# Amino Acids in Lyophilized Plasma
# UME CRM 1314

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## SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| $\Delta_m$ | Absolute difference between mean measured value and the certified value |
| ANOVA | Analysis of variance |
| CRM | Certified Reference Material |
| HPLC | High Performance Liquid Chromatography |
| ID-LCMS/MS | Isotope Dilution Liquid Chromatography Tandem Mass Spectrometry |
| ID-LCMS | Isotope Dilution Liquid Chromatography  Mass Spectrometry |
| IS | Internal Standard |
| ISO | International Standards Organisation |
| $k$ | Coverage factor |
| $MS_{between}$ | Mean of squares between-unit from an ANOVA |
| $MS_{within}$ | Mean of squares within-unit from an ANOVA |
| $n$ | Number of replicate measurements |
| NIST | National Institute for Standards and Technology |
| NIST SRM | Standard Reference Material produced by NIST |
| Q-NMR | Quantitative Nuclear Magnetic Resonance |
| $RSD_{stab}$ | Relative standard deviation of stability data |
| $s$ | Standard deviation |
| $s_{bb}$ | Between-unit standard deviation |
| SI | International System of Units |
| SRM | Standard Reference Material |
| SS | Sum of Squares |
| $u_\Delta$ | Combined measurement uncertainty for the difference between measurement result and certified value |
| $U_\Delta$ | Expanded measurement uncertainty for the difference between measurement result and certified value. |
| $u_{bb}$ | Standard uncertainty related to a possible between-unit inhomogeneity |
| $u^*_{bb}$ | Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by method repeatability |
| $u_{char}$ | Standard uncertainty of the material characterisation |
| $u_{CRM}$ | Combined standard uncertainty of CRM |
| $u_{sts}$ | Standard uncertainty for short term stability study |
| $u_{lts}$ | Standard uncertainty for long term stability study |
| $u_{meas}$ | Measurement uncertainty |
| $U_{CRM}$ | Expanded uncertainty of CRM |
| $\nu_{eff}$ | Effective degrees of freedom |
| $\nu_{MS_{within}}$ | $MS_{within}$ degrees of freedom |
| $t_i$ | Time for each repeated analysis |
| $\bar{t}$ | Average of all time periods |
| $t$ | Selected shelf life |

## ABSTRACT

The Certified Reference Material (CRM) is utilized in chemical measurements, as a useful tool for proving traceability of measurement result and enhances measurement quality. Amino acid concentrations are frequently measured for treatment and diagnosis purposes. Amino acid metabolism distortedness is evaluated according to the concentration of amino acids metabolites.

Number of available CRMs to be used in these measurements is very limited. The purpose of this project is production and certification of UME CRM 1314 “Amino Acids in Lyophilized Plasma”. SI traceability of the related method will be proved by utilization of this CRM in the measurements.

This report includes details for the certification of UME CRM 1314 in accordance with the requirements of ISO 17034:2016 standard [1]. The production facilities, chemical analyses, results of homogeneity assessment, stability and characterization studies, statistical evaluation of data and conclusions have been presented and the corresponding uncertainties (Table 1) have been calculated in accordance with the ISO Guide 35:2017 [2].

**Table 1.** Certified values and uncertainties for UME CRM 1314

| | Mass Fraction (mg/kg) | | Molar Concentration[3] (µmol/L) | |
|---|---|---|---|---|
| **Parameter (CAS No)** | **Certified Value[1]** | **Uncertainty[2]** | **Certified Value** | **Uncertainty** |
| Alanine (56-41-7) | 32.6 | 1.7 | 383 | 20 |
| 2-Aminoadipic acid (1118-90-7) | 5.18 | 0.92 | 33.7 | 6.0 |
| 2-Aminobutyric acid (1492-24-6) | 1.65 | 0.23 | 16.8 | 2.4 |
| Anserine (584-85-0) | 12.2 | 2.9 | 53 | 13 |
| Arginine (74-79-3) | 15.13 | 0.93 | 90.9 | 5.6 |
| Asparagine (70-47-3) | 12.9 | 2.5 | 102 | 20 |
| Aspartic acid (56-84-8) | 2.42 | 0.28 | 19.0 | 2.2 |

[1] Certified values are the mean of six measurement results obtained from two units of the CRM by ID-LC-MS technique. The certified values and the uncertainties are traceable to the International System of Units (SI) through a calibration hierarchy using high purity materials of each parameter that were value-assigned using TÜBİTAK UME qNMR purity assessment procedure.

[2] The expanded uncertainty of certified value includes characterization, homogeneity, stability components and is stated as the standard uncertainty of measurement multiplied by the coverage factor $k$ = 2, which for a normal distribution corresponds to a coverage probability of approximately 95%. The standard uncertainty of measurement has been determined in accordance with GUM “Guide to the Expression of Uncertainty in Measurement”.

[3] Certified values and the uncertainties in molar concentrations are calculated from the mass fraction (mg/kg) using density of the reconstituted material (mean: 1.04702 g/mL, SD: 0.00481 g/mL, $n$ = 15) measured at 22 °C and molecular weight of the analyte.

**Table 1.** (*Continued)* Certified values and uncertainties for UME CRM 1314

| | Mass Fraction (mg/kg) | | Molar Concentration[3] (µmol/L) | |
|---|---|---|---|---|
| **Parameter (CAS No)** | **Certified Value[1]** | **Uncertainty[2]** | **Certified Value** | **Uncertainty** |
| Beta-alanine (107-95-9) | 8.5 | 1.4 | 100 | 17 |
| Citrulline (372-75-8) | 15.8 | 1.2 | 94.4 | 7.2 |
| Cystathionine (56-88-2) | 2.94 | 0.11 | 13.85 | 0.52 |
| 4-Aminobutyric acid (56-12-2) | 1.57 | 0.21 | 15.9 | 2.2 |
| Glutamic acid (56-86-0) | 12.0 | 1.9 | 85 | 14 |
| Glycine (56-40-6) | 21.2 | 1.8 | 296 | 26 |
| Histamine (51-45-6) | 1.31 | 0.15* | 12.3 | 1.5* |
| Histidine (71-00-1) | 17.7 | 1.2 | 119.4 | 8.1 |
| Hydroxyproline (51-35-4) | 5.24 | 0.97 | 41.8 | 7.8 |
| Isoleucine (73-32-5) | 9.9 | 1.9 | 79 | 16 |
| Leucine (61-90-5) | 18.6 | 1.3 | 148 | 11 |
| Lysine (56-87-1) | 25.7 | 3.7 | 184 | 27 |
| *N*-methylhistidine (332-80-9) | 7.2 | 1.2 | 44.6 | 7.5 |
| Ornithine (70-26-8) | 19.7 | 1.7 | 156 | 14 |
| Phenylalanine (63-91-2) | 13.56 | 0.90 | 86.0 | 5.7 |
| Proline (147-85-3) | 29.4 | 2.8 | 267 | 26 |
| Serine (56-45-1) | 9.73 | 0.73 | 96.9 | 7.3 |
| Threonine (72-19-5) | 12.3 | 0.7 | 108.1 | 6.2 |
| Valine (72-18-4) | 31.1 | 1.5 | 278 | 14 |
| Ethanolamine (141-43-5) | 1.81 | 0.58 | 31 | 10 |
| Creatinine (60-27-5) | 12.50 | 0.67 | 115.7 | 6.2 |
| Sarcosine (107-97-1) | 0.94 | 0.12 | 11.1 | 1.5 |

[1] Certified values are the mean of 6 measurement results obtained from two units of the CRM by ID-LC-MS technique. The certified values and the uncertainties are traceable to the International System of Units (SI) through a calibration hierarchy using high purity materials of each parameter that were value-assigned using TÜBİTAK UME qNMR purity assessment procedure.

[2] The expanded uncertainty of certified value includes characterization, homogeneity, stability components and is stated as the standard uncertainty of measurement multiplied by the coverage factor $k$ = 2 (*except Histamine, $k$ = 2.32), which for a normal distribution corresponds to a coverage probability of approximately 95%. The standard uncertainty of measurement has been determined in accordance with GUM “Guide to the Expression of Uncertainty in Measurement”.

