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Arginine, as a Key Indicator for Real-Time Stability Monitoring of Quality Control in the Newborn Screening Test Using Dried Blood Spot

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Abstract: Dried blood spots (DBS) have advantages such as minimizing blood collection volume and the distress to neonate. DBS have been used for tandem mass spectrometry (MS/MS)-based newborn screening tests (NST) of amino acid (AA) and acylcarnitine. The Newborn Screening Quality Assurance Program (NSQAP) have been provided quality control (QC) materials for MS/MS, as DBS cards. The NSQAP is generally provided within 14 months of the shelf life and the recommended storage condition is at −10 °C to −30 °C. Previously, several accelerated degradation studies had been performed to determine the transportation stability and short-term stability of AAs and acylcarnitines in DBS. However, the experimental condition is markedly different to the storage condition. We performed long-term monitoring for the real-time stability of seven AAs and 14 acylcarnitines from three levels of 2012 NSQAP QC materials across a time period of 788 days. Arginine suddenly yielded a catastrophic degeneration pattern, which started around D300. When comparing this with previous accelerated degradation studies, methionine, tyrosine, citrulline, and acetylcarnitine did not show a remarkable measurand drift for the real-time stability, except for arginine. Our study showed that arginine would require intensive QC monitoring in routine practice, and should be used for the assessment of the stability in long-term storage of DBS samples for biobanking.

Keywords: arginine; dried blood spot; amino acid; acylcarnitine; real-time stability; accelerated degradation study; biobanking

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1. Introduction

Inborn errors of metabolism (IEM) are treatable disorders for which timely treatment is critical to prevent mortality and to improve the outcome [1–3]. The newborn screening test (NST) is helpful for early detection of IEM [4], and it has been performed annually for approximately 10 million neonates worldwide [5]. In the 1990's, tandem mass spectrometry (MS/MS)-based NST for amino acid (AA) and acylcarnitine (AC) was developed and was implemented as a routine test for the simultaneous screening of various IEM to be carried out in an efficient and cost-effective manner [6,7]. Dried blood spots (DBS) have advantages such as minimizing blood collection volume and the distress to neonate [8], therefore DBS has been used for MS/MS-based NST [9,10]. Biobanking for residual DBS specimens may be useful for additional diagnostic use in unexpected causes of IEM for the child and family, research projects, and in the development of new NBS assays [11].

MS/MS-based NST is easily modified according to the laboratory's demands [12]. These in-house modifications, which are categorized as laboratory-developed tests (LDTs) cause the difficulty of quality assurance (QA) and inter-laboratory comparison [13], because there are no reference methods of MS/MS-based NST [14]. The Centers for Disease Control and Prevention (CDC) have operated the Newborn Screening Quality Assurance Program (NSQAP) for quality management of NST [15]. The NSQAP have provided QA services including quality control (QC) materials to participating laboratories. The NSQAP QC materials for MS/MS had been provided as DBS cards containing a basal pool and a basal

pool rich in specific metabolite concentrations in a linear range [14]. The reliable QC materials with obtained values from NSQAP participating laboratories within a similar MS/MS method and instrumentation help to maintain QA by quantitative internal QC monitoring [8].

According to clinical and laboratory standard institute guideline (CLSI) NBS04, the QC material stability and storage conditions should be provided by the supplier [16]. The NSQAP generally provided 14 months from shipping date as the shelf life at −10 °C to −30 °C with storage in securely sealed all zip-closures on bags. Previously, accelerated degradation studies had been performed to predict the QC performance and the stability of AAs and ACs faster than those observed in real-time stability studies [17–20]. However, each AA and AC has a different degradation pattern [17,18,20] and the long term in-use stability of the NSQAP QC is still unclear. Furthermore, Ebrahim et. al., recently suggested that an accelerated stability model based on the Arrhenius Equation does not accurately predict the stability of diagnostic reagents [21]. Due to the humidity which may have a significant impact on the stability of AAs and ACs, use of the Arrhenius Model is not considered as discrepancies between the prediction from accelerated stability studies and observation from the real-time stability monitoring may occur. The integrity of QC material is critical to management of QA and to meeting requirements of proficiency testing [22]. Therefore, the laboratory should determine the stability to monitor the performance and ensure the absence of critical degradation during the QC material storage period [16].

In the current study, we measured the three level NSQAP QC materials for MS/MS, in consecutive routine practice for MS/MS-based NST up to 28 months from shipping date. We analyzed the degradation patterns of seven AAs consisting of phenylalanine (Phe), leucine (Leu), methionine (Met), tyrosine (Tyr), valine (Val), citrulline (Cit), and arginine (Arg), and 14 ACs consisting of free carnitine (C0), acetylcarnitine (C2), propionylcarnitine (C3), butyrylcarnitine (C4), isovalerylcarnitine (C5), glutarylcarnitine (C5DC), hydroxyisovalerylcarnitine (C5OH), hexanoylcarnitine (C6), octanoylcarnitine (C8), decanoylcarnitine (C10), dodecanolycarnitine (C12), myristoylcarnitine (C14), palmitoylcarnitine (C16), and stearoylcarnitine (C18), respectively. By this, we aimed to assess the long-term real-time stability of each analytes from NSQAP QC materials for MS/MS in clinical setting, and to discover a stability biomarker of AAs and ACs in DBS for sample storage and biobanking. The study was approved by the Institutional Review Board of Samsung Medical Center Hospital (IRB file no. 2021-02-078). A waiver of consent was obtained given the nature of the project.

2. Materials and Methods

2.1. Specimens and Storage

We enrolled a total of six NSQAP QC materials for AA consisting of Lot 221 (low), Lot 222 (medium), and Lot 223 (high), and materials for AC consisting of Lot 262 (low), Lot 263 (medium), and Lot 264 (high), respectively. The lots of filter paper used in the QC materials were W111 of Whatman 903 (GE Healthcare, Chicago, IL, USA) and 0120201 of PerkinElmer 226 (PerkinElmer, Waltham, MA, USA). Specimens were received on 16 July 2012 and were stored at −20 °C with securely sealed all zip-closure bags with desiccants, according for NSQAP recommendation.

2.2. Reagents

Methanol and acetonitrile were obtained from Burdick & Jackson (Pittsburg, PA, USA), and formic acid was obtained from Sigma-Aldrich (Saint Louise, MO, USA). 3 N HCL in N-Butanol was obtained from Regis Technologies (Morton Grove, IL, USA). As the internal standards (IS) amino acid reference standards (NSK-A-CE) and carnitine/acylcarnitine reference standards (NSK-B-CE) kits were obtained from Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA). As the QC solution, lot 0211, lot 2912 and lot 2613 of MassCheck Amino Acids, Acylcarnitines, Succinylacetone Dried Blood Spot Controls (Chromsystems, Germany) were used.

2.3. Sample Preparation for Extraction and Derivatization

The DBS cards were punched as a 3.2 mm spot disc using DBS puncher (PerkinElmer, Waltham, MA, USA). The punched spot was extracted with 100 µL IS working solution for 30 min at ambient temperature in 96-well microplates shaken using a plate shaker (Wallac, Turku, Finland). IS working solution was diluted with 100-fold IS stock solution with methanol, which was consisting of one vial of amino acid reference standards with 50% methanol 1 mL and one vial of carnitine/acylcarnitine reference standards kits with 100% methanol 1 mL. As the QC solution, MassCheck Amino Acids, Acylcarnitines, Succinylacetone Dried Blood Spot Controls were added into two empty wells of plate. After extraction, mixtures were transferred to new microplate and were dried by nitrogen gas for 15 min at 40 °C. Derivatization of analytes was performed by adding 50 µL of 3 N HCL in N-Butanol into each well and second drying for 20 min at 40 °C. After the derivatization procedure, 75 µL of redissolved solution was added into each well and centrifuged at 1500× *g* for 5 min at ambient temperature.

