUCTION

Newborn screening (NBS) of inborn errors of metabolism (IEM) is a comprehensive system consisting of screening, confirmatory testing, follow-up of abnormal test results, treatment and evaluation of outcome and efficiency. The ultimate goal of NBS and follow-up programs is to reduce morbidity and mortality from IEM (1). Dried blood spots (DBS) represent an alternative matrix for measurement of circulating biomarkers which require only a small volume, which is high potential to overcome difficulties associated with blood collection in neonates in newborn screening. Inborn errors of metabolism (IEM) are typically hereditary and lead to defects in one or more enzymes that are important for normal metabolic activity (2). Impairment of protein, carbohydrate & fat metabolism can result in variation of physiological concentrations of key markers like free amino acids (AA), acylcarnitines (AC), organic acids, free sugars & results in appearance of clinical symptoms, which are usually not specific for a single disorder. One or several molecules can become biomarkers for one or a group of disorders and screening of all these compounds is required in order to confirm or exclude an impairment of IEM. Thus, the diagnosis and the therapy assessment of inherited disorders of metabolism are based on the simultaneous detection and quantification of all these biomarkers in biological fluids, mainly plasma, urine, whole blood (3). Presently a number of techniques developed for analysis of clinically significant biomarkers, including high performance liquid chromatography (HPLC) (4, 5), gas chromatography-mass spectrometry (GC–MS) (6), and liquid chromatography–mass spectrometry (LC–MS) (7, 8, 9, and 10). Major drawbacks of the current methods are time consuming procedures, problems with retention, and MS-sensitivity. Tandem mass spectrometry with derivatization of amino acids & acylcarnitines i.e. butyl ester formation is rapidly being adopted by newborn screening programs to screen dried blood spots for >20 markers of disease in a single assay (17). Due to its simplicity and speed, this method has been applied with great success to high-throughput newborn screening programs (10, 11). However there are some limitations to this method, i.e. due to hydrolysis in preparation of acylcarnitines butyl esters, increased free carnitine values also increased *(12).* Due to lack of selectivity, specificity & due to the presence of isobaric contaminants, false positive test results have been reported (13, 14, 15,16).

Lab developed a standardized non derivatized protocol to quantify 25 amino acids & 30 acylcarnitines along with Succinyl acetone using

MS/MS with underivatised method having analysis time only 1.5 min. As per literature available, no single step analytical method has been developed and validated for simultaneous determination of 25 AA and 30 AC with Succinyl acetone (SUAC) in dried blood spot for neonatal screening of IEM. Method can specifically screen of Tyrosinemia I due to involvement of SUAC. To accurately quantify the underivatised AA & AC in DBS, MRM scan with labelled deuterated internal standard is introduced. The protocol is very simple, sensitive and cost effective which can be effectively applied to the screening of a large number of samples in clinics with its short run time and the simple processing of DBS samples.

II. MATERIAL & METHOD

A. Chemicals & Equipment's

All reagents, Unlabelled Amino acids, Solvents and chemicals of analytical grade were purchased from Sigma Aldrich (USA). We purchased isotopically labelled Succinyl acetone (SUAC), AA and AC standards from Cambridge Isotope Laboratories, USA. Unlabelled acylcarnitines standards were purchased from Madrid University, Spain. Automated DBS puncher (DELFIA 1296- 071) was purchased from PerkinElmer (USA). Micro well plates with Pierceable mat were purchased from Agilent Technologies (USA).

B. Sample collection & Sample preparation

The heel prick Venous and EDTA whole blood was collected on specially manufactured DBS card (Whatman 903) in 50 µl portions. This filter paper meets NCCLS and CDC specifications [18]. Dried blood spot (DBS) samples were dried for 3h before processing & stored at 4^{o}C until analysis.

We punched single 3.2 mm (1/8') disc by Wallac DBS puncher (PerkinElmer) from controls and samples into 96-well plate. Dried blood spots were extracted for SUAC along with acylcarnitines and amino acids with 50 µL of a methanol solution containing isotope labelled internal standards, 50 µL of hydrate hydrazine, 50 µL of oxalic acid, 50 µL of Acetonitrile: water solution including 0.1% formic acid (19).The plate was covered and the discs were eluted by mixing using an orbital rotator for 20 min at 120 rpm. After incubation, plate was centrifuged for 10 minutes and 100 µL eluates were transferred to round-bottom 96-well plate & cover it with pierceable mat & use for analysis by HPLC-MS/MS.