[3] Certified values and the uncertainties in molar concentrations are calculated from the mass fraction (mg/kg) using density of the reconstituted material (mean: 1.04702 g/mL, SD: 0.00481 g/mL, $n$ = 15) measured at 22 °C and molecular weight of the analyte.

## INTRODUCTION

An inborn error of metabolism (IEM) is a permanent and inherited biochemical disorder generally caused by lack of a functional enzyme, some of the amino and organic acids could not be metabolized, which causes accumulation of toxic intermediate organic compounds and emergence of the diseases. Phenylketonuria, Maple Syrup Urine and Tyrosinemia are common diseases that are generated from amino acid metabolism disorders [3]. Detection of IEM through screening is the key to early treatment. Early diagnosis of metabolic diseases is very critical and they should be evaluated through reliable screening tests [4]. Newborn screening (NS) is a public health activity, in terms of secondary prevention, aimed at the early identification of infants affected by certain conditions-genetic, metabolic, infectiousthat threaten their life and long-term health, for which a timely intervention can lead to a significant reduction of morbidity, mortality and associated disabilities [5].

In order to obtain reliable results from measurements of amino acids, before starting any routine analysis, system calibration and quality control measurements must be conducted. When the expression of measurement results has to be in reliable quantitative values, the use of certified reference materials (CRM) is a powerful tool to ensure the quality of the measurements. Particularly, it is important to use CRMs, having the same chemical compositions (matrix matched CRM), for the proper quantification of subject quantity in the mixtures (matrix), such as body fluids, containing multiple analytes. In this way, through the use of matrix CRMs in measurements, verification of the metrological traceability can be ensured. An SRM from NIST (NIST SRM 1950, Metabolites in Frozen Human Plasma) certified for 12 amino acids and reference value for 4 was available [6]. Covering more number of amino acids and obtaining a more stable and transferable material was the main motivation for this study.

Amino acid concentrations in lyophilized human plasma are certified in UME CRM 1314. This report presents the details of the production and certification stages including the data, utilized techniques and statistical analysis. UME CRM 1314 was produced to be used as a quality control material to verify the traceability and quality of LC-MS and LC-MS/MS measurements of amino acids.

Systematic description for UME CRM 1314 according to ISO 15194 is "Certified Reference Material-UME CRM 1314 - Amino acids in Lyophilized Plasma”.

**Table 2.** Specific properties of selected amino acids

| Short Name | IUPAC Name | Chemical Formula | CAS Number | Molecular Weight (g/mol) |
|---|---|---|---|---|
| 3-Methylhistidine | (2S)-2-amino-3-(1-methyl-1H-imidazol-5-yl)propanoic acid | $C_7H_{11}N_3O_2$ | 368-16-1 | 169.1811 |
| Alanine | (2S)-2-aminopropanoic acid | $C_3H_7NO_2$ | 56-41-7 | 89.0932 |
| 2-Aminoadipic acid | 2-aminohexanedioic acid | $C_6H_{11}NO_4$ | 1118-90-7 | 161.1558 |
| 2-Aminobutyric acid | (2S)-2-aminobutanoic acid | $C_4H_9NO_2$ | 1492-24-6 | 103.1198 |
| 2-Aminopimelic acid | 2-aminoheptanedioic acid | $C_7H_{13}NO_4$ | 627-76-9 | 175.1824 |
| Anserine | (2S)-2-(3-aminopropanamido)-3-(1-methyl-1H-imidazol-5-yl)propanoic acid | $C_{10}H_{16}N_4O_3$ | 584-85-0 | 240.2590 |

**Table 2.** *(Continued)* Specific properties of selected amino acids

| Short Name | IUPAC Name | Chemical Formula | CAS Number | Molecular Weight (g/mol) |
|---|---|---|---|---|
| Arginine | (2S)-2-amino-5-carbamimidamidopentanoic acid | $C_6H_{14}N_4O_2$ | 74-79-3 | 174.2010 |
| Argininosuccinic acid | (2S)-2-{3-[(4S)-4-amino-4-carboxybutyl]carbamimidamido} butanedioic acid | $C_{10}H_{18}N_4O_6$ | 2387-71-5 | 290.2731 |
| Asparagine | (2S)-2-amino-3-carbamoylpropanoic acid | $C_4H_8N_2O_3$ | 70-47-3 | 132.1179 |
| Aspartic acid | (2S)-2-aminobutanedioic acid | $C_4H_7NO_4$ | 56-84-8 | 133.1027 |
| Beta alanine | 3-aminopropanoic acid | $C_3H_7NO_2$ | 107-95-9 | 89.0932 |
| 3-Aminoisobutyric acid | (2S)-3-amino-2-methylpropanoic acid | $C_4H_9NO_2$ | 144-90-1 | 103.1198 |
| Citrulline | (2S)-2-amino-5-(carbamoylamino)pentanoic acid | $C_6H_{13}N_3O_3$ | 372-75-8 | 175.1857 |
| Cystathionine | (2S)-2-amino-4-{[(2R)-2-amino-2-carboxyethyl]sulfanyl}butanoic acid | $C_7H_{14}N_2O_4S$ | 56-88-2 | 222.2621 |
| 4-Aminobutyric acid | 4-aminobutanoic acid | $C_4H_9NO_2$ | 56-12-2 | 103.1198 |
| Glutamic acid | (2S)-2-aminopentanedioic acid | $C_5H_9NO_4$ | 56-86-0 | 147.1293 |
| Glycine | 2-aminoacetic acid | $C_2H_5NO_2$ | 56-40-6 | 75.0666 |
| Histamine | 2-(1H-imidazol-4-yl)ethan-1-amine | $C_5H_9N_3$ | 51-45-6 | 111.1451 |
| Histidine | (2S)-2-amino-3-(1H-imidazol-5-yl)propanoic acid | $C_6H_9N_3O_2$ | 71-00-1 | 155.1546 |
| Hydroxyproline | (2S,4R)-4-hydroxypyrrolidine-2-carboxylic acid | $C_5H_9NO_3$ | 51-35-4 | 131.1299 |
| Isoleucine | (2S,3S)-2-amino-3-methylpentanoic acid | $C_6H_{13}NO_2$ | 73-32-5 | 131.1729 |
| Leucine | (2S)-2-amino-4-methylpentanoic acid | $C_6H_{13}NO_2$ | 61-90-5 | 131.1729 |
| Lysine | (2S)-2.6-diaminohexanoic acid | $C_6H_{14}N_2O_2$ | 56-87-1 | 146.1876 |
| Methionine | (2S)-2-amino-4-(methylsulfanyl)butanoic acid | $C_5H_{11}NO_2S$ | 63-68-3 | 149.2113 |
| *N*-Methylhistidine | (2S)-2-amino-3-(1-methyl-1H-imidazol-4-yl)propanoic acid | $C_7H_{11}N_3O_2$ | 332-80-9 | 169.1811 |
| Ornithine | (2S)-2,5-diaminopentanoic acid | $C_5H_{12}N_2O_2$ | 70-26-8 | 132.1610 |
| Phenylalanine | (2S)-2-amino-3-phenylpropanoic acid | $C_9H_{11}NO_2$ | 63-91-2 | 165.1891 |
| Proline | (2S)-pyrrolidine-2-carboxylic acid | $C_5H_9NO_2$ | 147-85-3 | 115.1305 |
| Serine | (2S)-2-amino-3-hydroxypropanoic acid | $C_3H_7NO_3$ | 56-45-1 | 105.0926 |
| Threonine | (2S,3R)-2-amino-3-hydroxybutanoic acid | $C_4H_9NO_3$ | 72-19-5 | 119.1192 |
| Tryptophan | (2S)-2-amino-3-(1H-indol-3-yl)propanoic acid | $C_{11}H_{12}N_2O_2$ | 73-22-3 | 204.2252 |
| Tyrosine | (2S)-2-amino-3-(4-hydroxyphenyl)propanoic acid | $C_9H_{11}NO_3$ | 60-18-4 | 181.1885 |
| Valine | (2S)-2-amino-3-methylbutanoic acid | $C_5H_{11}NO_2$ | 72-18-4 | 117.1463 |
| Ethanolamine | 2-aminoethan-1-ol | $C_2H_7NO$ | 141-43-5 | 61.0831 |
| Creatinine | 2-imino-1-methylimidazolidin-4-one | $C_4H_7N_3O$ | 60-27-5 | 113.1179 |
| *N*-Acetyltyrosine | (2S)-2-acetamido-3-(4-hydroxyphenyl)propanoic acid | $C_{11}H_{13}NO_4$ | 537-55-3 | 223.2252 |
| Sarcosine | 2-(methylamino)acetic acid | $C_3H_7NO_2$ | 107-97-1 | 89.0932 |

## PARTICIPANTS

All production and certification stages of UME CRM 1314 have been performed at TÜBİTAK UME.