2.4. LC-MS/MS Analysis

The analytes in blood spots were analyzed in the same day at the sample preparation using the API 2000 triple quadrupole LC/MS/MS (AB SCIEX, Framingham, MA, USA) equipped with an electrospray ionization (ESI) source in positive scan mode. The samples in each well were injected 10 µL at a flow rate of 0.08 mL/min using autosampler with flow gradient, and mobile phase solution consisting of 80% acetonitrile and 0.01% formic acid. A 5.5 kV ion spray voltage at 300 °C was applied to the ESI system. Pressures for curtain gas, ion source gas 1, and ion source gas 2 were 20, 60, and 60 psi, respectively. Neutral loss scan mode of m/z 102 and of m/z 85.1 were used for the analysis of most AAs and ACs. The multiple reaction monitoring (MRM) scan mode was used for Arg (Q1/Q3: 231.2/70.1) and Cit (Q1/Q3: 232.2/113.1), respectively. ChemoView 1.4.2 software (AB SCIEX, Framingham, MA, USA) was used for AAs and ACs quantification.

2.5. Real-Time Stability Monitoring

Day zero (D0) was set to 3 September 2012, which was the 55th day after the samples were received and stored. According for NSQAP's recommendation, the shelf life of these material was considered until 31 August 2013 (D364). Single measurement of NSQAP QC materials per each batch was performed between 3 September 2012 and 31 October 2014 (D778). The initial data was obtained by collecting data during first two month runs, from D0 to D60. Using these data, the initial mean of each analyte was calculated and was compared with peer group data with derivatized-MS/MS non-kit. According to CLSI EP25, acceptable differences criteria was set to extra 20% of the initial mean [23]. We alternatively assessed the allowable drift of the real-time stability by the comparison of predicted value and measured value to acceptable differences criteria, when compared to the initial mean. Between D0 and D778, commercial QCs were changed twice, on D81 and on D413. Schematic time course of NSQAP QC materials and study design of real-time stability test were illustrated in Figure 1.

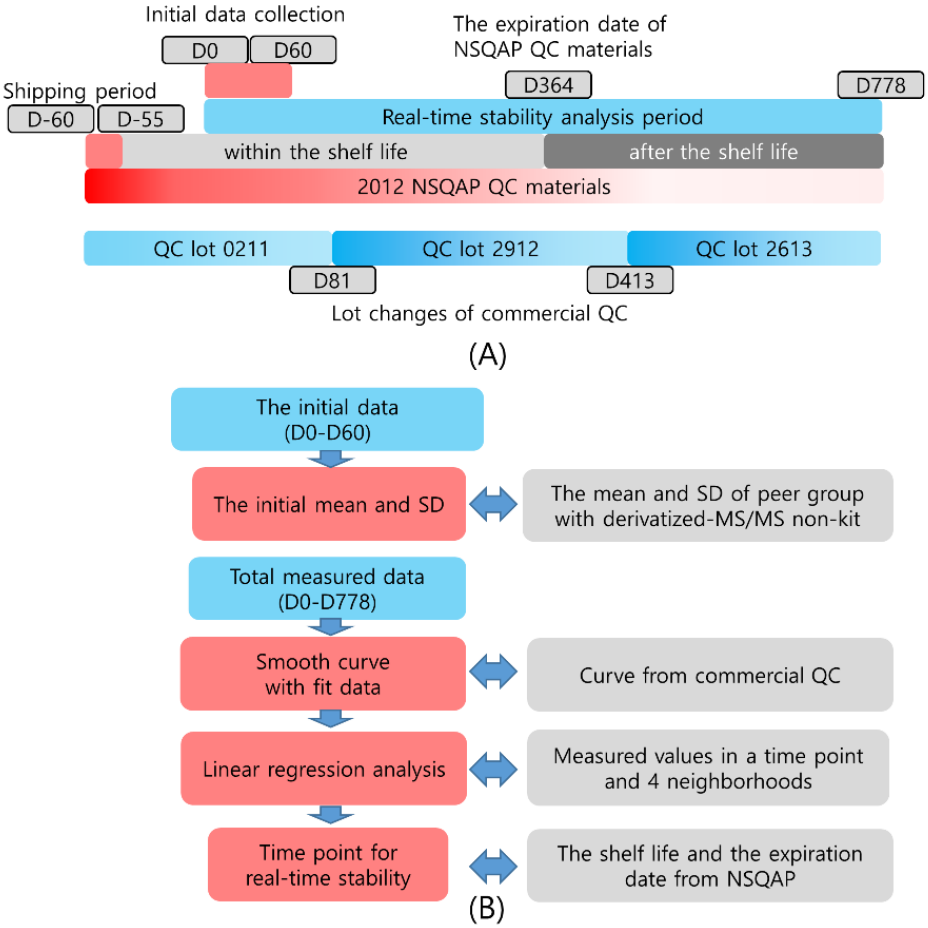

Figure 1. Time course of NSQAP QC marterials and study design of real-time stability test. Between D0 (3 September 2012) and D788 (31 October 2014), schematic time course of NSQAP QC marterials (**A**), and study design of real-time stability (**B**) in current study were illustrated.

2.6. Statistical Analysis

ROUT method was used for removing outliers. Student's *t*-test was used for comparing measured means between initial mean and within the shelf life or after expired shelf life. Linear regression was performed for predicting the time point of in-use stability metrics. Pearson correlation was used for assuming the relationship between time and measured value of each analyte. The Statistical Package for the Social Sciences version 25.0 (IBM Corporation, Armonk, NY, USA) and the GraphPad Prism (version 9.1.2.; GraphPad Software, La Jolla, CA, USA) were used for statistical analyses and graphs. *p* value of less than 0.05 was considered significant.

3. Results

3.1. Characteristics of Routine Practice

A total of 331 procedures were performed between September 2012 and October 2014. Of 331 data pieces, one outlier was removed for statistical analyses, using the ROUT method (Q = 0.5). 151 procedures were performed within the shelf life from D0 to D364 (week1 to week52), while 179 were performed after the shelf life from D365 to D788 (week53 to week113). Each interval of procedures was as same, 2.4 day regardless of the shelf life.

3.2. Method Validation

The repeatability and total imprecision were evaluated with replicated measures at three levels over 20 days. The repeatability of each analytes varied from 2.3% to 19.9% of coefficient of variation (CV), and total imprecision varied from 3.8% to 19.6% of CV,

respectively. The matrix effect of each analyte ranged from 76.7% to 121.6%, and extraction recovery ranged from 88.6% to 137.0%, respectively.

3.3. Comparison between the Initial Data and Peer Group Data with Derivatized-MS/MS Non-Kit

A comparison between the initial data and peer group data with derivatized-MS/MS non-kit [24] was performed. The initial mean and SD were calculated, using 25 obtained data from D0 to D60. The coefficient of variation (CV) of each analyte varied from 4.0% to 15.7%. Of 21 analytes, means of 20 were compared to peer group data, except for Arg. Among 20 analytes, 18 revealed standard deviation index (SDI) within ±2.0 in all three levels, except for Phe and C0 (Phe medium, 256.9 μmol/L vs. 190.8 μmol/L, SDI 2.40; C0 low, 39.7 μmol/L vs. 28.9 μmol/L, SDI 2.06; Phe medium, 56.8 μmol/L vs. 42.0 μmol/L, SDI 2.04) [24].

