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## Stability of Amino Acids in Stored Dried Blood Spots: Retrospective Analysis

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**Abstract:** Residual newborn screening dried blood spots (DBS) are a valuable resource for research in the retrospective diagnosis of inborn errors of metabolism and biomarker analysis. Many metabolites are subject to degradation depending on time and storage conditions, such as temperature and humidity. We studied the stability of 15 amino acids (AAs) in dried blood spots stored in a refrigerator after newborn screening. **Methods:** We retrospectively analyzed the levels of 15 AAs in 248 residual DBS from the Kazakhstan neonatal screening program using tandem mass spectrometry. DBS were stored at 4°C and 55-70% humidity and randomly selected during 2019-2022. Amino acid stability was assessed using linear regression and estimating the decrease in concentration of each metabolite during each year. **Results:** Retrospective analysis of dried blood spot samples stored for one to four years showed that the decrease in concentrations of 15 AA occurred in order from most stable to least stable: valine, proline, isoleucine, leucine, tyrosine, phenylalanine, alanine, arginine, hydroxyproline, methionine, citrulline, glutamate, ornithine, glycine, and glutamine. Alanine, arginine, hydroxyproline, glycine, and glutamate decayed in line with linear regression. **Conclusion:** Storing dried blood spots at 4°C and 55-70% humidity is not optimal for amino acid stability. Data obtained from measuring amino acid levels in dried blood spots stored over time should be corrected to reduce the incidence of negative and false positive results.

**Keywords:** Amino acids, Dried blood spot, Metabolite stability, Inborn errors of metabolism, Tandem mass spectrometry

### Introduction

Dried blood spots (DBS) allow for quick and easy collection and storage of human biospecimens (Freeman et al., 2018; Ward et al., 2021). DBS collection has many advantages over “standard” venous blood collection (Trifonova et al., 2019). DBS cards collected for newborn screening (NBS) are often stored for an extended period (Strnadova et al., 2007). DBS samples are collected from almost all of the more than 400 thousand children born in Kazakhstan annually. Unused parts of these samples (residual samples) are stored for three years after completion of testing.

Residual newborn screening DBS samples are a valuable resource for research (Benkendorf et al., 2010; Rothwell et al., 2019). Stored DBS can provide valuable samples for retrospective diagnosis of inborn errors of metabolism (IEM) and biomarker analysis, as well as for validation of NBS programs (Strnadová et al., 2007; Freeman et al., 2018; van Rijt et al., 2020; Dijkstra et al., 2020). 2023). This is especially important in resource-constrained countries (Ward et al., 2021; Ottosson et al., 2023).

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However, diagnostic uncertainties arising from the instability of metabolites during long-term storage have not been systematically and comprehensively studied (Strnadová et al., 2007; Fingerhut et al., 2009; van Rijt et al., 2020; Shimada et al., 2022; Dijkstra et al., 2023; Ottosson et al., 2023). When considering using DBS samples for clinical or research purposes, the data obtained from secondary use should be interpreted carefully (Murphy et al., 2018). Retrospective analyses using stored DBS are limited by the lack of information on the long-term stability of analytes in stored samples (Shimada et al., 2022).

Many metabolites are subject to time-dependent degradation. The stability of metabolites is assessed by the change in their concentration in DBS over time (van Rijt et al., 2020; Dijkstra et al., 2023). The stability of metabolites in dried blood spots must be assessed over the relatively short time intervals required to transport the sample to the laboratory as over the long period of sample storage with the possibility of re-analysis.

Factors influencing the stability of metabolites include storage time, temperature, and humidity. Humidity can cause or exacerbate analyte degradation, mainly due to hydrolysis reactions (Wagner et al., 2016). DBS requires low humidity and low-temperature conditions for transportation and storage (Adam et al., 2011; Golbahar et al., 2014). Adam et al. (2011) believe that most of the degradation of metabolic markers is due to the adverse effects of storage at high humidity and temperatures of 37°C. Among other things, it should be remembered that the quality of blood parameters analyzed from DBS samples depends on field conditions, especially the spot size. Smaller DBSs are associated with lower measured metabolite levels (Moat et al., 2020; Crimmins et al., 2020; Börsch-Supan et al., 2021; Groh et al., 2022).