**Table 3.** Participating institute and definition of the work

| Activity | Laboratory / Organization |
|---|---|
| Project management and data evaluation<br>Processing<br>Homogeneity study<br>Stability studies<br>Characterisation study | TÜBİTAK UME National Metrology Institute<br>Gebze-Kocaeli, TURKEY |

## MATERIAL PROCESSING

UME CRM 1314 was prepared by adding amino acid standards into the human plasma containing amino acids endogenously. 3-Methylhistidine, 2-Aminoadipic acid, 2-Aminobutyric acid, 2-Aminopimelic acid, Anserin, Arginine, Argininosuccinic acid, Asparagine, Aspartic acid, Beta alanine, 3-Aminoisobutyric acid, Citrulline, Cystathionine, 4-Aminobutyric acid, Glutamic acid, Glycine, Histamine, Histidine, Hydroxyproline, Methionine, *N*-Methylhistidine, Ornithine, Phenylalanine, Tryptophan, Tyrosine, Ethanolamine, Creatinine and *N*-Acetyltyrosine was added to the human plasma obtained from 48 healthy men donors. Human plasma was purchased from Türk Kızılay (Turkey) and amino acid standards were purchased from Medical Isotopes, Acros Organics, Santa Cruz Biotechnology and Sigma-Aldrich (USA).

The homogenised solution was filled into amber glass vials using an automated filling machine (FARMATEK, FTED 1-150, Turkey) as approximately 3 mL for each unit. Filling order was recorded by labelling after filling. Total of 1500 units were prepared. All vials were lyophilized at the same time. The vials were first lyo-capped in the lyophilizer (Millrock Technology, USA) under slightly vacuum-nitrogen atmosphere and then screw capped after being removed from the lyophilizer.

Units were classified in respect to CRM production stages (homogeneity, stability and characterization) with the random stratified sample selection approach by a software developed at TÜBİTAK UME (TRaNS) [7]. After classification into subgroups, samples were stored under selected test conditions. The units allocated for sales were stored at (-45 $\pm$ 5) $^{o}$C under controlled conditions, in the dark.

## HOMOGENEITY

Homogeneity study between the units is performed to show that the assigned values are valid for all units within the stated uncertainty. Homogeneity study between the units is performed with a number of samples representing the whole batch. In order to determine between unit heterogeneity for certification of amino acids, 15 units of UME CRM 1314 have been chosen by utilizing TRaNS [7]. Homogeneity study measurements were performed by validated ID-LCMS technique. Details of the applied technique are presented in Annex 1. Measurements were performed under repeatability conditions. The samples to be analysed were introduced to the instruments by random order to find out any trend arising from analytical and/or filling sequences.

Data were visually checked whether all data follow a unimodal distribution using histograms and normal probability plots. It was found that the distribution was normal and unimodal (except for arginine, aspartic acid, citrulline, cystathionine, gamma-aminobutyric acid, methionine, ornithine and ethanolamine). Minor deviations from unimodality of the individual values do not significantly affect the estimate of between-unit standard deviations.

The trend-corrected datasets were tested for consistency using Grubbs outlier tests on a confidence level of 99% on the individual results and the unit means. Some outlying individual results and outlying unit means were detected. Since no technical reason for the outliers could be found, all the data were retained for statistical analysis. The results of all statistical evaluations are given in Table 4.

**Table 4.** Results of the statistical evaluation of the homogeneity studies at the 99% confidence level

| Analyte | Any Trend? | | Any Outlier? | | Distribution |
|---|---|---|---|---|---|
| | Analytical sequence | Filling sequence | All data | Unit averages | All data |
| 3-Methylhistidine | No | No | No | No | Normal/unimodal |
| Alanine | No | No | No | No | Normal/unimodal |
| 2-Aminoadipic acid | No | No | No | No | Normal/unimodal |
| 2-Aminobutyric acid | No | No | No | No | Normal/unimodal |
| 2-Aminopimelic acid | No | No | No | No | Normal/unimodal |
| Anserine | No | No | No | No | Normal/unimodal |
| Arginine | No | No | No | No | Not Normal/unimodal |
| Argininosuccinic acid | No | No | No | No | Normal/unimodal |
| Asparagine | No | No | No | No | Normal/unimodal |
| Aspartic acid | No | No | No | No | Not Normal/unimodal |
| Beta alanine | No | No | No | No | Normal/unimodal |
| 3-Aminoisobutyric acid | No | No | No | No | Normal/unimodal |
| Citrulline | No | No | No | No | Not Normal/unimodal |
| Cystathionine | No | No | Yes | No | Not Normal/unimodal |

**Table 4.** *(Continued)* Results of the statistical evaluation of the homogeneity studies at the 99% confidence level

| Analyte | Any Trend? | | Any Outlier? | | Distribution |
|---|---|---|---|---|---|
| | **Analytical sequence** | **Filling sequence** | **All data** | **Unit averages** | **All data** |
| 4-Aminobutyric acid | No | No | No | No | Not Normal/unimodal |
| Glutamic acid | No | No | No | No | Normal/unimodal |
| Glycine | No | No | No | No | Normal/unimodal |
| Histamine | No | No | No | No | Normal/unimodal |
| Histidine | No | No | No | No | Normal/unimodal |
| Hydroxyproline | No | No | No | No | Normal/unimodal |
| Isoleucine | No | No | No | No | Normal/unimodal |
| Leucine | No | No | No | No | Normal/unimodal |
| Lysine | No | No | No | No | Normal/unimodal |
| Methionine | No | No | No | No | Not Normal/unimodal |
| *N*-Methylhistidine | No | No | No | No | Normal/unimodal |
| Ornithine | No | No | No | No | Not Normal/unimodal |
| Phenylalanine | No | No | No | No | Normal/unimodal |
| Proline | No | No | No | No | Normal/unimodal |
| Serine | No | No | No | No | Normal/unimodal |
| Threonine | No | No | No | No | Normal/unimodal |
| Tryptophan | No | No | No | No | Normal/unimodal |
| Tyrosine | No | No | No | No | Normal/unimodal |
| Valine | No | No | No | No | Normal/unimodal |
| Ethanolamine | No | No | No | No | Not Normal/unimodal |
| Creatinine | No | No | No | No | Normal/unimodal |
| *N*-Acetyltyrosine | No | No | No | No | Normal/unimodal |
| Sarcosine | No | No | No | No | Normal/unimodal |

The ANOVA allowed the calculation of the within- ($s_{wb}$) and between-unit homogeneity ($s_{bb}$), estimated as standard deviations, according to the following equations:

$$s_{wb} = \sqrt{MS_{within}} \quad (1)$$

$MS_{within}$ : Mean squares within-unit

$s_{wb}$ is equivalent to the *s* of the method, provided that subsamples are representative for the whole unit.

$$s_{bb} = \sqrt{\frac{MS_{between} - MS_{within}}{n}} \quad (2)$$

$MS_{between}$ : Mean squares between-unit

$n$ : Number of replicates per unit

When $MS_{between}$ is smaller than $MS_{within}$, $s_{bb}$ cannot be calculated. Instead, $u^*_{bb}$, the heterogeneity that can be hidden by the method repeatability, is calculated, according to the following equation [8]:

$$u^*_{bb} = \frac{s_{wb}}{\sqrt{n}} \sqrt[4]{\frac{2}{\nu_{MSwithin}}} \quad (3)$$

$\nu_{MSwithin}$ : Degrees of freedom of $MS_{within}$

The occurrence of $MS_{between} < MS_{within}$ can be seen, if material heterogeneity is smaller than that can be detected by the analytical methodology used.