3.4. Comparison of the Initial Data and Data within the Shelf Life or after the Shelf Life

The consecutive measured values of each analyte from NSQAP QC materials are summarized in Table 1 and illustrated in Figure S1. CVs of each analyt within the shelf life varied from 6.7% to 12.4%. When comparing with the initial mean, Leu, Met, and Arg showed differences in the mean within the shelf life (Leu low, 161.8 μmol/L vs. 153.2 μmol/L, $p = 0.003$; Leu high, 584.7 μmol/L vs. 560.6 μmol/L, $p = 0.030$; Met medium, 78.8 μmol/L vs. 82.0 μmol/L, $p = 0.049$; Arg low, 9.9 μmol/L vs. 9.0 μmol/L, $p < 0.001$), while most analytes did not. After the shelf life from D365 to D788, CVs of each analyte from NSQAP QC materials varied from 6.1% to 12.1%. Among 14 ACs, C0, C2, C3, C5DC, and C12 showed differences in the mean after the shelf life compared to the initial mean (C0 low, 41.3 μmol/L vs. 39.7 μmol/L, P = 0.009; C2 high, 41.5 μmol/L vs. 44.0 μmol/L, $p = 0.030$; C3 low, 5.2 μmol/L vs. 5.0 μmol/L, $p = 0.010$; C5DC high, 1.7 μmol/L vs. 1.9 μmol/L, $p < 0.001$; C12 high, 1.6 μmol/L vs. 1.5 μmol/L, $p = 0.029$), while other ACs did not. However, most AAs, except for Tyr, showed differences in the mean after the shelf life compared to the initial mean.

Table 1. The consecutive measured values of each analyte from NSQAP QC materials, according for the shelf life.

Analyte	Level	Initial			Within the Shelf Life				After the Shelf Life				Total			
		D0 to D60 (n = 25)			D0 to D364 (n = 151)				D365 to D788 (n = 179)				D0 to D788 (n = 330)			
(μmol/L)		Mean	SD	CV	Mean	SD	CV	*p*	Mean	SD	CV	*p*	Mean	SD	CV	Range
Phe	low	89.7	10.5	11.7%	91.5	8.1	8.9%	N.S	95.9	7.5	7.8%	<0.001	93.9	8.1	8.6%	76.0 to 116.4
	medium	256.9	35.3	13.7%	256.7	24.4	9.5%	N.S	269.5	22.7	8.4%	0.017	263.7	24.3	9.2%	205.0 to 336.8
	high	459.7	50.2	10.9%	471.5	39.9	8.5%	N.S	495.1	47.5	9.6%	<0.001	484.3	45.8	9.4%	377.0 to 596.1
Leu	low	153.2	15.1	9.9%	161.8	13.1	8.1%	0.003	169.1	13.9	8.2%	<0.001	165.8	14.0	8.5%	135.0 to 212.4
	medium	343.6	37.1	10.8%	347.8	32.5	9.3%	N.S	358.7	31.9	8.9%	0.031	353.7	32.5	9.2%	276.0 to 436.4
	high	560.6	48.6	8.7%	584.7	51.3	8.8%	0.030	599.1	57.0	9.5%	0.002	592.5	54.7	9.2%	472.0 to 727.1
Met	low	23.6	2.4	10.3%	23.1	2.3	9.9%	N.S	23.6	2.1	9.1%	N.S	23.4	2.2	9.5%	19.0 to 27.9
	medium	82.0	8.9	10.9%	78.8	7.2	9.2%	0.049	78.4	7.8	10.0%	0.035	78.6	7.5	9.6%	59.0 to 103.2
	high	191.2	18.7	9.8%	189.4	17.9	9.5%	N.S	184.2	17.6	9.5%	N.S	186.6	17.9	9.6%	151.0 to 225.1
Tyr	low	66.6	6.7	10.0%	65.7	5.9	9.0%	N.S	66.7	6.4	9.5%	N.S	66.2	6.2	9.3%	51.0 to 84.5
	medium	313.2	31.4	10.0%	310.0	26.9	8.7%	N.S	316.3	27.3	8.6%	N.S	313.4	27.3	8.7%	253.0 to 395.9
	high	502.5	46.4	9.2%	500.2	42.3	8.5%	N.S	507.8	48.0	9.4%	N.S	504.3	45.6	9.0%	391.0 to 624.8
Val	low	173.3	16.8	9.7%	179.0	16.4	9.2%	N.S	182.7	15.2	8.3%	0.005	181.0	15.9	8.8%	149.0 to 235.0
	medium	384.1	37.7	9.8%	385.5	36.6	9.5%	N.S	395.7	39.6	10.0%	N.S	391.0	38.6	9.9%	302.0 to 488.9
	high	644.2	56.7	8.8%	663.0	57.4	8.7%	N.S	669.7	62.7	9.4%	N.S	666.6	60.3	9.0%	537.0 to 831.6
Cit	low	26.9	2.2	8.0%	27.5	1.9	7.0%	N.S	28.0	1.7	6.3%	0.004	27.8	1.8	6.7%	21.0 to 33.6
	medium	78.7	6.7	8.5%	78.7	5.9	7.5%	N.S	78.7	4.9	6.2%	N.S	78.7	5.4	6.9%	58.0 to 97.3
	high	169.7	12.4	7.3%	174.5	12.6	7.2%	N.S	172.9	10.5	6.1%	N.S	173.7	11.5	6.6%	135.0 to 207.0
Arg	low	9.0	0.9	9.7%	9.9	0.9	9.4%	<0.001	10.6	1.0	9.1%	<0.001	10.3	1.0	9.8%	7.3 to 12.5
	medium	28.4	3.3	11.8%	29.2	2.7	9.2%	N.S	27.5	2.4	8.9%	N.S	28.3	2.7	9.5%	21.0 to 34.4
	high	40.0	3.7	9.2%	39.6	4.2	10.5%	N.S	30.8	2.8	9.2%	<0.001	34.8	5.6	16.2%	23.0 to 49.1
C0	low	39.7	3.6	9.0%	40.3	3.1	7.7%	N.S	41.3	2.7	6.5%	0.009	40.9	2.9	7.2%	34.0 to 48.6
	medium	56.8	5.0	8.8%	57.5	5.0	8.7%	N.S	57.7	4.6	7.9%	N.S	57.6	4.8	8.3%	45.0 to 72.1
	high	76.5	5.4	7.0%	76.8	5.7	7.4%	N.S	76.7	7.0	9.1%	N.S	76.8	6.4	8.4%	60.0 to 97.4
C2	low	25.2	1.9	7.6%	25.2	1.9	7.7%	N.S	25.1	1.8	7.2%	N.S	25.1	1.9	7.4%	20.0 to 30.0
	medium	34.2	2.3	6.8%	34.1	2.5	7.4%	N.S	33.6	2.8	8.3%	N.S	33.8	2.7	7.9%	27.0 to 42.3
	high	44.0	2.9	6.5%	43.2	2.6	5.9%	N.S	41.5	3.6	8.7%	0.002	42.3	3.3	7.8%	34.0 to 51.6
C3	low	5.0	0.5	9.4%	5.1	0.4	7.7%	N.S	5.2	0.3	6.5%	0.010	5.1	0.4	7.1%	4.2 to 6.1
	medium	10.3	0.8	7.5%	10.4	0.8	7.8%	N.S	10.2	0.8	7.5%	N.S	10.3	0.8	7.7%	8.4 to 13.0
	high	15.5	1.0	6.7%	15.6	1.0	6.7%	N.S	15.3	1.2	8.2%	N.S	15.4	1.2	7.6%	13.0 to 18.7
C4	low	0.9	0.1	9.2%	0.9	0.1	10.5%	N.S	0.9	0.1	11.4%	N.S	0.9	0.1	11.0%	0.7 to 1.1
	medium	1.9	0.3	13.6%	2.0	0.2	10.4%	N.S	1.9	0.2	10.3%	N.S	2.0	0.2	10.4%	1.5 to 2.5
	high	3.9	0.5	11.8%	3.8	0.4	10.9%	N.S	3.7	0.4	10.5%	N.S	3.7	0.4	10.8%	2.8 to 4.6