According to Ottosson et al. (2023), the metabolomics of DBS samples during long-term storage at -20 °C in biobanks is suitable for retrospective epidemiological studies. Palmer et al. (2019) recommend collecting and transporting DBS samples within 28 days at room temperature and storing for more extended periods at -20 or -80 °C.

Michopoulos et al. (2011) suggest that the stability of metabolites in DBS samples is reduced if the cards are not stored at a temperature of at least -20°C, and preferably -80°C. In addition, untreated cards are recommended to minimize background interference incurred. Thus, factors influencing the stability of metabolites in DBS samples include storage time, temperature, humidity, and the type of paper used to collect DBS samples (Michopoulos et al., 2011; Wagner et al., 2016). Assessing annual metabolite decline may allow retrospective diagnosis of IEM in DBS stored over long periods (Strnadová et al., 2007). Van Rijt et al. (2020) recommend including follow-up DBS in diagnostic retrospective cohorts and validation studies using fresh samples and repeatedly reestimating cut-off values.

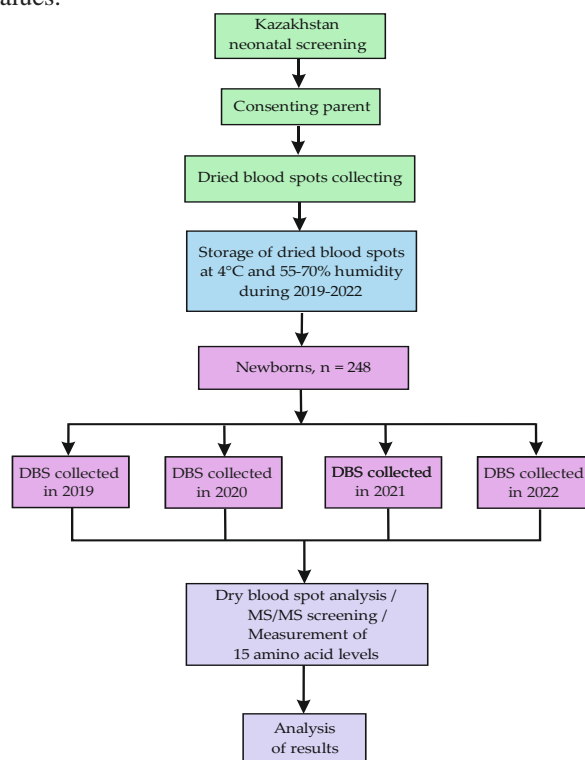


Figure 1. Flowchart of retrospective analysis of dried blood spots.

## Objectives

The study aims to determine the stability of 15 amino acids in DBS stored in a refrigerator after NBS.

## Methods

### Data Sources

The data used in our analysis were obtained by studying the level of 15 AAs in 248 DBS obtained from healthy newborns aged 1-3 days during the Kazakhstan neonatal screening (Figure 1). The DBS stored in the archives were randomly selected. DBS stored at 4°C and 55-70% humidity were randomly selected during 2019-2022 (Table 1).

Table 1. Total storage time and storage conditions of DBS employed in this study.

Group	2019	2020	2021	2022	2023	Storage Time (yrs)
2019	Collection and storage at +4° C				Transport to the laboratory + storage at +4° C	3.5-4.5
2020	Collection and storage at +4° C					2.5-3.5
2021	Collection and storage at +4° C				AA measurements	1.5-2.5
2022	Collection and storage at +4° C					0.5-1.5

In Kazakhstan, neonatal screening is carried out nowadays for two hereditary diseases - phenylketonuria and congenital hypothyroidism, the most represented screening diseases in most countries (Therrell et al., 2018). However, the MS/MS tandem mass spectrometry method is not used in the state newborn screening program, and ENBS for AAD, OA, and FAOD is not carried out in Kazakhstan. Blood samples from newborns on Guthrie cards were previously (until 2019) stored for one year in a refrigerator at 4°C, then at room temperature. Since 2019, DBS samples have been stored permanently at 4°C. The study was approved by the Bioethics Committee of the West Kazakhstan Marat Ospanov Medical University (Ref. No. 7, 09/09/2020.) Written informed consent (IC) was obtained from the parents and/or legal guardians of children after birth to collect a DBS sample.