For the parameters for which ANOVA was applied, the larger value of $s_{bb}$ or $u^*_{bb}$ is taken as uncertainty contribution for homogeneity, $u_{bb}$ (Table 5).

**Table 5.** Results of the homogeneity study

| Analyte | $s_{wb,rel}$ % | $s_{bb,rel}$ % | $u^*_{bb,rel}$ % | $u_{bb,rel}$ % |
|---|---|---|---|---|
| 3-Methylhistidine | 13.37 | $MS_{between} < MS_{within}$ | 5.71 | 5.71 |
| Alanine | 1.06 | 1.20 | 0.45 | 1.20 |
| 2-Aminoadipic acid | 14.49 | 4.57 | 6.19 | 6.19 |
| 2-Aminobutyric acid | 5.01 | 3.36 | 2.14 | 3.36 |
| 2-Aminopimelic acid | 4.19 | 2.17 | 1.79 | 2.17 |
| Anserine | 12.73 | 8.56 | 5.44 | 8.56 |
| Arginine | 1.27 | 1.03 | 0.54 | 1.03 |
| Argininosuccinic acid | 4.16 | $MS_{between} < MS_{within}$ | 1.78 | 1.78 |
| Asparagine | 20.22 | $MS_{between} < MS_{within}$ | 8.64 | 8.64 |
| Aspartic acid | 9.68 | $MS_{between} < MS_{within}$ | 4.13 | 4.13 |
| Beta alanine | 6.50 | 4.31 | 2.78 | 4.31 |
| 3-Aminoisobutyric acid | 14.92 | 6.57 | 6.37 | 6.57 |
| Citrulline | 0.76 | 1.58 | 0.32 | 1.58 |
| Cystathionine | 1.34 | 0.85 | 0.57 | 0.85 |
| 4-Aminobutyric acid | 9.08 | $MS_{between} < MS_{within}$ | 3.88 | 3.88 |
| Glutamic acid | 9.86 | $MS_{between} < MS_{within}$ | 4.21 | 4.21 |
| Glycine | 2.41 | 1.20 | 1.03 | 1.20 |

**Table 5.** *(Continued)* Results of the homogeneity study

| **Analyte** | $s_{wb,rel}$ % | $s_{bb,rel}$ % | $u^*_{bb,rel}$ % | $u_{bb,rel}$ % |
|---|---|---|---|---|
| Histamine | 0.87 | 0.81 | 0.37 | 0.81 |
| Histidine | 3.52 | 0.87 | 1.50 | 1.50 |
| Hydroxyproline | 2.98 | 1.51 | 1.27 | 1.51 |
| Isoleucine | 11.37 | $MS_{between} < MS_{within}$ | 4.86 | 4.86 |
| Leucine | 2.88 | 0.81 | 1.23 | 1.23 |
| Lysine | 7.01 | 2.23 | 3.00 | 3.00 |
| Methionine | 11.32 | 0.94 | 4.84 | 4.84 |
| *N*-Methylhistidine | 13.38 | $MS_{between} < MS_{within}$ | 5.72 | 5.72 |
| Ornithine | 0.74 | 1.50 | 0.32 | 1.50 |
| Phenylalanine | 4.42 | $MS_{between} < MS_{within}$ | 1.89 | 1.89 |
| Proline | 2.47 | 1.87 | 1.05 | 1.87 |
| Serine | 5.06 | 0.83 | 2.16 | 2.16 |
| Threonine | 2.00 | 1.34 | 0.85 | 1.34 |
| Tryptophan | 14.36 | 3.01 | 6.13 | 6.13 |
| Tyrosine | 1.37 | 1.30 | 0.59 | 1.30 |
| Valine | 2.49 | 1.33 | 1.06 | 1.33 |
| Ethanolamine | 5.28 | $MS_{between} < MS_{within}$ | 2.25 | 2.25 |
| Creatinine | 2.48 | 0.58 | 1.06 | 1.06 |
| *N*-Acetyltyrosine | 9.42 | 6.06 | 4.03 | 6.06 |
| Sarcosine | 1.83 | 1.28 | 0.78 | 1.28 |

The plotted data for the homogeneity is presented in Annex 2.

## STABILITY

The stability studies were carried out using an isochronous design [9]. In this approach, samples are stored for a certain time at different temperature conditions. Afterwards, the samples are moved to conditions where further degradation can be assumed to be negligible ("reference conditions"), effectively "freezing" the degradation status of the materials. At the end of the isochronous storage, the samples are analysed simultaneously under repeatability conditions. Two different stability tests, for UME CRM 1314, have been conducted. Short term stability test has been performed to simulate transportation conditions and long term stability test to simulate long term storage conditions.

Short term stability study was performed with 10 units of CRM and long term stability study was conducted by 26 units of CRM. Samples were selected by TRaNS software [7].

**Short Term Stability Results**

For the Short Term Stability (STS) test, three different temperatures (4 °C, 18 °C and 45 °C) and four time points (0, 1, 2, and 4 weeks) were tested. Ten samples were randomly selected. Test samples were moved to -45 °C (reference temperature) after completion of the test time. All samples were analysed under repeatability conditions at the same time.

The results obtained from isochronous measurements were first grouped according to the time period and then evaluated for each time point. These evaluations were carried out for three temperatures, separately.

The results were screened for outliers by applying the single Grubbs' test at confidence levels of 95 %. The measured concentration values were plotted against time and the regression lines were calculated to check for significant trends indicating possible changes in the concentrations of the analytes by time. The calculated slope values were tested for significance using a *t*-test, with $t_{\alpha.df}$ being the critical *t*-value (two-tailed) for a significance level $\alpha$ = 0.05 (95 % confidence level). The graphs are given in Annex 3. Outliers (shown in Table 6) were identified in the statistical evaluation (Grubbs' test) of the data; nevertheless, as there was no technical reason to exclude them from evaluation, they remained in the data set. The data evaluation results for the short-term stability at +4 °C, +18 °C and +45 °C are summarised in Table 6.

Uncertainty calculations are done using equation (4) [9]. Maximum time for transfer is chosen as one week.

$$u_{sts,rel} = \frac{RSD}{\sqrt{\Sigma\left(t_i - \bar{t}\right)^2}} \times t \qquad (4)$$

where.

| | | |
|---|---|---|
| *RSD* | : | relative standard deviation of the points on the regression line |
| $t_i$ | : | time point for each replicate |
| $\bar{t}$ | : | mean of all time points |
| *t* | : | maximum time suggested for transfer (one week) |

Results obtained from short term stability tests are given in Table 6.