Table 1. *Cont.*

Analyte	Level	Initial			Within the Shelf Life				After the Shelf Life				Total			
		D0 to D60 (n = 25)			D0 to D364 (n = 151)				D365 to D788 (n = 179)				D0 to D788 (n = 330)			
(μmol/L)		Mean	SD	CV	Mean	SD	CV	*p*	Mean	SD	CV	*p*	Mean	SD	CV	Range
C5	low	0.6	0.1	15.7%	0.6	0.1	11.7%	N.S	0.6	0.1	10.7%	N.S	0.6	0.1	11.1%	0.4 to 0.7
	medium	1.5	0.2	14.5%	1.5	0.2	10.7%	N.S	1.5	0.2	10.4%	N.S	1.5	0.2	10.5%	1.2 to 1.8
	high	2.7	0.2	9.1%	2.7	0.3	9.2%	N.S	2.8	0.3	9.7%	N.S	2.8	0.3	9.5%	2.1 to 3.3
C5DC	low	0.4	0.1	14.3%	0.4	0.0	11.8%	N.S	0.4	0.0	11.8%	N.S	0.4	0.0	11.9%	0.3 to 0.5
	medium	0.7	0.1	11.0%	0.7	0.1	10.3%	N.S	0.7	0.1	12.1%	N.S	0.7	0.1	11.3%	0.5 to 0.9
	high	1.9	0.2	10.8%	1.8	0.2	11.1%	N.S	1.7	0.2	11.5%	<0.001	1.7	0.2	11.7%	1.2 to 2.2
C5OH	low	0.8	0.1	11.1%	0.8	0.1	9.8%	N.S	0.8	0.1	10.1%	N.S	0.8	0.1	10.0%	0.6 to 1.0
	medium	1.2	0.2	14.7%	1.2	0.2	12.4%	N.S	1.2	0.1	10.1%	N.S	1.2	0.1	11.2%	0.9 to 1.5
	high	1.7	0.2	12.3%	1.7	0.2	10.8%	N.S	1.7	0.2	10.3%	N.S	1.7	0.2	10.5%	1.3 to 2.1
C6	low	0.5	0.1	12.7%	0.5	0.1	12.1%	N.S	0.5	0.1	11.6%	N.S	0.5	0.1	11.8%	0.4 to 0.6
	medium	0.8	0.1	14.1%	0.9	0.1	12.0%	N.S	0.9	0.1	11.5%	N.S	0.9	0.1	11.7%	0.7 to 1.1
	high	2.0	0.2	10.2%	2.1	0.2	10.5%	N.S	2.0	0.2	10.1%	N.S	2.0	0.2	10.3%	1.6 to 2.5
C8	low	0.6	0.1	9.2%	0.6	0.1	10.5%	N.S	0.6	0.1	10.6%	N.S	0.6	0.1	10.5%	0.5 to 0.9
	medium	1.1	0.1	12.4%	1.1	0.1	10.8%	N.S	1.1	0.1	10.0%	N.S	1.1	0.1	10.4%	0.9 to 1.4
	high	2.8	0.3	10.5%	2.8	0.3	10.1%	N.S	2.7	0.3	11.1%	N.S	2.8	0.3	10.7%	2.1 to 3.5
C10	low	0.4	0.0	13.4%	0.4	0.0	11.1%	N.S	0.4	0.0	11.5%	N.S	0.4	0.0	11.3%	0.3 to 0.5
	medium	1.1	0.1	13.3%	1.1	0.1	11.0%	N.S	1.1	0.1	11.2%	N.S	1.1	0.1	11.1%	0.8 to 1.4
	high	2.2	0.3	13.4%	2.2	0.2	11.1%	N.S	2.2	0.2	11.1%	N.S	2.2	0.2	11.1%	1.6 to 2.8
C12	low	0.4	0.1	14.7%	0.4	0.0	10.7%	N.S	0.4	0.0	11.8%	N.S	0.4	0.0	11.5%	0.3 to 0.5
	medium	0.7	0.1	11.8%	0.7	0.1	11.1%	N.S	0.7	0.1	10.3%	N.S	0.7	0.1	10.9%	0.5 to 0.9
	high	1.5	0.1	9.1%	1.5	0.2	10.1%	N.S	1.6	0.2	10.2%	0.029	1.5	0.2	10.3%	1.1 to 1.9
C14	low	0.6	0.1	10.7%	0.6	0.1	9.4%	N.S	0.6	0.1	11.3%	N.S	0.6	0.1	10.4%	0.5 to 0.8
	medium	1.6	0.3	15.8%	1.7	0.2	11.4%	N.S	1.7	0.2	9.9%	N.S	1.7	0.2	10.7%	1.3 to 2.1
	high	3.3	0.4	10.5%	3.3	0.3	8.9%	N.S	3.4	0.4	10.4%	N.S	3.4	0.3	9.8%	2.7 to 4.2
C16	low	4.6	0.4	8.4%	4.8	0.4	8.8%	N.S	4.8	0.4	9.1%	N.S	4.8	0.4	8.9%	3.9 to 5.9
	medium	8.2	0.8	9.2%	8.5	0.8	9.1%	N.S	8.5	0.8	9.9%	N.S	8.5	0.8	9.5%	6.6 to 10.5
	high	11.5	1.4	12.3%	11.8	1.2	9.7%	N.S	12.0	1.2	10.1%	N.S	11.9	1.2	9.9%	8.4 to 14.9
C18	low	1.9	0.2	11.3%	1.9	0.2	9.8%	N.S	1.9	0.2	10.0%	N.S	1.9	0.2	9.9%	1.5 to 2.4
	medium	3.0	0.4	12.1%	3.0	0.3	10.6%	N.S	3.0	0.3	10.6%	N.S	3.0	0.3	10.6%	2.2 to 3.7
	high	6.1	0.7	11.0%	6.2	0.6	9.7%	N.S	6.3	0.6	9.4%	N.S	6.2	0.6	9.5%	5.0 to 7.7

Amino acid; low: Lot 221; medium: Lot 222; high: Lot 223 of NSQAP QC materials; Acylcarnitine; low: Lot 262; medium: Lot 263; high: Lot 264 of NSQAP QC materials; Phe: phenylalanine; Leu: leucine; Met: methionine; Tyr: tyrosine; Val: valine; Cit: citrulline; Arg: arginine; C0: free carnitine; C2: acetylcarnitine; C3: propionylcarnitine; C4: butyrylcarnitine; C5: isovalerylcarnitine; C5DC: glutarylcarnitine; C5OH: hydroxyisovalerylcarnitine; C6: hexanoylcarnitine; C8: octanoylcarnitine; C10: decanoylcarnitine; C12: dodecanolycarnitine; C14: myristoylcarnitine; C16: palmitoylcarnitine; C18: stearoylcarnitine.