C. Instrumentation and Analysis

Samples were analysed using Shimadzu 8030 LCMS system (Shimadzu, Kyoto, Japan) operated in the positive electrospray mode with MRM peaks of AA, AC & SUAC, with their internal standard. Analyte separation is based on direct injection method under binary gradient conditions of mobile phases A (water with 0.1% formic acid) and B (100% acetonitrile) at ratio 70:30 over a run time of 1.5 minutes only. Gradient profile was set with flow rate 200 μL/min for initial 0.25 min, for next 1 min it was gradually decrease up to 0.03 μL/min. same solvent condition was set at 0.2 mL/min rate for next 0.25 min for next injection system preparation. The oven temperature was set to 30^{o}C and the flow rate was 200 μL/min with injection volume of 10 μL. The ionization source settings optimized to give maximum signal intensity as desolation line temperature 250^{O}C; capillary voltage, 4.5KV nebulizer gas flow 3 L/min. The protonated molecular ion $[M+H]^{+}$ of biomarkers was monitored using MRM mode. The software programme Lab solution 5.6 SP2 (Shimadzu Technologies) was used to operate the system and acquire all data & Neonatal Solution 2.1 software used for analyses & quantification of data. Deuterated labelled IS's intensity & concentration is used for quantification of unknown concentration.

D. Preparation of Calibrators and Controls

Adult whole blood was obtained from Biological Specialty Corporation (Colmar, PA.) and the haematocrit adjusted to 50%. We prepared the DBS as multiple-analyte mixtures. Individual analyte stocks of unlabelled calibrators and QCs were prepared by dissolving respective analyte at a concentration of 1 mg/mL into ultrapure water. Each solution was used to make the standard and QC working stocks to be diluted for the appropriate DBS concentration. We prepared a stock of analytes mixture & with dilution multiple levels of DBS calibrators & QCs prepared which covering the analytical measurement range for measured MS/MS. A stock analyte mixture was used to monitor linearity and recovery of the dilution series by current MS/MS methods. We applied aliquots of 50 µL of analyte-enriched blood on strips of Whatman 903 and the spotted cards dried for 3 hrs. at room temperature. After drying DBS were transferred to a zip-lock bag with desiccator & humidity indicators and stored at 20 °C until use. Spiked DBS of analyte mixture was further used for calibration, linearity, recovery, stability, and imprecision studies.

E. Validation study

Validation was carried out according to the NCCLS approved Guidelines for bioanalytical assays [20-22]. Important items to show are accuracy and precision of the assay, matrix effect (ME), recovery (RE) and process efficiency (PE) as well as general stability of QCs and clinical samples.

Linearity

AA, AC & SUAC calibration standards were processed in replicate ($n = 3$) and analysed on three separate days with same method mention above. A linearity plot of analyte/IS peak area ratio against

nominal analyte concentration was produced and an equally weighted linear regression was applied. The limit of detection (LOD) were calculated by examining blank filter paper samples and low-enrichment QC specimens over a three-day period. LOD of each Analyte was determined based on the S/N-ratio for each Analyte in QC-low. LOD was calculated as three times signal to noise (S/N)-ratio, Obtained values were the mean of three independent experiments.

Precision, Recovery & accuracy of analytes from Dried Blood Spots

The assay precision was assessed by analysing a calibration with 3 concentration levels in triplicate. Intraday & Interday precision was performing with 3 concentration for 10 days.

The overall efficiency of the extraction method was investigated at three level control DBS of analyte mixture. The 3.2mm DBS was subsequently punched out and extracted with extraction solution. Recovery was determined by comparing the peak area obtained with DBS extracts to those obtained from analytes in solvent containing an amount which assumes 100% recovery. Recoveries were calculated from the following equation:

Recovery % = Response in DBS extract/Response in pure solvent× 100.

Accuracy of the method was determined by replicated analysis of samples containing known amounts of each analyte in DBS calibrators through the Newborn Screening Quality Assurance Program (NSQAP) from CDC.

Sample Stability

Stability was assessed on two sets of two level DBS controls. One set evaluated after long term storage (three months at −20 °C), second set was evaluated for short-term storage (stored for 7days) at ambient room temperature, 37^0C, 4^0C,. Compare these results with those obtained from freshly prepared samples. The spots were stored in a sealed plastic bag with a desiccant.

F. *Evaluation of method with CDC quality control material*

The cross verification of method performance was done through the Newborn Screening Quality Assurance Program (NSQAP) & proficiency testing External quality assurance provided by Centers for Disease Control and Prevention (CDC) Atlanta. CDC was provided the QC specimen lots as 6-month supplies of DBSs on filter paper. The materials were enriched with predetermined quantities of selected analytes and dispensed in 100 µL aliquots on GE Healthcare Bio-Sciences Corporation (Westborough, MA) Grade 903 filter paper. The QC shipment for Amino acids, SUAC, and the acylcarnitines consisted of blood spot cards from four lots. The QC materials were supplied for use as external controls in quantities sufficient to maintain continuity and transcend changes in production lots of routinely used method & control materials. The external QC materials were intended to supplement method & control materials at periodic intervals which help to monitor the long-term stability of our method assays.

G. Method comparison

We compared the LC-MS/MS non derivatized method with MassChrom amino acids & acylcarnitines non-derivatization based kit method *(7)* by analyzing 30 random DBS specimens & CDC proficiency testing samples in which the concentration of all analytes had been measured by the derivatized kit method assay, provided by the Chromsystems Instruments & Chemicals GmBH, Germany.