### Mass Spectrometry Analysis

The Neobase2™ Non-derivatized MSMS kit (PerkinElmer, Wallac Oy, Turku, Finland) were used to quantify 15 amino acids in DBS according to the manufacturer's instructions. Vial with lyophilized isotope-labeled internal standards (IS) containing 2H3-Alanine (Ala IS), 2H4, 13C-Arginine (Arg IS), 2H2-Citrulline (Cit IS), 13C5-Glutamine (Gln IS), 13C5-Glutamic acid (Glu IS), 15N,2-13C-Glycine (Gly IS), 2H3-Leucine (Leu IS), 2H3-Isoleucine (Leu IS), 2H3-Hydroxyproline (Leu IS), 2H3-Methionine (Met IS), 2H6-Ornithine (Orn IS), 13C6-Phenylalanine (Phe IS), 13C5-Proline (Pro IS), 13C6-Tyrosine (Tyr IS), 15N-13C5-Valine (Val IS) was being recovered by adding 1.4 ml of the extraction solution that has been included in the Neobase 2 kit. The Extraction Working Solution (EWS) IS was prepared by diluting the recovered internal standards with the extraction solution of 1:100 (v/v).

DBS were analyzed using a Shimadzu LCMS-8050 Triple Quadrupole Mass Spectrometer (Shimadzu Corporation, Kyoto, Japan). Sample preparation was based on extraction followed by derivatization into oil esters. Level I and Level II (low standard and high standard) dried blood drops were included with each assay lot of the Neobase2™ Non-derivatized MSMS kit to monitor system accuracy and precision.

To analyze amino acids and acylcarnitines, stored DBS card samples were brought to room temperature (+18 to +25°C) before extraction. A 3.2 mm disc (equivalent to ~3.1 µl of whole blood) was punched out of one dried blood spot with a diameter of 3.2 mm using a Wallac DBS Puncher (PerkinElmer, Wallac Oy, Mustionkatu 6, FI-20750 Turku, Finland) into the well of the 96-well polystyrene U-bottom microplate supplied with the Neobase2™ Non-derivatized MSMS kit. After adding 125 µL of working extraction solution to each well of the microplate, the plate was covered with an adhesive aluminum film and incubated for 30 minutes at room temperature on a microplate shaker with a shaking speed of 650 rpm. After incubation, 100 µL of the supernatant

was transferred to a new 96-well U-bottom microplate, covered with aluminum foil to reduce evaporation, and incubated for 1 hour. The plate was then placed into the Shimadzu LCMS-8050 Triple Quadrupole Mass Spectrometer autosampler, and 5  $\mu$ L of supernatant was injected into the LCMS for analysis.