3.5. Real-Time Stability Analysis

Using data obtained from D0 to D788, we predicted the allowable time point as D542 for real-time stability of NSQAP QC materials. We calculated the predicted measurand drift of D542 (eD542) and the predicted percent difference of D542 compared with the initial mean (e%D542). The real-time stability of NSQAP QC materials was reviewed by the comparison between e%D542 and the measured percent difference of D542 compared with the initial mean (%D542). The results of real-time stability analysis are summarized in Table 2.

Table 2. The results of real-time stability analysis, using data obtained from D0 to D788.

Analyte (μmol/L)	Level	Slope	Intercept	SE Slope	F	*p* Value	eD542 (μmol/L)	%eD542	$\%D542_{adj}$
Phe	low	0.012	89.4	0.002	38.6	<0.001	95.6	6.6%	2.0%
	medium	0.029	252.3	0.006	25.7	<0.001	267.9	4.3%	1.4%
	high	0.056	462.2	0.011	27.7	<0.001	492.6	7.2%	1.5%
Leu	low	0.022	157.2	0.003	46.8	<0.001	169.0	10.3%	0.1%
	medium	0.032	341.1	0.008	17.3	<0.001	358.4	4.3%	−0.5%
	high	0.048	573.4	0.013	13.9	<0.001	599.7	7.0%	0.3%
Met	low	0.001	23.2	0.001	1.0	0.329	23.4	−0.9%	0.3%
	medium	−0.003	79.6	0.002	2.0	0.158	78.2	−4.6%	−1.8%
	high	−0.010	190.3	0.004	4.9	0.027	185.1	−3.2%	0.5%
Tyr	low	0.004	64.8	0.001	5.8	0.016	66.7	0.2%	−7.2%
	medium	0.019	306.1	0.007	8.1	0.005	316.2	1.0%	−12.1%
	high	0.025	494.3	0.011	5.3	0.022	508.0	1.1%	−5.8%
Val	low	0.014	175.4	0.004	14.3	0.000	183.1	5.6%	13.4%
	medium	0.025	381.2	0.009	7.3	0.007	394.7	2.8%	1.6%
	high	0.041	650.2	0.014	8.3	0.004	672.7	4.4%	−4.8%
Cit	low	0.002	27.2	0.000	12.3	0.001	28.0	3.9%	7.8%
	medium	0.002	78.0	0.001	1.6	0.203	78.9	0.3%	−1.8%
	high	0.001	173.1	0.003	0.3	0.616	173.9	2.4%	−6.9%
Arg	low	0.001	9.8	0.000	23.6	<0.001	10.5	15.9%	30.2%
	medium	−0.004	30.0	0.001	51.5	<0.001	27.7	−2.7%	0.6%
	high	−0.019	42.5	0.001	507.3	<0.001	32.0	−20.1%	−24.5%
C0	low	0.003	39.7	0.002 to 0.004	20.5	<0.001	41.3	4.1%	1.6%
	medium	0.001	57.2	−0.001 to 0.003	1.0	0.314	57.8	1.7%	0.1%
	high	0.003	75.7	−0.000 to 0.006	3.0	0.082	77.2	0.9%	−2.8%
C2	low	−0.001	25.5	−0.002 to 0.000	4.2	0.041	25.0	−1.0%	2.0%
	medium	−0.003	34.9	−0.004 to −0.001	17.6	<0.001	33.4	−2.4%	3.1%
	high	−0.004	43.9	−0.006 to −0.003	29.6	<0.001	41.7	−5.2%	−3.4%
C3	low	0.000	5.0	0.000	7.6	0.006	5.2	3.9%	5.6%
	medium	0.000	10.4	−0.001	3.8	0.051	10.2	−1.0%	2.0%
	high	0.000	15.5	−0.001	0.8	0.374	15.4	−0.8%	−3.4%
C4	low	0.000	0.9	0.000	0.7	0.397	0.9	3.0%	8.9%
	medium	0.000	2.0	0.000	0.6	0.445	2.0	1.8%	2.3%
	high	0.000	3.8	0.000	7.6	0.006	3.7	−5.0%	−10.6%
C5	low	0.000	0.6	0.000	1.0	0.328	0.6	−0.4%	−0.7%
	medium	0.000	1.5	0.000	0.1	0.717	1.5	−1.0%	3.8%
	high	0.000	2.7	0.000	5.1	0.025	2.8	4.0%	4.5%
C5DC	low	0.000	0.4	0.000	18.0	<0.001	0.4	−5.6%	−6.2%
	medium	0.000	0.7	0.000	15.2	<0.001	0.7	−4.2%	0.9%
	high	0.000	1.8	0.000	31.7	<0.001	1.7	−10.8%	−14.2%
C5OH	low	0.000	0.8	0.000	0.1	0.778	0.8	0.7%	5.9%
	medium	0.000	1.2	0.000	0.1	0.706	1.2	−0.5%	6.9%
	high	0.000	1.7	0.000	1.3	0.258	1.7	0.8%	4.9%
C6	low	0.000	0.5	0.000	3.5	0.064	0.5	3.9%	5.4%
	medium	0.000	0.9	0.000	0.2	0.660	0.9	4.6%	7.0%
	high	0.000	2.1	0.000	1.5	0.215	2.0	0.5%	6.3%
C8	low	0.000	0.6	0.000	1.1	0.306	0.6	−1.7%	−6.0%
	medium	0.000	1.1	0.000	0.4	0.506	1.1	4.4%	5.3%
	high	0.000	2.8	0.000	1.6	0.204	2.7	−0.8%	−8.0%
C10	low	0.000	0.4	0.000	0.1	0.729	0.4	5.7%	6.3%
	medium	0.000	1.1	0.000	0.4	0.513	1.1	0.3%	11.1%
	high	0.000	2.2	0.000	1.3	0.250	2.2	−2.8%	−0.9%
C12	low	0.000	0.4	0.000	22.7	<0.001	0.4	8.2%	10.6%
	medium	0.000	0.7	0.000	25.0	<0.001	0.7	9.6%	14.9%
	high	0.000	1.5	0.000	21.2	<0.001	1.6	4.7%	5.5%
C14	low	0.000	0.6	0.000	0.0	0.975	0.6	−0.4%	−0.7%
	medium	0.000	1.7	0.000	4.7	0.032	1.7	4.9%	6.6%
	high	0.000	3.3	0.000	6.2	0.013	3.4	1.4%	−1.6%
C16	low	0.000	4.7	0.000	3.1	0.077	4.8	3.1%	9.4%
	medium	0.000	8.5	0.000	0.0	0.962	8.5	3.0%	8.3%
	high	0.001	11.7	0.000 to 0.001	4.4	0.036	12.0	4.4%	3.2%
C18	low	0.000	1.9	0.000	2.9	0.090	1.9	0.6%	−0.8%
	medium	0.000	3.0	0.000	0.0	0.878	3.0	−0.9%	3.5%
	high	0.000	6.1	0.000 to 0.001	6.9	0.009	6.3	3.4%	1.4%

Intercept: Y-intercept; SE: standard error; eD542: estimated value on D542; %eD542: the percent difference between initial value and estimated value on D542; $\%D542_{adj}$: the percent difference between initial value and mean of measured values on D542 and 4 neighborhoods; Amino acid; low: Lot 221; medium: Lot 222; high: Lot 223 of NSQAP QC materials; Acylcarnitine; low: Lot 262; medium: Lot 263; high: Lot 264 of NSQAP QC materials; Phe: phenylalanine; Leu: leucine; Met: methionine; Tyr: tyrosine; Val: valine; Cit: citrulline; Arg: arginine; C0: free carnitine; C2: acetylcarnitine; C3: propionylcarnitine; C4: butyrylcarnitine; C5: isovalerylcarnitine; C5DC: glutarylcarnitine; C5OH: hydroxyisovalerylcarnitine; C6: hexanoylcarnitine; C8: octanoylcarnitine; C10: decanoylcarnitine; C12: dodecanolycarnitine; C14: myristoylcarnitine; C16: palmitoylcarnitine; C18: stearoylcarnitine.