III. RESULT

A. Linearity

Our LCMSMS-ESI positive based mass spectrometric method enables the simultaneous detection of 25 amino acids and 30 acylcarnitines & SUAC in only 1.5 min. We have successively optimize 56 analytes in MRM positive mode.

Fig 1 shows that increasing amounts of standard unlabelled analyte added to biological samples (DBS) display a linear relationship with the analyte response detected & with all the slopes and linear regression coefficient values very close to 1. Fig. 1 also express the results as the amount of acylcarnitines detected is directly proportional to the added concentration of spiked acylcarnitines. This result proves that acylcarnitines was not hydrolysed. We added increasing concentrations of the 30 acylcarnitines to aliquots of samples of normal human whole blood and assayed for acylcarnitines.

The linearity of rest of calibrators was assessed following NCCLS Approved guidelines (21). Table no 1 summarizes the results of Linearity studies with AA, AC & SUAC. DBS calibrators of all analytes at 7 different concentrations showed detectable and reproducible signals with a linear response. An r^2 of 0.98 or higher was considered acceptable. The LOD range 0.01–0.15 µmol/L (data not shown) were calculated and were low enough to clear cut diagnosis of IEM disorder, thereby fulfilling the criteria of analytical sensitivity.

B. Precision, Recovery & accuracy

Precision and Recovery were assessed according to NCCLS guidelines for general bioanalytical matrices (20). Three replicates of each of the three prepared QC levels were extracted and analyzed against a standard calibration curve consisting of seven levels of individual calibration range. This was repeated in three separate analytical runs to determine both

inter- and intra-accuracy and precision (% coefficient of variation (CV)). Assay was considered valid if both of these parameters were within 15% at both the levels. Table 2A & 2B gives a summary of inter- and intra-assay accuracy and precision.

For this non derivatized method, the average intra-assay precisions analysis of 10 replicate analyses on 3 specimens within the same day yielded mean for AA and SUAC at three concentrations were 8.36% (level I), 8.11% (level II), and 7.70 % (level III). The average intra-assay precisions for AC at three concentrations were 9.23% (level I), 8.95% (level II), and 7.58% (level III). Results we get for inter-assay precession, the average inter-assay precisions for AA and SUAC at three concentrations were 10.45% (level I), 10.13% (level II), and 9.62 % (level III). The average inter-assay precisions for 18 AC at three concentrations were 11.54% (level I), 11.18% (level II), and 9.47 % (level III). Overall precision of the assay calculated by replicate analyses was approximately 8.5 % of the same normal blood sample on the same day and on different days. This is sufficient for the correct differentiation between normal and pathologic samples.

The analytical recoveries of analytes added to blood were determined in triplicate analysis. These results show good recovery (Table 2A & 2B). The direct quantification via a corresponding internal standard resulted in recovery rates between 80 and 101% for 25 amino acids & Succinyl acetone. Similar results were shown for acylcarnitines & Succinyl acetone recovery. For Homocysteine (73%) & histidine (73%) underestimation were observed.

Accuracy of the method was determined by replicated analysis of samples containing known amounts of each analyte in DBS calibrators from CDC. The accuracy data obtained were in the range of 83.95 to 116.15 % for AAs, of 92.0 to 112.7% for ACs and was 96.6 % for SUAC (Table 2A & 2B).

C. Sample stability

The stability tests were designed to finalize retention time & suitable storage condition of handling of the real samples. Interday precision of QC DBS processed samples were found to be acceptable as per pre-defined 15 % acceptance criteria. Compare to freshly prepared QC samples, at long storage at −20 °C for 6 months showing average 9.01% loss was observed in Amino acids while 9.52% loss was observed for acylcarnitines. Average 12-14% loss was observed during storage at ambient temp & -20^{0}C for 1 month.,

To further investigate the stabilities of various AAs in DBSs for short period i.e. 1 month, as affected by sample storage at different temperatures figure no 2A & B shown that all analytes did not show significant concentration decreases at -20^{o}C, 4^{o}C & at ambient temperature for 30 days. His, 4-hydroxy-L-Proline, Succinylacetone, C0, C2, C12OH, and C18:1 were showing concentration decrease at 37 ^{o}C in 1 month storage. Histidine & Hydroxy proline were the least stable amino acids in DBS. All the analytes showing significant response during long storage at -20^{o}C for 180 days. This demonstrated that DBS samples were stable when stored under these conditions. 4- Hydroxyproline & hydroxyl derivatives of acylcarnitines were the least stable AA & AC under the conditions tested.

D. Method Evaluation with External quality control material

The reported QC comparison data are summarized in tables no 3, which show the analyte by series of QC lots, the number of measurements (N) and the mean values by our analytic method. The QC mean of external controls are determined by non derivatized non kit method. The results which we get from replicate analysis of material by our non derivatized method are 80-100 % comparable with the expected manufacturer's ranges.