**Table 6.** Results of short term stability tests for 1 week

| Analyte | 4 °C $u_{sts,rel}$ (%) | 18 °C $u_{sts,rel}$ (%) | 45 °C $u_{sts,rel}$ (%) | Number of outliers in 95% confidence interval* | | | Is there a significant trend in 95% confidence interval? | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 4 °C | 18 °C | 45 °C | 4 °C | 18 °C | 45 °C |
| 3-Methylhistidine | 1.36 | 1.24 | **3.26** | - | - | - | No | No | Yes |
| Alanine | 0.46 | 0.28 | **1.28** | - | - | - | No | No | Yes |
| 2-Aminoadipic acid | 1.56 | 0.88 | **1.55** | - | - | - | No | No | No |
| 2-Aminobutyric acid | 0.37 | 0.21 | **1.64** | - | - | - | No | No | Yes |
| 2-Aminopimelic acid | 0.71 | 0.48 | **0.75** | - | - | - | No | No | Yes |
| Anserine | 3.2 | 2.76 | **3.08** | - | 1 | - | No | No | No |
| Arginine | 0.31 | 0.16 | **0.48** | - | - | - | No | No | Yes |
| Argininosuccinic acid | 1.21 | 1.14 | **0.82** | - | - | - | Yes | No | No |
| Asparagine | 0.97 | 1.2 | **2.11** | 1 | - | - | No | No | No |
| Aspartic acid | 0.53 | 0.55 | **0.93** | - | - | - | No | Yes | Yes |
| Beta alanine | 1.37 | 2.18 | **2.92** | 1 | - | - | No | No | No |
| 3-Aminoisobutyric acid | 1.15 | 0.52 | **2.87** | - | - | - | No | No | Yes |
| Citrulline | 0.30 | 0.23 | **1.34** | - | - | - | No | No | Yes |
| Cystathionine | 0.22 | 0.16 | **1.27** | - | - | - | No | No | Yes |
| 4-Aminobutyric acid | 0.82 | 0.9 | **3.25** | - | - | - | No | No | Yes |
| Glutamic acid | 1.43 | 1.96 | **1.51** | - | - | - | No | No | No |
| Glycine | 0.81 | 0.7 | **2.28** | - | - | - | No | No | Yes |
| Histamine | 0.22 | 0.34 | **4.35** | - | 1 | - | No | Yes | Yes |
| Histidine | 0.28 | 0.18 | **1.21** | - | - | - | No | No | Yes |
| Hydroxyproline | 1.33 | 1.28 | **1.70** | - | - | - | No | Yes | No |
| Isoleucine | 0.49 | 0.71 | **1.89** | - | - | - | No | No | Yes |
| Leucine | 0.29 | 0.18 | **1.52** | - | - | - | No | No | Yes |
| Lysine | 0.62 | 0.62 | **3.68** | 1 | - | - | No | No | Yes |
| Methionine | 0.8 | 1.19 | **2.28** | - | - | - | No | Yes | Yes |
| *N*-Methylhistidine | 1.37 | 1.25 | **3.22** | - | - | - | No | No | Yes |
| Ornithine | 0.27 | 0.31 | **3.36** | - | - | - | No | No | Yes |
| Phenylalanine | 0.64 | 0.58 | **1.78** | - | - | - | No | No | Yes |
| Proline | 3.11 | 2.81 | **3.17** | - | 1 | - | No | No | No |
| Serine | 0.49 | 0.64 | **0.99** | - | 1 | - | No | No | No |
| Threonine | 0.83 | 0.64 | **0.67** | - | - | - | No | No | No |
| Tyrptophan | 3.65 | 2.9 | **4.43** | 1 | - | 1 | No | No | No |
| Tyrosine | 0.15 | 0.21 | **0.59** | - | - | - | No | No | Yes |
| Valine | 1.13 | 1.48 | **1.01** | - | - | 1 | No | No | No |
| Ethanolamine | 1.64 | 1.50 | **7.64** | - | - | - | No | No | Yes |
| Creatinine | 0.59 | 0.54 | **0.88** | - | - | - | No | No | No |
| *N*-Acetyltyrosine | 4.09 | 3.93 | **3.97** | - | 1 | 1 | No | No | No |
| Sarcosine | 0.34 | 0.55 | **1.73** | - | - | - | No | No | Yes |

*SGT: Single Grubbs' Test

The material is found to be stable at 45 °C for up to one week. Thus, the samples can be safely dispatched under conditions where the temperatures do not exceed 45 °C for up to one week, i.e. at ambient temperature without applying any cooling elements.

**Long Term Stability Results**

Shelf life of the CRM has been determined through long term stability measurements. For the measurements, two units for each of the months of 1, 3, 6 and 9 have been stored at -20 °C, +4°C and +18 °C and transferred to reference temperature (-45 °C) after each period of time to be measured isochronously afterwards. Two units, designated as reference units, were stored at -45 °C. The data for each time point has been calculated from 2 replicate measurements for each of two units. Thus, the average of 4 measurements for each time point is given in Annex 4. The error bars on each time point are calculated as the standard deviation of 2 unit averages.

No outlying data was detected by Grubbs' test. The graphs were plotted against time and the regression lines were calculated. The relative long term stability uncertainty, $u_{lts.rel}$ for each parameter is then calculated using equation (5) for the required shelf life as [9]:

$$u_{lts,rel} = \frac{RSD}{\sqrt{\Sigma(t_i - \bar{t})^2}} \times t \quad (5)$$

where;

*RSD* : the relative standard deviation of the points on the regression line

$t_i$ : being the time point for each replicate

$\bar{t}$ : being the average of all time points

$t$ : being the proposed shelf life at -20 °C, 4 °C and 18 °C (12 months)

The uncertainty contribution $u_{lts}$ was calculated for 9 months (t) at -20 °C. This uncertainty was one of the four parameters of the overall uncertainty budget of the certified values. The results are given in Table 7. The graphs for long term stability are given in Annex 4.

**Table 7.** Results of the long-term stability tests for 12 months

| Analyte | Slope significantly different from zero at -20 °C?* | $u_{lts,rel}$ [%] for shelf-life of 12 months at -20 °C | Slope significantly different from zero at 4 °C?* | $u_{lts,rel}$ [%] for shelf-life of 12 months at 4 °C | Slope significantly different from zero at 18 °C?* | $u_{lts,rel}$ [%] for shelf-life of 12 months at 18 °C |
|---|---|---|---|---|---|---|
| 3-Methylhistidine | No | **4.88** | No | 6.17 | No | 5.31 |
| Alanine | No | **1.73** | No | 1.66 | No | 1.98 |
| 2-Aminoadipic acid | No | **4.96** | Yes | 11.03 | No | 8.64 |
| 2-Aminobutyric acid | No | **1.33** | No | 1.35 | Yes | 3.53 |
| 2-Aminopimelic acid | No | **1.84** | No | 2.65 | No | 2.01 |
| Anserine | No | **2.83** | No | 7.12 | No | 8.48 |
| Arginine | Yes | **1.51** | No | 1.02 | No | 1.27 |
| Argininosuccinic acid | No | **4.34** | No | 6.03 | No | 5.51 |
| Asparagine | No | **2.91** | Yes | 7.17 | No | 4.54 |
| Aspartic acid | No | **3.20** | No | 3.12 | No | 6.91 |
| Beta alanine | No | **5.68** | No | 6.44 | No | 8.13 |
| 3-Aminoisobutyric acid | Yes | **4.35** | No | 3.07 | No | 4.53 |
| Citrulline | No | **0.94** | No | 1.60 | Yes | 2.51 |
| Cystathionine | No | **0.51** | Yes | 1.70 | Yes | 2.26 |
| 4-Aminobutyric acid | No | **2.87** | No | 3.39 | No | 2.78 |
| Glutamic acid | No | **5.33** | Yes | 6.24 | No | 6.38 |
| Glycine | No | **1.92** | No | 2.65 | No | 1.92 |
| Histamine | No | **0.77** | Yes | 2.95 | Yes | 6.01 |
| Histidine | No | **2.62** | No | 2.18 | Yes | 3.41 |
| Hydroxyproline | No | **8.16** | No | 10.40 | No | 9.89 |
| Isoleucine | No | **7.28** | No | 9.90 | No | 14.74 |
| Leucine | No | **0.88** | Yes | 2.57 | Yes | 2.59 |
| Lysine | No | **4.97** | No | 6.73 | No | 5.21 |
| Methionine | No | **2.96** | No | 3.49 | Yes | 6.62 |
| *N*-Methylhistidine | No | **4.90** | No | 6.17 | No | 5.35 |
| Ornithine | No | **0.79** | Yes | 1.72 | Yes | 3.43 |
| Phenylalanine | No | **1.95** | No | 3.86 | No | 4.16 |
| Proline | No | **1.07** | No | 1.71 | No | 3.05 |
| Serine | No | **1.16** | No | 2.02 | Yes | 2.50 |
| Threonine | No | **1.94** | No | 1.66 | No | 1.58 |
| Tyrptophan | No | **12.91** | No | 16.77 | No | 19.46 |
| Tyrosine | No | **1.06** | No | 1.34 | Yes | 2.08 |
| Valine | No | **0.94** | No | 1.97 | Yes | 3.61 |
| Ethanolamine | Yes | **3.25** | No | 2.55 | No | 2.59 |
| Creatinine | No | **1.49** | Yes | 3.16 | Yes | 3.86 |
| *N*-Acetyltyrosine | No | **2.28** | Yes | 10.83 | No | 7.89 |
| Sarcosine | No | **2.86** | Yes | 3.65 | No | 3.41 |

*Data are evaluated at confidence level of 95%.