Of 21 analytes, most analytes except for Arg were predicted that eD542 met within the allowable acceptance criteria, ±20% difference compared with the initial mean. Among 21 analytes, 18 analytes were predicted that e%D542 was within ±10% difference compared with the initial mean, while three analytes, Leu, Arg, and C5DC were predicted that e%D542 was over ±10% (Leu low, 10.3%; Arg low, 15.9%; Arg high, −20.1%; C5DC high, −10.8%, respectively) (Table 3 and Figure 2). Each mean of %D542 and 4 neighborhoods between D538 and D546 (%$D542_{adj}$) was compared with paired e%D542. Overall, e%D542 yielded 0.2% lower estimations rather than %$D542_{adj}$. Among 21 analytes, 20 analytes could meet the allowable acceptance criteria, while Arg could not meet the criteria (Arg high, −24.5%; Arg low, 30.2%).

Table 3. The results of correlation analysis between time and measured values of each analyte.

Analyte	Level	ρ	95% Confidence Interval	*p*
Phe	low	0.32	0.22 to 0.42	<0.001
	medium	0.27	0.17 to 0.37	<0.001
	high	0.28	0.18 to 0.38	<0.001
Leu	low	0.35	0.26 to 0.44	<0.001
	medium	0.22	0.12 to 0.32	<0.001
	high	0.20	0.10 to 0.30	<0.001
Met	low	0.05	−0.05 to 0.16	N.S
	medium	−0.08	−0.18 to 0.03	N.S
	high	−0.12	−0.23 to -0.01	0.027
Tyr	low	0.13	0.02 to 0.24	0.016
	medium	0.16	0.05 to 0.26	0.005
	high	0.13	0.02 to 0.23	0.022
Val	low	0.20	0.10 to 0.31	<0.001
	medium	0.15	0.04 to 0.25	0.007
	high	0.16	0.05 to 0.26	0.004
Cit	low	0.19	0.08 to 0.29	<0.001
	medium	0.07	−0.04 to 0.18	N.S
Arg	low	0.26	0.16 to 0.36	<0.001
	high	−0.78	−0.82 to -0.73	<0.001
C0	low	0.24	0.14 to 0.34	<0.001
	medium	0.06	−0.05 to 0.16	N.S
	high	0.10	−0.01 to 0.20	N.S
C2	low	−0.11	−0.22 to 0.00	0.041
	medium	−0.23	−0.33 to -0.12	<0.001
	high	−0.29	−0.38 to -0.19	<0.001
C3	low	0.15	0.04 to 0.25	0.006
	medium	−0.11	−0.21 to 0.00	0.051
	high	−0.05	−0.16 to 0.06	0.374
C4	low	−0.05	−0.15 to 0.06	0.397
	medium	−0.04	−0.15 to 0.07	0.445
	high	−0.15	−0.25 to -0.04	0.006
C5	low	0.05	−0.05 to 0.16	N.S
	medium	−0.02	−0.13 to 0.09	N.S
	high	0.12	0.02 to 0.23	0.025
C5DC	low	−0.23	−0.33 to -0.12	<0.001
	medium	−0.21	−0.31 to -0.11	<0.001
	high	−0.30	−0.39 to -0.20	<0.001
C5OH	low	0.02	−0.09 to 0.12	N.S
	medium	−0.02	−0.13 to 0.09	N.S
	high	0.06	−0.05 to 0.17	N.S
C6	low	−0.10	−0.21 to 0.01	N.S
	medium	−0.02	−0.13 to 0.08	N.S
	high	−0.07	−0.18 to 0.04	N.S
C8	low	−0.06	−0.16 to 0.05	N.S
	medium	0.04	−0.07 to 0.14	N.S
	high	−0.07	−0.18 to 0.04	N.S
C10	low	0.02	−0.09 to 0.13	N.S
	medium	−0.04	−0.14 to 0.07	N.S
	high	−0.06	−0.17 to 0.04	N.S
C12	low	0.25	0.15 to 0.35	< 0.001
	medium	0.27	0.16 to 0.36	< 0.001
	high	0.25	0.14 to 0.35	< 0.001
C14	low	0.00	−0.11 to 0.11	N.S
	medium	0.12	0.01 to 0.22	0.032
	high	0.14	0.03 to 0.24	0.013
C16	low	0.10	−0.01 to 0.20	N.S
	medium	0.00	−0.11 to 0.11	N.S
	high	0.12	0.01 to 0.22	0.036
C18	low	0.09	−0.01 to 0.20	N.S
	medium	0.01	−0.10 to 0.12	N.S
	high	0.14	0.04 to 0.25	0.009

Amino acid; low: Lot 221; medium: Lot 222; high: Lot 223 of NSQAP QC materials; Acylcarnitine; low: Lot 262; medium: Lot 263; high: Lot 264 of NSQAP QC materials; Phe: phenylalanine; Leu: leucine; Met: methionine; Tyr: tyrosine; Val: valine; Cit: citrulline; Arg: arginine; C0: free carnitine; C2: acetylcarnitine; C3: propionylcarnitine; C4: butyrylcarnitine; C5: isovalerylcarnitine; C5DC: glutarylcarnitine; C5OH: hydroxyisovalerylcarnitine; C6: hexanoylcarnitine; C8: octanoylcarnitine; C10: decanoylcarnitine; C12: dodecanolycarnitine; C14: myristoylcarnitine; C16: palmitoylcarnitine; C18: stearoylcarnitine.