E. Method comparison

As part of the validation procedure, the described method was compared with LC-MS/MS non-derivatization based MassChrom kit method (7.) The average method differences resulting from derivatization and no derivatization methods in 100 sample analysis was 6.62% for 12 AA in which Arginine shows significant difference (8.71%). The average method differences of 18 AC was 10.5% in which C0, C2 & C8:1 shows significant difference 11.54, 12.98 & 14.82 respectively. The small bias is of no clinical importance and therefore the differences were accepted. Inter method comparison conclude that two methods were highly correlated. Figure no 3 shows the method comparison six representative analytes.

Among these 100 samples, with inter-method comparison we screen & confirm 11 patients with an established diagnosis of MSUD (4 cases), PKU (2 cases), organic academia (5 cases) & 3-Methylcrotonyl-CoA Carboxylase Deficiency (3MCC) (1 case).

IV. Discussion

Current newborn screening of amino acids & acylcarnitines involves a derivatization step before MS analysis. All the acylcarnitines are first converted into the corresponding methyl or n-butyl ester [23]. The derivatives have a net positive charge, which is expected to prevent the formation of zwitterions during ESI so that both stability and sensitivity of the analysis are improved. However, there are some

problems that are associated with the process, such as incomplete derivatization or subsequent hydrolysis, in addition to the time involved in sample preparation [24].

Our main objective in the current study was to take further steps toward establishing ESI-MS/MS as a more efficient with high-sample-throughput neonatal screening method. We got success to increase sample throughput by simplifying sample preparation. We converted the single step sample preparation like extraction, centrifugation & direct injection to a more efficient and cost-effective batch-type process by using the convenience of 96-well microplates. The application of our mass spectrometric method enables the simultaneous detection of about 25 amino acids and 30 acylcarnitines along with SUAC in only 1.5 min.

In present study, first time we are include SUAC along with AA & AC in single extraction method because of Newborn screening for TYR 1 is highly desirable due to the availability of effective treatment for this otherwise life-threatening condition. However, tyrosine is a poor marker for TYR 1, and not every laboratory has the ability to provide testing for SUAC either as a primary screen or as a 2-tiered approach. It was therefore included in the American College of Medical Genetics' core panel of conditions that every newborn should be screened for *[25]*.

To evaluate LC-MS/MS method performance, we validated our method DBS having Precisions showed CVs <10% for high abundant metabolites and CVs <15% for low-abundant compounds. Accuracies displayed CVs of ±10% with a few exceptions. This is a major improvement over previously published methods, for which up to 30% variation was reported in plasma [26]), or for which no measures were reported at all [27]. We observed that DBS samples were stable for 6 months when stored at -20^0 temperature & for 1 month at RT, 4^0C. Method has significant LODs and LOQs those are sufficient to determine analytes in DBS.

In developing a new screening method, the probability of both false-positive and false-negative results. A high rate of false positives will both increase the cost of the screening program and put an emotional burden on the parents, when they are requested to submit repeat samples [28]. As pointed out by Rashed et al. *[28]*, and our results presented here, the Precision, recovery and accuracy provided by our method over conventional screening methods should reduce the rate of false-positives. Incorporation of Inter- method comparison & CDC proficiency testing program, we got success in the reduction or elimination of false-negatives cases because missing of false negative cases could seriously undermine the screening program. To the best of our knowledge, we have not encountered a false-negative case. Sample preparation is only single extraction of the DBS sample, which raises concern as it may decrease the recovery of analytes. But after comparing the results of 100 samples analyzed by derivatized kit method and after implementation of the new method revealed mostly negligible mean difference in recovery of amino acids and acylcarnitines.

V. Conclusion

The non-derivatized method performance characteristics shown provide preliminary evidence of the method's suitability for high-throughput neonatal screening for inborn errors of metabolism. Clinically significant & acceptable differences in group means were observed for amino acids, acylcarnitines & Succinyl acetone. Our results indicated that the recoveries of all the assayed biomarkers were comparable to the results obtained from the well-known kit methods. The addition of SUAC along with AA and AC with deuterated labeled internal standards provides a simple approach to implementing neonatal screening by laboratories. This will increases throughput without an increase in instrument time & test cost per specimen, reduces labor cost as one extraction method, and allows for simultaneous detection of Amino acids, acylcarnitines & SUAC quantitation during newborn screening of DBS extracts.

In conclusion, we have developed and validated a new assay that successfully combines existing methods for newborn screening for disorders of fatty acid, organic acid, and amino acids metabolism including TYR 1. This method is sensitive, rapid, and cost-effective, with no increased risk of either false-positive or false-negative results and could be implemented by all laboratories.