Based on the results obtained. Temperature of -20 °C was found to be suitable to keep the samples for up to 12 months. In addition, to ensure stability beyond the initially determined shelf life, stability will be re-evaluated in certain periods, based on the results of post-certification monitoring.

## CHARACTERIZATION

According to ISO 17034, the characterization and the value assignment can be carried out in different ways. In this project, characterization study for UME CRM 1314 were performed with ID-LCMS which is a single reference measurement procedure applied in one laboratory.

Two units of the candidate CRM were measured with the ID-LCMS technique. Isotope labelled amino acids were used as internal standards for ID-LCMS method. Method details are presented in Annex 1. Three replicate measurements were performed from each unit on two different days. Units to be analyzed were selected randomly by TRaNS. Standards used for the calibration of the method were obtained from commercial suppliers and purity values were determined by Q-NMR analysis at TÜBİTAK UME. NIST SRM 1950 was used as the quality control material for the method performance (Annex 1. Table A5). As Tyrosine and Methionine recoveries of the NIST 1950 was not acceptable, assigned values for these two analytes are given as informative value. Data obtained from characterization study revealed normal distribution and measurement uncertainties were calculated according to the “Guide to the Expression of Uncertainty in Measurements (GUM)” and “EURACHEM/CITAC Guide Quantifying Uncertainty in Analytical Measurement” documents.

$$u_{char} = \sqrt{u_{LC-IDMS}{}^2} \qquad (6)$$

where.

$u_{char}$ : standard uncertainty of the characterization study

$u_{LC\text{-}IDMS}$: standard uncertainty of the ID-LCMS method.

## PROPERTY VALUE AND UNCERTAINTY ASSIGNMENT

Assigned values and uncertainties of the CRM were evaluated by applying approach in the characterization and uncertainty data that contribute to the homogeneity and stability assessments.

The certified value is the mean of the ID-LCMS results, which is a reference method traceable to the SI via Q-NMR purity analysis and gravimetric preparation. Applied method was validated in respect to the quality system set up at TÜBİTAK UME.

The uncertainties of the certified values contains contributions of the characterisation $u_{\mathrm{char}}$, the homogeneity $u_{\mathrm{bb}}$, the long-term stability $u_{\mathrm{lts}}$ and the short term stability $u_{\mathrm{sts}}$ .

The different contributions to the CRM uncertainty are combined using equation (7):

$$U_{\mathrm{CRM}} = k \, . \sqrt{u_{\mathrm{char}}^2 + u_{\mathrm{bb}}^2 + u_{\mathrm{lts}}^2 + u_{\mathrm{sts}}^2} \qquad (7)$$

The expanded uncertainty of the certified value $U_{\mathrm{CRM}}$ is calculated with a coverage factor of $k$ = 2, representing a confidence level of approximately 95% considering the effective degrees of freedom values which were found to be greater than 10 for all analytes (except Histamine) using Welch‐Satterthwaite formula (8) [10].

$$\nu_{eff} = \frac{u_{CRM}^4}{\frac{u^4{}_{char}}{n_{char^{-1}}}+\frac{u^4{}_{bb}}{n_{bb^{-1}}}+\frac{u^4{}_{lts}}{n_{lts^{-1}}}+\frac{u^4{}_{sts}}{n_{sts^{-1}}}} \quad (8)$$

The certified and assigned values and uncertainties are summarised in Table 8.

**Table 8.** Summary of certified values and their uncertainties

| Analyte | Certified Value (mg/kg) | $U_{CRM}$ (mg/kg) ($k$ = 2) | $U_{CRM,rel}$ (%) ($k$ = 2) | $u_{char,rel}$ (%) | $u_{bb,rel}$ (%) | $u_{lts,rel}$ (%) | $u_{sts,rel}$ (%) |
|---|---|---|---|---|---|---|---|
| Alanine | **32.6** | **1.7** | **5.11** | 0.68 | 1.20 | 1.73 | 1.28 |
| 2-Aminoadipic acid | **5.18** | **0.92** | **17.60** | 3.48 | 6.19 | 4.96 | 1.55 |
| 2-Aminobutyric acid | **1.65** | **0.23** | **13.78** | 5.63 | 3.36 | 1.33 | 1.64 |
| Anserine | **12.2** | **2.9** | **23.73** | 7.07 | 8.56 | 2.83 | 3.08 |
| Arginine | **15.13** | **0.93** | **6.10** | 2.39 | 1.03 | 1.51 | 0.48 |
| Asparagine | **12.9** | **2.5** | **18.93** | 1.41 | 8.64 | 2.91 | 2.11 |
| Aspartic acid | **2.42** | **0.28** | **11.57** | 2.31 | 4.13 | 3.20 | 0.93 |
| Beta alanine | **8.5** | **1.4** | **15.99** | 2.15 | 4.31 | 5.68 | 2.92 |
| Citrulline | **15.8** | **1.2** | **7.17** | 2.77 | 1.58 | 0.94 | 1.34 |
| Cystathionine | **2.94** | **0.11** | **3.71** | 0.92 | 0.85 | 0.51 | 1.27 |
| 4-Aminobutyric acid | **1.57** | **0.21** | **13.13** | 3.04 | 3.88 | 2.87 | 3.25 |
| Glutamic acid | **12.0** | **1.9** | **15.41** | 3.31 | 4.21 | 5.33 | 1.51 |
| Glycine | **21.2** | **1.8** | **8.25** | 2.59 | 1.20 | 1.92 | 2.28 |
| Histamine | **1.31** | **0.15*** | **9.31*** | 1.22 | 0.81 | 0.77 | 4.35 |
| Histidine | **17.7** | **1.2** | **6.61** | 0.58 | 1.50 | 2.62 | 1.21 |
| Hydroxyproline | **5.24** | **0.97** | **18.46** | 3.67 | 1.51 | 8.16 | 1.70 |
| Isoleucine | **9.9** | **1.9** | **18.30** | 1.89 | 4.86 | 7.28 | 1.89 |
| Leucine | **18.6** | **1.3** | **6.60** | 2,51 | 1.23 | 0.88 | 1.52 |
| Lysine | **25.7** | **3.7** | **14.38** | 2.10 | 3.00 | 4.97 | 3.68 |
| *N*-Methylhistidine | **7.2** | **1.2** | **16.61** | 1.37 | 5.72 | 4.90 | 3.22 |
| Ornithine | **19.7** | **1.7** | **8.28** | 1.72 | 1.50 | 0.79 | 3.36 |
| Phenylalanine | **13.56** | **0.90** | **6.58** | 0.52 | 1.89 | 1.95 | 1.78 |
| Proline | **29.4** | **2.8** | **9.50** | 2.81 | 1.87 | 1.07 | 3.17 |
| Serine | **9.73** | **0.73** | **7.50** | 2.66 | 2.16 | 1.16 | 0.99 |
| Threonine | **12.3** | **0.7** | **5.23** | 0.91 | 1.34 | 1.94 | 0.67 |
| Valine | **31.1** | **1.5** | **4.55** | 1.23 | 1.33 | 0.94 | 1.01 |
| Ethanolamine | **1.81** | **0.58** | **31.83** | 13.39 | 2.25 | 3.25 | 7.64 |
| Creatinine | **12.50** | **0.67** | **5.29** | 1.70 | 1.06 | 1.49 | 0.88 |
| Sarcosine | **0.94** | **0.12** | **12.33** | 5.02 | 1.28 | 2.86 | 1.73 |

***** For Histamine, coverage factor; $k$ = 2.32 is used for the calculation of expanded uncertainty ($\nu_{eff}$≈ 9)

## INFORMATIVE VALUES

The assigned values and uncertainties given as informative are summarised in Table 9.