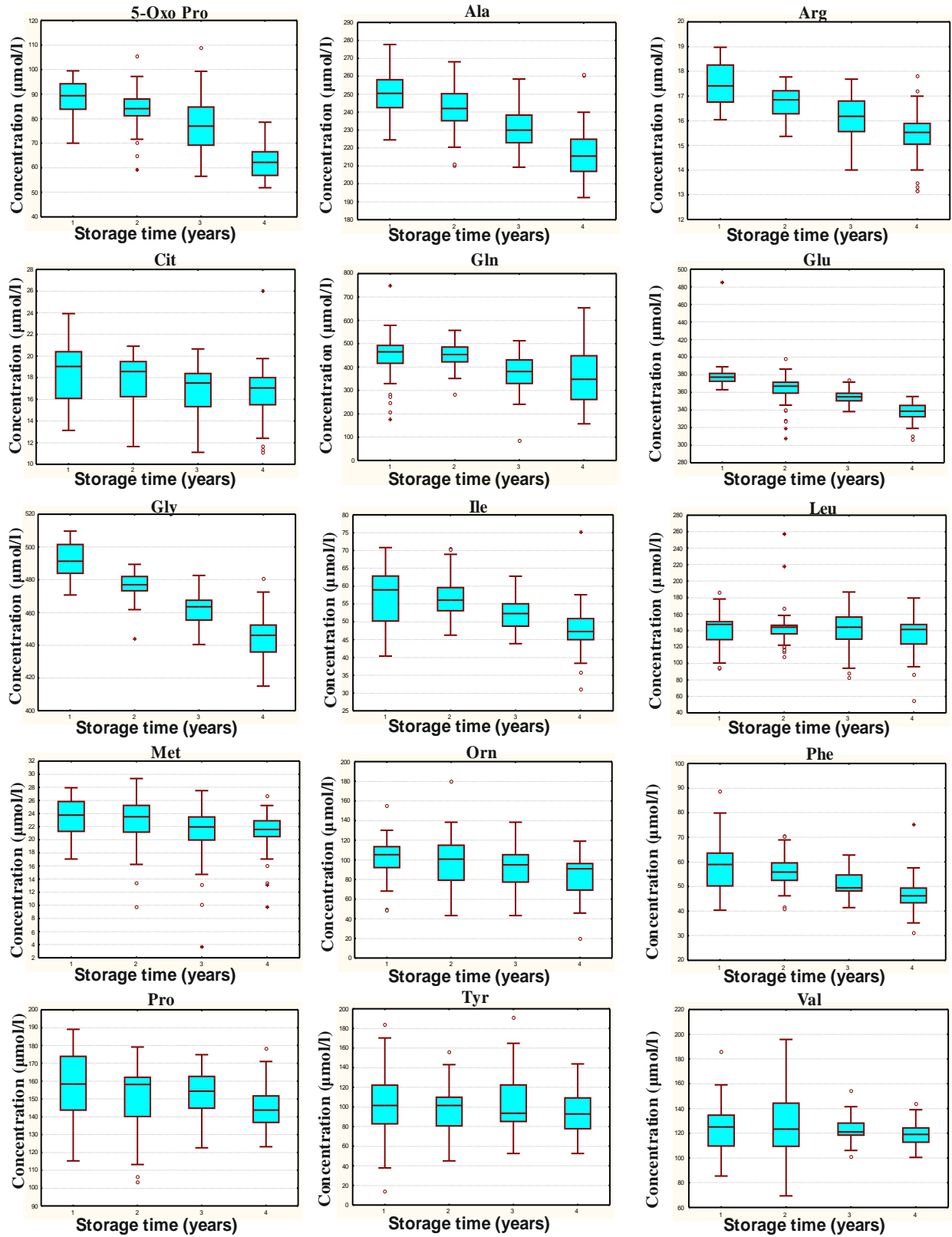


Figure 2. Changes in the concentration of amino acids in dried blood spots at a temperature of 4°C with humidity 55-70% during storage for four years.

(The boxplots represent 1st quartile, median, and 3rd quartile. The whiskers extend to the non-outlier range. Circles = non-extreme outliers, asterisks = extreme outliers).

## Statistical Analysis

Shapiro-Wilk and Kolmogorov-Smirnov tests were used to check the normality of the distribution. The data obtained in the study demonstrated that the distribution of amino acids in DBS differs from normal. Me (median) and quartiles (IQR, interquartile range) were used for descriptive statistics of the samples. Nonparametric tests (Kruskal-Wallis H-test) were used to check differences between AA concentrations depending on storage time. Amino acid stability was assessed using linear regression and estimating the decrease in concentration of each metabolite during each year. Two-sided levels  $<0.05$  are assumed to be statistically significant. Statistical analysis was carried out using the statistical packages IBM SPSS v. 23.0 (IBM, Armonk, NY, USA) and Statistica (StatSoft, Inc., Tulsa, OK, USA, v. 10).

## Results and Discussion

The study results of the amino acids stability in DBS stored for one to four years in a refrigerator at a temperature of  $4^{\circ}\text{C}$  and 55-70% humidity are presented in a series of box plots in Fig. 2, and linear diagrams of amino acid degradation in Fig. 3.

Retrospective analysis of DBS samples stored for one to four years showed that the decrease in concentrations of 15 AA occurred in the order from most stable to least stable: valine, proline, isoleucine, leucine, tyrosine, phenylalanine, alanine, arginine, hydroxyproline, methionine, citrulline, glutamate, ornithine, glycine, and glutamine.