Figures and Tables

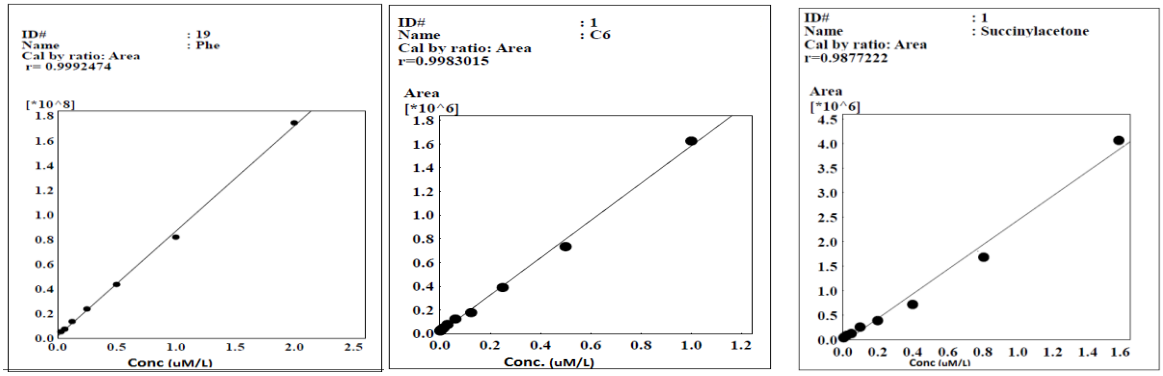

Figure No.1 Calibration curve (dose–response) for Phe, C6 & SA from blood spot calibrators (other data for AA & AC not shown). The correlation coefficient (r2) were = 0.999, 0.998 & 0.987 respectively.

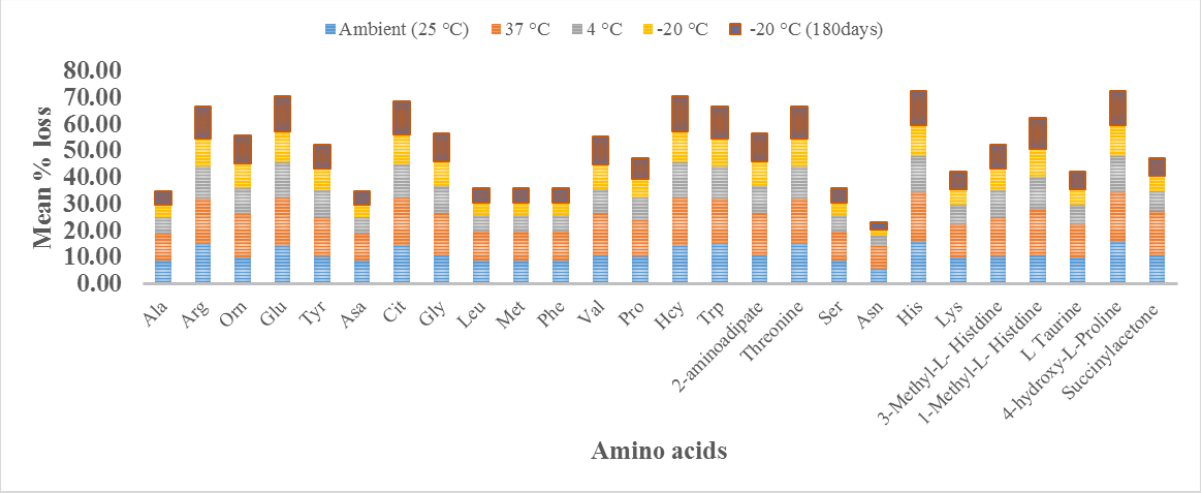

Figure 2A. The mean percentage loss of amino acids after short & long storage at different temperatures.

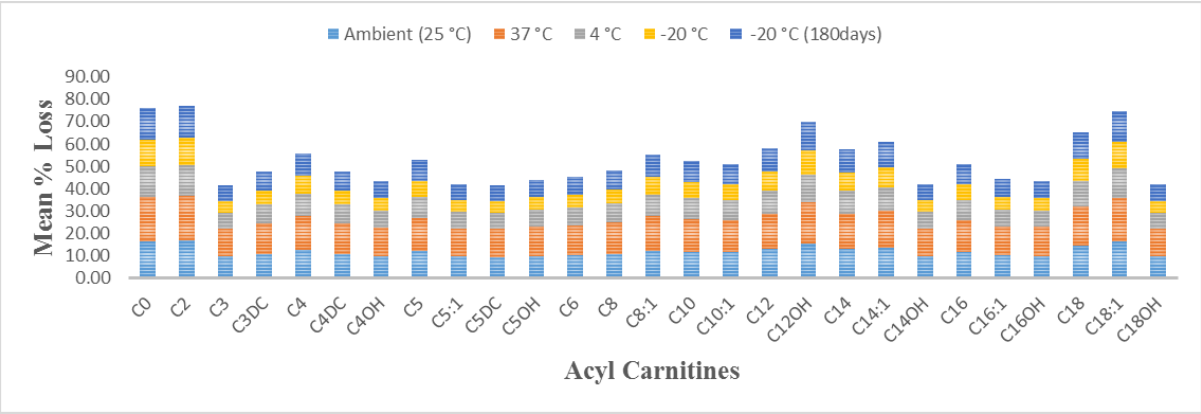

Figure 2B. The mean percentage loss of acylcarnitines after short & long storage at different temperatures.