**Table 9.** Summary of assigned values and their uncertainties

| Analyte | Assigned Value (mg/kg) | $U_{CRM}$ (mg/kg) ($k$ = 2) | $U_{CRM,rel}$ (%) ($k$ = 2) | $u_{char,rel}$ (%) | $u_{bb,rel}$ (%) | $u_{lts,rel}$ (%) | $u_{sts,rel}$ (%) |
|---|---|---|---|---|---|---|---|
| Argininosuccinic acid | **12.8** | **2.0** | **15.68** | 6.23 | 1.78 | 4.34 | 0.82 |
| 3-Methylhistidine | **9.6** | **1.7** | **16.77** | 1.79 | 5.71 | 4.88 | 3.26 |
| 2-Aminopimelic acid | **1.06** | **0.21** | **19.52** | 9.31 | 2.17 | 1.84 | 0.75 |
| 3-Aminoisobutyric acid | **1.78** | **0.39** | **21.39** | 6.64 | 6.57 | 4.35 | 2.87 |
| Methionine | **7.52** | **0.98** | **12.93** | 2.10 | 4.84 | 2.96 | 2.28 |
| Tyrosine | **5.46** | **0.37** | **6.66** | 2.81 | 1.30 | 1.06 | 0.59 |
| Tyrptophan | **7.1** | **2.2** | **30.49** | 2.94 | 6.13 | 12.91 | 4.43 |
| *N*-Acetyltyrosine | **45.4** | **7.3** | **16.05** | 2.59 | 6.06 | 2.28 | 3.97 |

For five of the analytes (presented in Table 9 and Table10), internal standards which are not the isotope labelled molecules of the analytes were used for the measurements. Purity assessment by Q-NMR could not be achieved for Argininosuccinic acid. Recoveries of the NIST SRM 1950 were not acceptable for Methionine and Tyrosine (Annex1 Table A5).Therefore these values are reported as informative.

**Table 10.** Informative values and uncertainties for UME CRM 1314

| Parameter (CAS No) | Mass Fraction (mg/kg) | | Molar Concentration[3] (µmol/L) | |
|---|---|---|---|---|
| | Assigned Value[1] | Uncertainty[2] | Assigned Value | Uncertainty |
| Argininosuccinic acid (2387-71-5) | 12.8 | 2.0 | 46.0 | 7.3 |
| 3-Methylhistidine (368-16-1) | 9.6 | 1.7 | 59 | 11 |
| 2-Aminopimelic acid (3721-85-5) | 1.06 | 0.21 | 6.3 | 1.3 |
| 3-Aminoisobutyric acid (144-90-1) | 1.78 | 0.39 | 18.1 | 4.0 |
| Methionine (63-68-3) | 7.52 | 0.98 | 52.8 | 6.9 |
| Tyrosine (60-18-4) | 5.46 | 0.37 | 31.6 | 2.2 |
| Tryptophan (73-22-3) | 7.1 | 2.2 | 36 | 12 |
| *N*-Acetyltyrosine (537-55-3) | 45.4 | 7.3 | 213 | 35 |

[1] Assigned values are the mean of six measurement results obtained from two units (total of 18 measurements with triplicate instrumental repetitions) of the CRM by LC-MS technique.

[2] The expanded uncertainty of assigned value includes characterization, homogeneity, stability components and is stated as the standard uncertainty of measurement multiplied by the coverage factor $k$ = 2, which for a normal distribution corresponds to a coverage probability of approximately 95%. The standard uncertainty of measurement has been determined in accordance with GUM “Guide to the Expression of Uncertainty in Measurement”.

[3] Assigned values and the uncertainties in molar concentrations are calculated from the mass fraction (µg/kg) using density of the reconstituted material (mean: 1.04702 g/mL. SD: 0.00481 g/mL, $n$ = 15) measured at 22 °C and molecular weight of the analyte.

## COMMUTABILITY

The intended use of this reference material is to check method performance and validation of amino acid measurements in plasma with LC-MS and LC-MS/MS methods.

Commutability of the material, with routine in vitro diagnostic amino acid kit methods has not been assessed. The user would need to assess the commutability, if UME CRM 1314 is going to be used for evaluating the accuracy of routine amino acid kit methods.

## TRACEABILITY

Stock solutions and calibrations solutions used in the in-house validated methods were gravimetrically prepared from the solid materials for which the purity values were assigned by Q-NMR measurements at TÜBİTAK UME. All weighing operations were performed with balances calibrated at TÜBİTAK UME with E2 Class weigh sets traceable to national standards. The assigned values of the amino acid calibrants are traceable to SI through Q-NMR measurements using SI traceable calibrants. Purity assessment capability of TÜBİTAK UME by Q-NMR was demonstrated with international comparison studies and publications [11].

As a quality control for the amino acids quantification in plasma samples conducted at TÜBİTAK UME. NIST SRM 1950 “Metabolites in Frozen Human Plasma” was used. TUBİTAK UME’s measurement capability was demonstrated with international comparison studies; CCQM-K12.2-Determination of Creatinine in Human Serum and CCQM K159-Amino acids in Human Plasma [12].

Absence of bias for the assigned value of Phenylalanine was further confirmed by dataset obtained from the international comparison study “CCQM-K159 “Amino acids in human plasma” organized by CIPM CCQM Organic Analysis Working Group. Graph of the results reported by the participating laboratories to the key comparison study for Phenylalanine is given in Annex 5.

## INSTRUCTIONS FOR USE

### Intended Use

This material is intended to be used for method performance check and validation purposes of amino acid measurements in plasma by LC-MS and LC-MS/MS methods.

### Scope of Application

UME CRM 1314 is suitable for use in evaluating the accuracy of procedures for determination of amino acid concentrations in human plasma using LC-MS and LC-MS/MS methods.

The material is not suitable for evaluating the accuracy of amino acid measurements in human plasma using routine *in vitro* diagnostic amino acid kit methods unless the commutability of the material is proven by the user.

### Safety Precautions

Raw material: frozen human plasma (origin: Turkey) was tested by the manufacturer (Türk Kızılay) for Anti HCV (-), Anti HIV (-), HBsAg (-), HBV DNA (-), HSV RNA (-), HIV RNA (-), Sifilis (-). However, no known test method can offer complete assurance that the reported or any other infectious agents are absent from this material. As a matter of fact, the material should be treated as a biohazard capable of transmitting infectious disease. This human blood-based product should be handled at the Biosafety Level 2 or higher as recommended for any potentially infectious human plasma or blood specimen. The material is suitable for *in-vitro* use only. Safety Data Sheet (SDS) should be read before use.

**Storage Conditions**

The material should be stored at temperatures equal to or lower than -20 °C in a dry and dark place. Solutions of UME CRM 1314 should not be exposed to direct sunlight or UV light. User should take necessary precautions against evaporation or sublimation of the sample.

TÜBİTAK UME cannot be held responsible for changes that might happen to the material at customer's premises due to noncompliance of the instructions for use, and the storage conditions given in the certificate.

**Reconstitution of the Material**

All content of the unit should be reconstituted at once according to the recommended protocol presented below:

- UME CRM 1314 and the deionized water should be kept at room temperature at least for one hour in the room of balance before weighing.
- Before opening, the vial should be tapped gently to table or benchtop in order to collect the whole content at the bottom of the vial.
- Screw cap is opened. The vial, with the inner cap, is placed to the balance and tare is pressed. Unit is opened gently at vertical position by balancing the inner pressure with outer pressure. Care should be taken in order not to lose any sample on cap or elsewhere. 2750 µL of water is then added gently with a calibrated pipette into the sample. Then the inner cap is closed.
- Vial, with its content and inner cap is weighed ($m$).
- The average amount of water added to the analysed units at TÜBİTAK UME was

$$m_{avg} = (2.76682 \pm 0.00562)\ \text{g}\ (k = 2)$$

If $m$ is deviating from $m_{avg}$, then the corrected value of the analyte concentration can be calculated as:

$$\text{Corrected analyte concentration} = \text{Certified value} \times \frac{m_{avg}}{m}$$

The vial with added water should be gently shaken with its cap and inner cap closed. If material is stacked on to the inner cap, user can gently rotate the vial upside down several times to dissolve those particles. Solid particles should be inspected by eye and dissolving procedure should be repeated until all of the content is dissolved. User should perform the measurement on the dissolved material as quickly as possible. If it is necessary to keep the dissolved material for later measurements, it should be portioned into the small volumes and stored at temperatures equal to or lower than -20 °C in the dark. Subsamples should be stored in the vials resistive to volatilization and sun light.