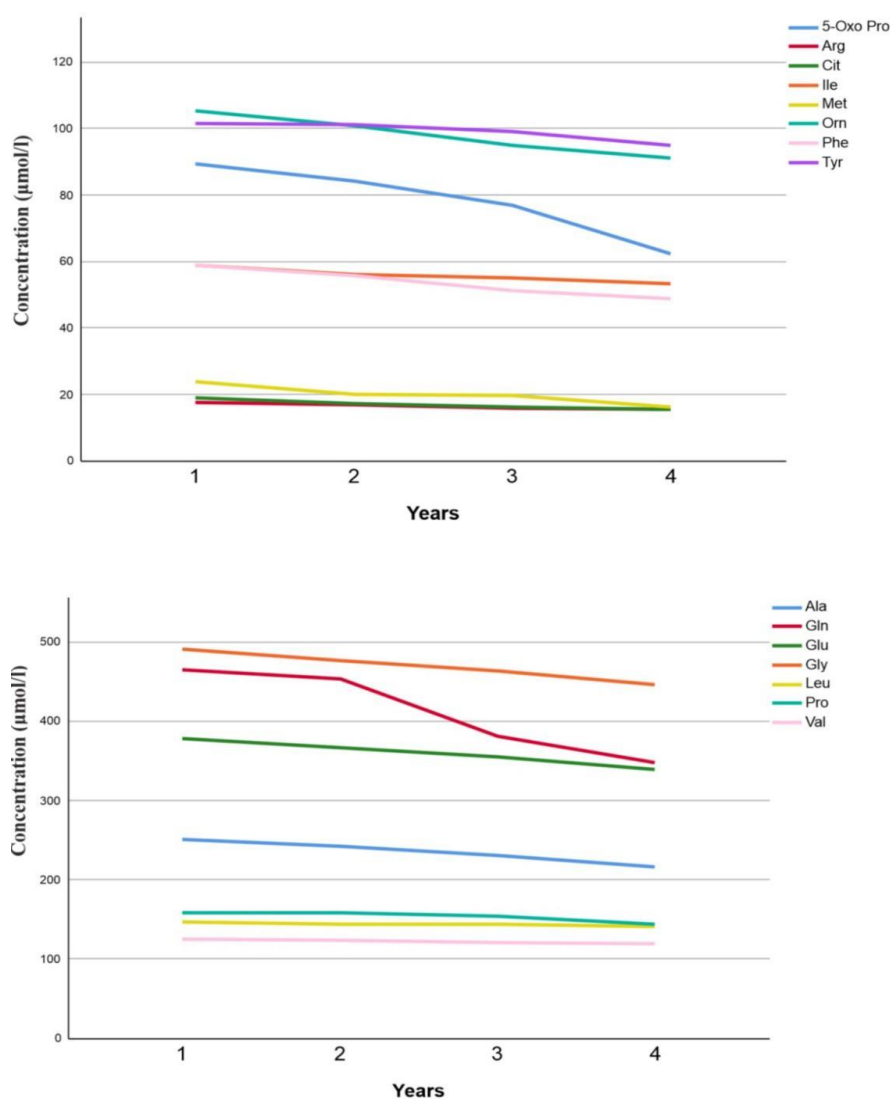


Figure 3. Degradation of amino acids stored at  $4^{\circ}\text{C}$  and humidity 55-70% within four years.

The results of linear regression analysis, where the concentrations of amino acids in DBS were considered dependent variables and the storage time of samples was considered independent variables, are presented in Table 3. Alanine, arginine, hydroxyproline, glycine, and glutamate decayed in line with linear regression.