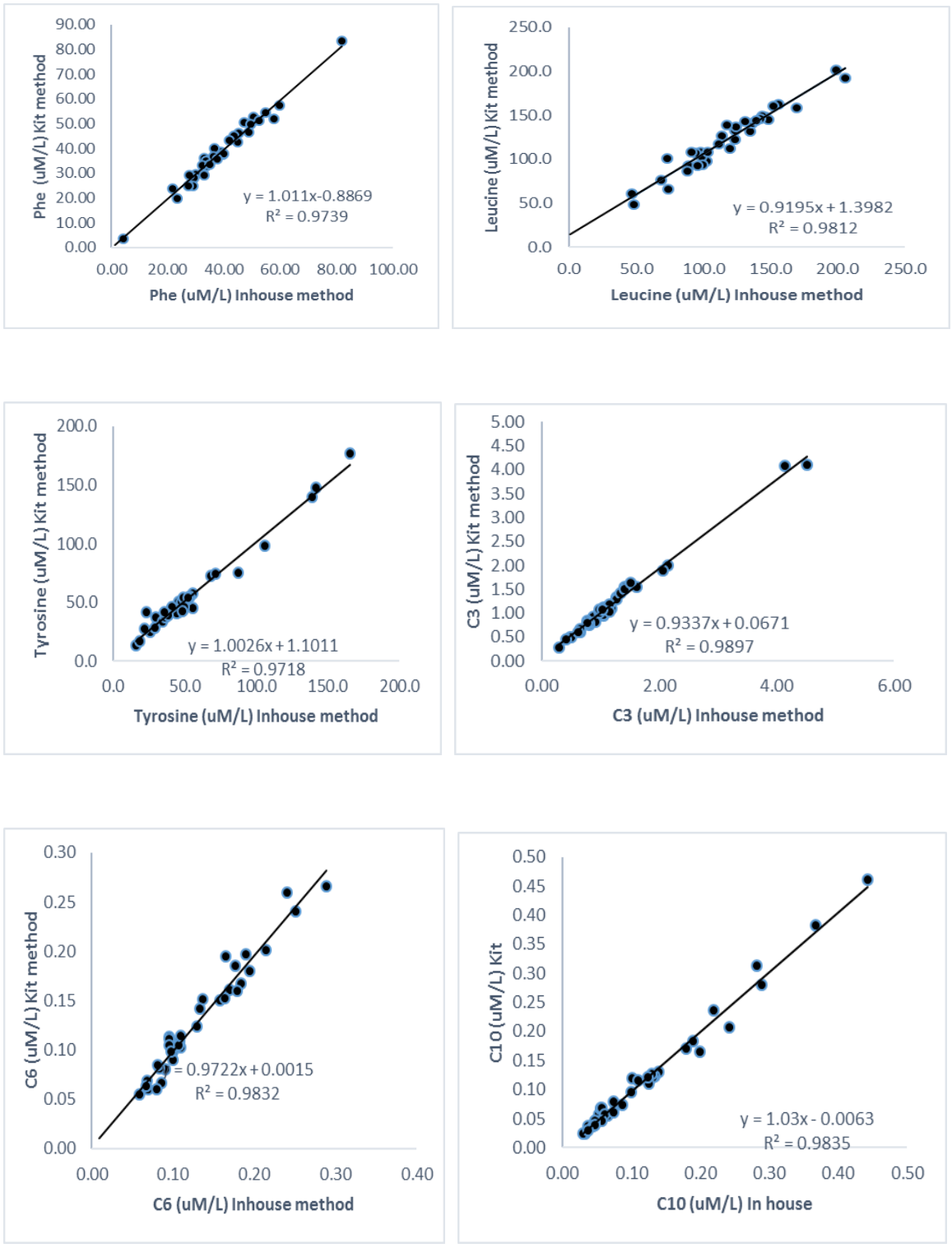

Figure no 3 Method comparison six representative analytes.