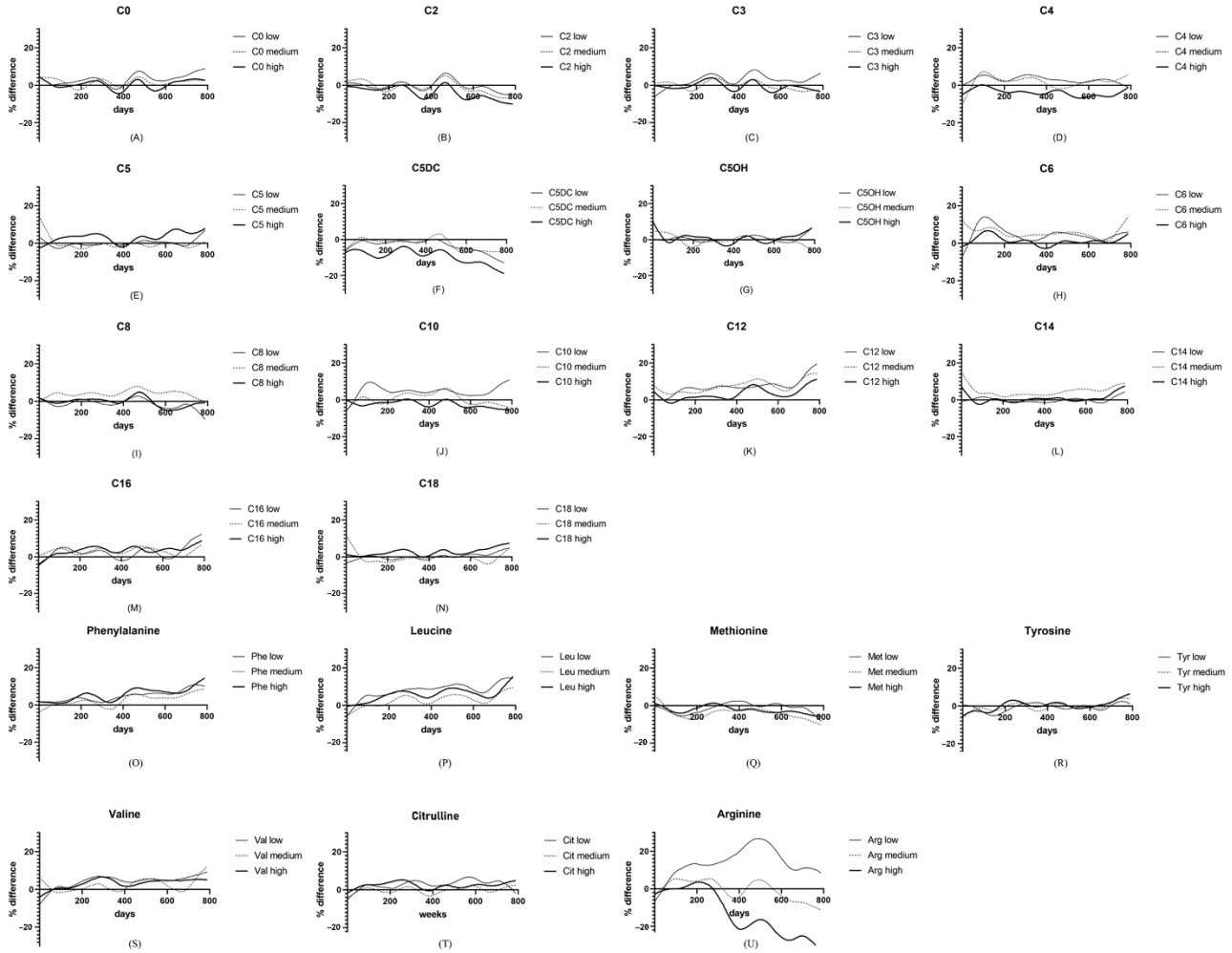

Figure 2. Nomogram for the percent difference between measured value and the initial mean between D0 and D788. Smooth curves derived from the percent difference between consecutive measured values and initial means of each analytes. According for level of NSQAP QC materials, thin solid lines, spotted lines, and bold sold lines represented low, medium, and high level, respectively. Amino acid; low: Lot 221; medium: Lot 222; high: Lot 223 of NSQAP QC materials; Acylcarnitine; low: Lot 262; medium: Lot 263; high: Lot 264 of NSQAP QC materials; C0: free carnitine (**A**); C2: acetylcarnitine (**B**); C3: propionylcarnitine (**C**); C4: butyrylcarnitine (**D**); C5: isovalerylcarnitine (**E**); C5DC: glutarylcarnitine (**F**); C5OH: hydroxyisovalerylcarnitine (**G**); C6: hexanoylcarnitine (**H**); C8: octanoylcarnitine (**I**); C10: decanoylcarnitine (**J**); C12: dodecanolycarnitine (**K**); C14: myristoylcarnitine (**L**); C16: palmitoylcarnitine (**M**); C18: stearoylcarnitine (**N**); Phe: phenylalanine (**O**); Leu: leucine (**P**); Met: methionine (**Q**); Tyr: tyrosine (**R**); Val: valine (**S**); Cit: citrulline (**T**); Arg: arginine (**U**).

3.6. Correlation Analysis Aetween Aime and Analytes

The results of correlation analysis between time and measured values of each analyte are summarized in Table 3. Of 21 analytes, 18 analytes showed a negligible correlation within ± 0.3 of pearson's ρ, except for Phe, Leu, and Arg. Among these three analytes, Arg showed a strong negative correlation with lower -0.7 of pearson's ρ ($\rho = -0.78$, $p < 0.001$), while Phe and Leu showed low positive correlations within ± 0.5 of pearson's ρ, respectively (Phe low, $\rho = 0.32$, $p < 0.001$; Leu low, $\rho = 0.35$, $p < 0.001$, respectively).

4. Discussion

Quantitation of AAs and ACs for MS/MS-based NST using the DBS requires a degree of uniformed absorbance volume of analytes on the DBS card [25]. Hematocrit and filter paper are most critical factors for determining absorbance volume and the chromatographic

distribution of the analytes on the DBS [26]. Hematocrit might influence flux and diffusion properties of the blood because it has a profound effect on viscosity [27]. When hematocrit was increased from 40% to 65%, serum volume per 3.2 mm disk decreased by 27%, while a diameter of spot decreased by less than 8% [28]. Filter paper might show different levels of imprecision according to individual products and their lots [29]. To overcome the hematocrit effect, a calibration strategy for quantitation in the DBS of patients with limited blood volume less than 50 µL and unknown hematocrit level had been suggested [27]. Moreover, the DBS spot made from verified filter paper with intact blood of fixed volume and fixed hematocrit level could reveal the acceptable quality and homogeneity, by controlling the hematocrit effect. The NSQAP QC materials were made using 100 µL of washed intact red blood cells at a 55% hematocrit with FDA approved filter papers [30]. The mean serum absorbance volumes for used lots of filter paper were ranged from 1.44 µL to 1.49 µL per 3.2 mm disk for intact red blood cells, in the 2012 NSQAP QC materials [24]. Thesevalues were within the acceptable range from CLSI NBS01 ED7, 1.454 ± 0.11 µL [25]. In current study, the concentration of analytes from the qualified DBS cards was measured, using the ratio between the peak intensity of analyte and that of the stable isotope labeled IS.

Verification of the real-time stability in the clinical laboratory is generally disturbed by bias or variability due to various factors such as instrument hardware changes and laboratory environment fluctuations over the study duration [23]. We compared commercial QCs and Arg high, and reviewed the historical chart and the nomogram (Figure 3). The results revealed that there was no definitive measurement error during the study, such as calibration failure or rejected QC, except for one procedure. One occurred by a specimen injection problem, and was removed by an outlier identification process. Our data showed periodic fluctuation patterns, which may highlight or mask the effect of measurand drift, without an instrument replacement. Lot 2912 and lot 2613 of commercial QC for AAs showed the similar fluctuation pattern of Arg high, while no remarkable measurand drift or correlation with time was observed in the commercial QCs, contrast to Arg high (Figure 3). In the current study, the discrepancies between real-time measured values and predicted values from a linear regression analysis might be results from variability due to laboratory environment fluctuations.