Table 3. The linear regression formulae and coefficient correlation of R<sup>2</sup>

Amino acid, $\mu\text{mol/l}$	R <sup>2</sup>	linear regression formulae	Durbin-Watson statistic
5-Oxo Pro	0.585	$y = -8,80x + 99.86$	1.28
Ala	0.504	$y = -11.08x + 252.52$	1.73
Arg	0.549	$y = -0.76x + 18.25$	1.69
Cit	0.214	$y = -0.96x + 19.03$	1.91
Gln	0.163	$y = -34.94x + 496.91$	1.87
Glu	0.573	$y = -13.04x + 391.14$	2.07
Gly	0.745	$y = -16.08x + 508.95$	1.85
Ile	0.075	$y = -1.29x + 58.79$	1.81
Leu	0.004	$y = -1.18x + 144.20$	1.81
Met	0.430	$y = -2.26x + 25.49$	1.98
Orn	0.096	$y = -6.33x + 109.99$	1.95
Phe	0.217	$y = -2.97x + 61.21$	1.85
Pro	0.058	$y = -3.38x + 159.52$	2.01
Tyr	0.012	$y = -2.21x + 104.82$	1.67
Val	0.008	$y = -1.34x + 125.87$	2.08

\*The shaded rows indicate the items maintaining a linear correlation with R<sup>2</sup>  $\geq$  0.5.

This study distinguishes three conditional groups of amino acids: those demonstrating the most excellent stability under storage conditions at a temperature of +4°C and a humidity of 55-70%, amino acids with high degradation rates, and a group of amino acids with intermediate rates. The first group included valine, proline, isoleucine, and leucine; the intermediate group had tyrosine, phenylalanine, alanine, arginine, hydroxyproline, and the most unstable were methionine, citrulline, glutamate, ornithine, glycine, and glutamine. According to various studies, the most significant factors influencing the stability of amino acids in DBS are storage time, temperature, and humidity.

Different DBS storage conditions hamper comparative analysis of studies describing amino acid stability in DBS. Some studies describe the stability of the metabolome in DBS under long-term storage conditions. Thus, Prentice et al. (2013) evaluated the stability of metabolites in DBS stored at various temperatures (+21, -20, and -80°C) for two years. Shimada et al. (2022) studied the characteristic stability profiles of amino acids in DBS stored in a refrigerator at 5° C after newborn screening at 1, 3, 6 months, one, and two years of storage. Ottosson et al. (2023) found that most (71%) of the metabolome of neonatal DBS was stable for ten years of storage at -20° C. Dijkstra et al. (2023) investigated the five-year stability of 23 amino acids in residual DBS stored for five years (one year at +4° C and four years at room temperature). Some authors described the stability of amino acids under short-term storage conditions. Ward et al. (2021) found that more than 80% of metabolites are chemically stable in DBS stored at room temperature for a week. Golbahar et al. (2014) examined the short-term effect of heat and humidity on the levels of 7 amino acids for eight days. Han et al. (2018) showed that most amino acids are stable in DBS samples during 4 hours of sunlight exposure.

The relative stability of proline (Shimada et al., 2022, Dijkstra et al., 2023), isoleucine (Dijkstra et al., 2023), alanine (Shimada et al., 2022, Dijkstra et al., 2023), phenylalanine (Shimada et al., 2022, Dijkstra et al., 2023), and valine (Dijkstra et al., 2023) are confirmed by the results of this study. Research data from Han et al. (2018), Moat et al. (2020), and Dijkstra et al. (2023) confirmed the instability of methionine, both under short-term and long-term storage conditions. Methionine is one of the most unstable amino acids in DBS, and at room temperature, it is degraded by at least 50% in six months (Han et al. 2018). Rapidly degrading amino acids also include citrulline, glycine, ornithine (Strnadová et al., 2007; Dijkstra et al., 2023), and glutamine (Dijkstra et al., 2023). In our study, glutamate also showed a high level of degradation.

## Conclusion

Storing dried blood spots at +4°C and 55-70% humidity is not optimal to ensure the stability of amino acids. Some of the AA in DBS from neonatal screening stored under these conditions can have undergone significant

degradation. This may lead to misinterpretation of test results for retrospective biomarker studies and IEM diagnosis.

## **Recommendations**

Data obtained from measuring amino acid levels in DBS stored for a specific time should be adjusted to reduce the incidence of negative and false positive results. Conducting retrospective analyses from DBS samples requires the development of reference values established for similarly stored blood samples from DBS of healthy newborns.

## **Scientific Ethics Declaration**

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

## **Acknowledgements or Notes**

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\*The authors declare no conflict of interest.

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