Table no 1 summarizes the results of Linearity studies with AA, AC & SA

Ref DBS Standard	Correlation coefficient (r2)	Ref DBS Standard	Correlation coefficient (r2)
Ala	0.98	C2	0.98
Arg	0.99	C3	0.98
Orn	0.99	C3DC	0.99
Glu	0.98	C4	0.98
Tyr	0.99	C4DC	0.99
Asa	0.98	C4OH	0.99
Cit	0.99	C5	0.99
Gly	0.95	C5:1	0.99
Leu	0.99	C5DC	0.99
Met	0.98	C5OH	0.99
Phe	0.99	C6	0.99
Val	0.98	C8	0.98
Pro	0.98	C8:1	0.99
Hcy	0.98	C10	0.99
Trp	0.98	C10:1	0.99
2-aminoadipate	0.98	C12	0.98
Threonine	0.99	C12OH	0.99
Ser	0.99	C14	0.98
Asn	0.99	C14:1	0.99
His	0.99	C14OH	0.98
Lys	0.99	C16	0.99
3 -Methyl-L-Histidine	0.99	C16:1	0.99
1- Methyl-L-Histidine	0.99	C16OH	0.99
Succinylacetone	0.99	C18	0.98
C0	0.98	C18:1	0.99
		C18OH	0.99

Table 2 A Precision & Recovery of Amino Acid & Succinyl Acetone in the Ms/Ms Assay On Blood Spots Samples

Analytes spiked in whole blood	Level I			Level II			Level III			Recovery (%)
	Mean Conc. (uM/L)	Intraday Precision (CV %)	Interday Precision (CV %)	Mean Conc. (uM/L)	Intraday Precision (CV %)	Interday Precision (CV %)	Mean Conc. (uM/L)	Intraday Precision (CV %)	Interday Precision (CV %)	
Ala	256	5.71	7.14	384.00	6.62	8.28	512	7.9	9.88	96
Arg	32	6.32	7.90	48.00	7.34	9.18	64	9.4	11.75	87
Orn	250	10.58	13.23	375.00	9.42	11.78	500	10.39	12.99	87
Glu	500	10.83	13.54	750.00	8.93	11.16	1500	9.7	12.13	98
Tyr	32	8.88	11.10	48.00	8.54	10.68	64	7.65	9.56	85
Asa	128	9.41	11.76	192.00	7.42	9.28	512	8.7	10.88	95
Cit	31.25	8.82	11.03	46.88	7.24	9.05	125	6.9	8.63	82
Gly	128	8.06	10.08	192.00	9.76	12.20	512	10.19	12.74	90
Leu	250	8.07	10.09	375.00	6.52	8.15	500	7.65	9.56	91
Met	128	9.42	11.78	192.00	7.52	9.40	256	5.69	7.11	86
Phe	64	9.34	11.68	96.00	7.52	9.40	256	5.12	6.40	91
Val	125	7.3	9.13	187.50	8.2	10.25	500	5.7	7.13	88
Pro	160	6.8	8.50	240.00	5.6	7.00	320	9.7	12.13	101
Hcy	2.5	9.78	12.23	3.75	8.35	10.44	10	6.52	8.15	73

Trp	40	9.76	12.20	60.00	9.85	12.31	80	10.54	13.18	96
2-aminoadipate	1.25	9.32	11.65	1.88	10.52	13.15	2.5	9.48	11.85	96
Threonine	1	7.52	9.40	1.50	8.98	11.23	2	8.26	10.33	98
Ser	160	4.8	6.00	240.00	5.2	6.50	320	3.16	3.95	100
Asn	40	8.65	10.81	60.00	9.45	11.81	80	6.2	7.75	97
His	40	8.51	10.64	60.00	9.45	11.81	80	8.55	10.69	73
Lys	160	5.18	6.48	240.00	5.37	6.71	320	4.29	5.36	92
3-Methyl-L-Histdine	2	8.6	10.75	3.00	10.6	13.25	4	5.75	7.19	92
1-Methyl-L-Histdine	4	7.21	9.01	6.00	8.95	11.19	8	8.54	10.68	86
L Taurine	64	9.49	11.86	96.00	6.3	7.88	100	7.42	9.28	89
4-hydroxy-L-Proline	256	8.74	10.93	384.00	8.74	10.93	1000	7.04	8.80	82
Succinylacetone	2	10.3	12.88	3.00	8.5	10.63	8	9.85	12.31	84

Table 2 B Precision & Recovery of Acylcarnitines in the Ms/Ms Assay on Blood Spots Samples