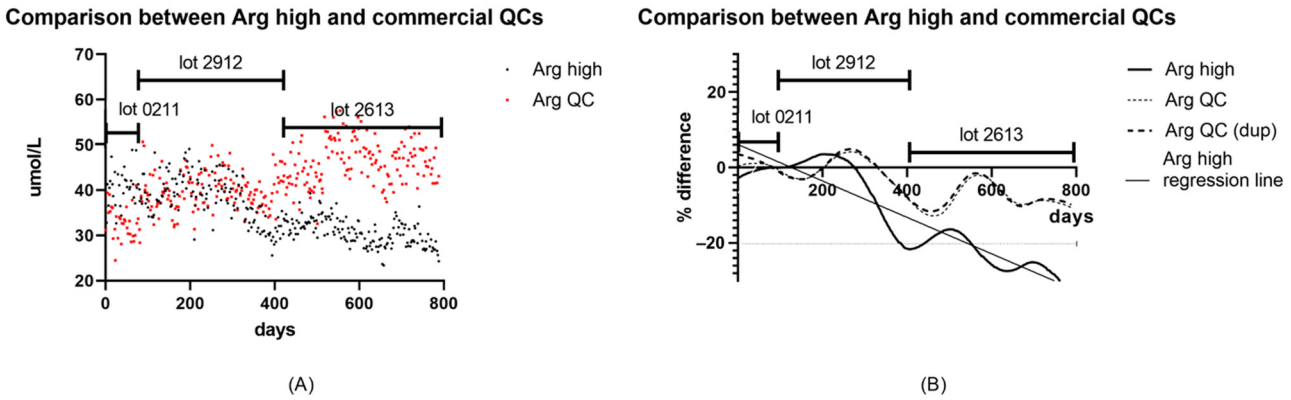

Figure 3. Comparison between consecutive measured values and percent difference of arginine high from NSQAP QC material and commercial QCs. Consecutive values of arginine high (black) and commercial QCs (red) were scattered (**A**), and each whisker means the usage periods of each commercial QC. On the comparison between arginine high and commercial QCs (**B**), bold solid line, spotted line, dashed line, and thin sold line represented arginine high, duplicated commercial QCs, and the regression line of arginine, respectively. Each curve was smoothed using difference percentages between measured values and each initial means. The meeting point of curves of arginine high (bold solid line) and the regression line of arginine (thin sold line) at the around −20% of difference was approximately D542.

Temperature and humidity are factors that affect the denaturation of analytes in DBS during storage or transport to the laboratory [17–20,31]. NSQAP QC materials were repeatedly exposed to ambient temperature and altered humidity during each routine procedure,

though exposure time and manner were minimized. While several excursions to room temperature were not affected for stability of DBS [32], the high humidity might have a critical role for stability of analytes from DBS. The accelerated degradation study had been conducted at 37 °C for predetermined intervals in low-humidity and high-humidity environments [17]. According for the research by Adam et al., Arg had been reported as the most sensitive AA to the effects of high humidity, and Arg lost 95% of its concentration at 37 °C within 35 days in the over 50% humidity condition [17]. The research also showed that galactose-1-phosphate uridyltransferase, biotinidase, succinylacetone, C2, malonylcarnitine, decenoylcarnitine, and tetradecenoylcarnitine lost 95% of their concentration within 25 to 35 days in the same condition [17]. However, C2 showed a negligible negative correlation with time, and other analytes were not measured in our study (Table 4). The gaps between accelerated degradation study and real-time stability monitoring were significantly observed in this study. Therefore, we agree with the suggestion of a recent study that it is necessary to highlight the limitations of the accelerated stability model [21].

Table 4. Comparison between real-time monitoring and accelerated degradation studies.

Study	This Study	Adam et al.	Golbahar et al.
Publication year	2021	2011	2014
Method	real-time monitoring	accelerated degradation study	accelerated degradation study
Analytes	7 AAs, 14 ACs	7 AAs, 18 ACs, 8 hormones and metabolites	7AAs, 10 ACs
Study length	778 days	35 days	8 days
Compared temperature	−20 °C	37 °C	37 °C
Compared humidity	<30%	>50%	>70%
Compared analytes		7 AAs, 9 ACs	
most sensitive analytes	Arg, Phe, Leu	Arg, C2, C3	Met, Tyr, Arg
least sensitive analytes	C5OH, C6, C8, C10	Val, C5, C16, C18	C0, C5, Phe
Reference		[17]	[20]

AA: Amino acid; Acylcarnitine: AC Phe: phenylalanine; Leu: leucine; Met: methionine; Tyr: tyrosine; Val: valine; Arg: arginine; C0: free carnitine; C2: acetylcarnitine; C3: propionylcarnitine; C5: isovalerylcarnitine; C5OH: hydroxyisovalerylcarnitine; C6: hexanoylcarnitine; C8: octanoylcarnitine; C10: decanoylcarnitine; C16: palmitoylcarnitine; C18: stearoylcarnitine.

The storage stability of DBS specimens is an important issue for biobanking or second usage of residual samples. However, the long-term stability studies which were conducted at −20 °C for AAs and ACs in the DBS were limited [33,34]. Our data showed that most AAs and ACs have storage stability at −20 °C at least 2 years, and these results were in line with the previous studies [33,34]. However, the studies had not included Arg, while high susceptibility of Arg in DBS to heat-related and humidity-related degradation was reported [17].

In the current study, Arg showed different degeneration patterns between a low and high level. To evaluate the initial mean, we compared the initial mean and Y-intercept from each linear regression equation. All of the CVs were within 15% for the three level of 21 analytes over 778 days, including CV of Arg low. Most analytes showed no significant difference between the initial mean and Y-intercept, except for Arg low (initial mean vs. Y-intercept, 9.0 μmol/L vs. 9.8 μmol/L, $p = 0.0001$). Furthermore, Arg low showed a negligible positive correlation with time ($\rho = 0.26$, $p < 0.001$). These figures indicated that the predicted measurand drift of Arg low was overestimated due to the undervalued initial mean of Arg low (Figure 2U).

The current study has a limitation in assessing sample storage stability, because the study was performed using readymade QC material, not freshly prepared DBS. To assess the storage stability, sources of variation related to sample collection, transport, and storage would be clearly evaluated [35]. However, uncertainties around transport and pre-storage duration might disturb the assessment of short-term stability for DBS. A recent study showed that the decline of AAs and ACs in the freshly prepared DBS primarily occurred from one to three months of storage [36]. However, the storage instability of DBS samples in a short time period could not be observed in this study, because D0 was the 55th day after

the samples were received and stored at −20 °C. According for the nomograms, a linear regression line or a quadratic regression curve is not suitable for reflecting a degeneration pattern of AAs and ACs. High order kinetics including humidity factor with a polynomial curve [37] may be appropriate for the interpolation of continuous measured values of 21 analytes. Notably, Arg high suddenly yielded a catastrophic degeneration pattern, which started around D300 (Figure 2U). Further trend analysis using other lots of NSQAP QC materials for AAs high should be performed for elucidating whether this pattern and trend are observed at similar times. Taken together, these results indicate that there is a need for QC monitoring of MS/MS-based NST, including Arg high, and, furthermore, more intensive monitoring is required for Arg.

5. Conclusions

In conclusion, we showed that the real-time stability of NSQAP QC materials was allowable until D542, longer than 14 months from shipping date, as the shelf life from NSQAP's recommendation. There was a discrepancy of relatively sensitive analytes between our real-time stability monitoring and previous accelerated degradation study. In the current study, Arg showed the remarkable measurand drift from D300 to D778, therefore Arg would require intensive QC monitoring, in routine practice of MS/MS-based NST. Additionally, our study suggests that Arg should be used for the assessment of the stability in long-term storage DBS samples for biobanking.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/separations8110201/s1, Figure S1: Consecutive measured values of 21 analytes from NSQAP QC materials between D0 to D788.

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Informed Consent Statement: A waiver of consent was obtained given the nature of the project.

Data Availability Statement: Refer to Supplementary Materials Section.

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