Analytes spiked in whole blood	Level I			Level II			Level III			Recovery (%)
	Mean Conc. (uM/L)	Intraday Precision (CV %)	Interday Precision (CV %)	Mean Conc. (uM/L)	Intraday Precision (CV %)	Interday Precision (CV %)	Mean Conc. (uM/L)	Intraday Precision (CV %)	Interday Precision (CV %)	
C0	30.8	7.81	9.76	46.20	7.66	9.58	60.8	7.93	9.91	96
C2	7.6	6.89	8.61	11.40	9.93	12.41	15.2	4.31	5.39	98
C3	1.52	8.31	10.39	2.28	8.94	11.18	3.04	8.21	10.26	89
C3DC	0.4	7.9	9.88	1.07	6.5	8.13	1.6	5.29	6.61	84
C4	1.52	9.61	12.01	2.28	10.31	12.89	3.04	6.57	8.21	88
C4DC	0.3	7.4	9.25	0.93	6.9	8.63	1.4	9.01	11.26	86
C4OH	1.52	6.22	7.78	2.28	7.1	8.88	3.04	6.61	8.26	90
C5	1.52	10.6	13.25	2.28	7.9	9.88	3.04	5.4	6.75	85
C5:1	0.25	10.14	12.68	1.33	10.04	12.55	2	8.12	10.15	81
C5DC	0.76	10.89	13.61	1.14	10.43	13.04	1.52	11.72	14.65	87
C5OH	0.38	8.89	11.11	0.57	8.45	10.56	0.76	7.84	9.80	81
C6	0.7	8.1	10.13	2.20	5.7	7.13	3.3	6.9	8.63	84
C8	1.52	10.8	13.50	2.28	8.8	11.00	3.04	5.11	6.39	89
C8:1	0.125	10.67	13.34	0.19	12.08	15.10	0.25	5.17	6.46	84
C10	0.33	7.14	8.93	1.61	4.52	5.65	2.41	6.06	7.58	96
C10:1	0.25	10.67	13.34	0.67	8.45	10.56	1	9.14	11.43	85
C12	0.38	8.8	11.00	0.57	6.7	8.38	1.3	6.1	7.63	96
C12OH	1	12.45	15.56	1.50	10.66	13.33	2	10.59	13.24	89
C14	1.52	10.7	13.38	2.28	10.8	13.50	3.04	8.09	10.11	89
C14:1	1	8.58	10.73	1.50	11.84	14.80	2	10.59	13.24	84
C14OH	0.049	11.28	14.10	0.53	9.45	11.81	0.79	7.11	8.89	80
C16	3.04	6.6	8.25	4.56	6.4	8.00	6.08	5.5	6.88	92
C16:1	0.12	9.64	12.05	0.67	10.97	13.71	1	9.01	11.26	81
C16OH	0.25	9.02	11.28	0.38	7.52	9.40	0.5	6.05	7.56	86
C18	0.76	10.2	12.75	1.14	10.66	13.33	1.52	9.34	11.68	89
C18:1	0.125	10.67	13.34	0.19	12.08	15.10	0.25	10.17	12.71	81
C18OH	2	9.3	11.63	3.00	10.9	13.63	4	8.8	11.00	82

Table No 3-Evaluation of Method Performance by External QC Material (CDC)

Analyte	Level I		Level II		Level III		Level IV	
	Expected mean	Observed mean	Expected mean	Observed mean	Expected mean	Observed mean	Expected mean	Observed mean
Ala	232.50	220.88	430.50	412.62	603.40	584.97	778.60	763.03
Arg	5.90	5.07	70.70	61.40	144.00	126.64	211.10	187.88
Cit	10.30	8.34	31.80	26.03	91.60	75.98	207.70	174.47
Gly	232.60	207.01	460.30	413.56	680.30	618.71	910.10	837.29
Leu	68.90	62.01	154.80	140.63	271.70	249.82	472.50	439.43
Met	9.50	8.08	44.00	37.77	112.20	97.55	191.00	168.08
Orn	94.50	81.27	157.30	136.61	218.40	192.07	287.30	255.70
Phe	21.70	19.53	99.60	90.48	174.10	160.08	254.70	236.87
SUAC	0.20	0.17	0.90	0.75	2.40	2.04	4.90	4.21
Tyr	24.00	20.16	166.70	141.44	310.50	266.86	460.30	400.46
Val	58.40	50.81	201.30	176.83	305.40	271.64	414.50	373.05
C0	15.80	15.01	25.70	24.63	35.50	34.42	46.10	45.18
C2	9.90	9.60	18.00	17.61	26.00	25.73	34.50	34.50
C3	1.10	0.97	4.30	3.82	7.60	6.84	11.00	10.01
C4OH	0.00	0.00	0.30	0.27	0.60	0.55	1.30	1.20
C4	0.10	0.09	0.80	0.70	2.20	1.96	3.60	3.24
C5	0.10	0.08	0.40	0.34	1.20	1.03	2.30	2.00
C5DC	0.00	0.00	0.40	0.35	0.80	0.70	2.00	1.78
C5OH	0.60	0.48	1.50	1.21	2.30	1.88	3.10	2.57
C6	0.00	0.00	0.30	0.25	0.70	0.59	1.60	1.38
C8	0.00	0.00	0.40	0.36	0.80	0.72	2.10	1.91
C10	0.10	0.10	0.50	0.48	1.00	0.97	2.50	2.45
C12	0.00	0.00	0.90	0.86	1.70	1.65	2.70	2.65
C14	0.00	0.00	0.50	0.44	1.30	1.17	2.70	2.46
C16	0.80	0.73	4.10	3.77	7.10	6.60	10.50	9.87
C16OH	0.00	0.00	0.30	0.26	1.10	0.96	1.60	1.41
C18	0.70	0.62	1.70	1.51	3.60	3.24	5.70	5.19
C18OH	0.00	0.00	0.10	0.08	0.50	0.41	0.80	0.67

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