**Minimum Sample Intake**

The minimum sample intake suggested for the reconstituted sample is 100 µL.

**Use of the Certified Value**

For assessing the method performance, the measured values of the CRMs are compared with the certified values [13]. The procedure can be described briefly as follows:

- Calculate the absolute difference between mean measured value and the certified value ($\Delta_m$).
- Combine measurement uncertainty ($u_{meas}$) with the uncertainty of the certified value ($u_{CRM}$):

$$u_\Delta = \sqrt{u_{meas}^2 + u_{CRM}^2}$$

- Calculate the expanded uncertainty ($U_\Delta$) from the combined uncertainty ($u_\Delta$) using a coverage factor of two ($k = 2$), corresponding to a confidence level of approximately 95%.

If $\Delta_m \leq U_\Delta$, then it is assumed that there is no significant difference between the measurement result and the certified value at 95% confidence level.

An online application: CRM Result Evaluation-CRM RE to evaluate your measurement results and automatically create quality control charts is available through the link https://rm.ume.tubitak.gov.tr/en/crm_re/

## ACKNOWLEDGEMENTS

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Murat Çelik and Gökhan Günay (former Zivak Technologies employees) are gratefully acknowledged for their contribution to the project.

We would like to thank Dr. Byungjo Kim for valuable discussions and comments during the ISO 17034 accreditation assessment of the project.

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## REVISION HISTORY

| Date | Remarks |
|---|---|
| 31.05.2019 | First issue. |
| 12.02.2021 | Intended use, commutability and traceability information is revised.<br>Anserine, Asparagine, Glutamic acid, Leucine, Serine and N-Acetyltyrosine assigned values and uncertainties are revised.<br>$U_{CRM}$ for histamine is recalculated and revised.<br>Argininosuccinic acid is moved from certified values to the Informative values section.<br>Results of the statistical evaluation of the homogeneity studies revised at the 99% confidence level.<br>More information about the method performance is added to Annex 1.<br>Editorial changes are made. |
| 28.05.2021 | CRM uncertainty values are revised.<br>Methionine and Tyrosine are moved from certified values to the Informative values section.<br>Information about CCQM K159-Amino acids in Human Plasma is added to the Traceability section.<br>CCQM K159 Study plot for Phenylalanine is added as Annex 5 to the report. |

**Annex 1. Details of ID-LCMS Technique**

CRM sample was dissolved with 2750 µL of deionized water (≥18 MΩ). 100 µL portion of this solution was transferred into an Eppendorf tube by weighing. 100 µL isotopes labeled internal standards (IS) solution TAU-methyl-histidine (Methyl-D3) (10.84 mg/kg), Alanine (2,3-13C2, 99%) (28.71 mg/kg), 2-Aminoadipic acid-2,5,5-D3 (98%D) (5.03 mg/kg), 2-Aminobutyric-D6 acid (98%D) (1.76 mg/kg), Phenylalanine (RING-D5, 98%) (14.43 mg/kg), Anserin-D4 (6.22 mg/kg), Arginine:HCl (U-13C6, 99%) (19.57 mg/kg), Argininosuccinic acid-13C6 (%99)-15N7 (%99) (1.80 mg/kg), Asparagine:H2O (15N2, 98%) (16.85 mg/kg), Aspartic acid (2,3,3-D3, 98%) (4.70 mg/kg), Beta alanine-2,2,3,3-D4 (98%D) (5.54 mg/kg), Citrulline (Uredio-13C, 99%) (12.54 mg/kg), Cystathionine (3,3,4,4-D4 98%D) (4.54 mg/kg), 4-Aminobutyric-2,2,3,3,4,4-D6 acid, 98%D (2.21 mg/kg), Glutamic acid (2,3,3,4,4-D5. 98%) (18.19 mg/kg), Glycine (2,2-D2, 98%) (21.95 mg/kg), Histamine:2HCl (A,A,B,B-D4. 98%) (2.94 mg/kg), Histidine:HCl:H2O (<5% D)(RING-2-13C) (25.56 mg/kg), 4-Hydroxyproline-2,5,5-D3(99%D) (5.42 mg/kg), Isoleucine (15N, 98%) (8.05 mg/kg), Leucine (1-13C, 99%) (16.46 mg/kg), Lysine:2HCl (alpha-15N, 95-99%) (26.64 mg/kg), Methionine (1-13C, 99%) (6.38 mg/kg), Ornithine:HCl (3,3,4,4,5,5-D6, 98%) (26.78 mg/kg), Proline (15N, 98%) (26.49 mg/kg), Serine (2,3,3-D3, 98%) (12.13 mg/kg), Threonine (4-13C, 97%; 2,3-D2, 98%) (15.32 mg/kg), Tyrosine (ring-3,5-D2, 98%) (17.16 mg/kg), Valine (1-13C, 99%) (23.53 mg/kg), Ethanolamine (D4, 98%) (1.50 mg/kg), Creatinine (N-methyl-D3, 98%) (6.17 mg/kg) and Sarcosine (N-methyl-D3-glycine HCl) (2.59 mg/kg) was added. The content was mixed with vortex for 10 s. 200 µL 1-propanol:pyridine (4:1) solution was added and was mixed with vortex for 10 s. 200 µL isooctane:pyropyl chloroformate (5:1) -derivatization reagent- was added and was mixed with vortex for 10 s. Resultant mixture was waited for 2 minutes and was vortexed again for 10s. 200 µL chloroform:isooctane (3:2) solution was added and vortexed for 10 s and waited for 1 minute. The content was centrifuged for 3 minute at 12000 rpm (HETTICH, Micro 120, Germany). 200 µL was taken from the upper layer in to a vial and 2 µL of this solution was injected to LC-HRMS.

**Table A1.** Properties of LC-MS Instrument

| Name of the Component | Producer | Model |
|---|---|---|
| HRMS | Thermo | Q Exactive Orbitrap (Germany) |
| HPLC | Thermo | Dionex Ultimate 3000 (Germany) |
| HPLC column | Phenomenex | Phenomenex EZ:faast 4u AAA-MS (250 x 2.0 mm) |

**Table A2.** LC parameters

| Name | Value | | | |
|---|---|---|---|---|
| **Column Temperature** | 40° C | | | |
| **Mobile Phase** | A: Methanol: Water (1% 1 M Ammonium formate) (50:50)<br>B: Methanol (1% 1 M Ammonium formate) | | | |
| **Flow rate** | 0.25 mL/min. Gradient | | | |
| **Gradient Pump Program** | 00:00 min | 38 % B | 14:01 min | 38 % B |
| | 12:00 min | 65 % B | 20:00 min | 38 % B |
| | 12:01 min | 95 % B | | |
| | 14:00 min | 95 % B | | |

**Table A3.** MS Parameters

| Parameter | Value |
|---|---|
| Ionization Mode | ESI |
| Sheath Gas Flow Rate | 35 |
| Aux Gas Flow | 10 |
| Sweep Gas | 0 |
| Discharge Current (µA) | 5 |

**Table A4.** LC-MS Parameters

| Analyte | RT (min) | MH+- m/z | Internal Standard | RT (min) | MH+- m/z |
|---|---|---|---|---|---|
| 3-Methylhistidine | 4.10 | 298.175 | TAU-methyl-histidine (Methyl-D3) | 4.45 | 301.194 |
| Alanine | 5.15 | 218.138 | Alanine (2,3-13C2, 99%) | 5.15 | 220.145 |
| 2-Aminoadipic acid | 9.32 | 332.20563 | 2-aminoadipic acid-2,5,5-D3 (98%D) | 9.26 | 335.22437 |
| 2-Aminobutyric acid | 6.40 | 232.154 | 2-aminobutyric-D6 acid (98%D) | 6.34 | 238.191 |
| 2-Aminopimelic acid | 11.02 | 346.22125 | Phenylalanine (RING-D5, 98%) | 9.77 | 299.201 |
| Anserine | 7.50 | 369.19818 | Anserin-D4 | 6.55 | 374.22827 |
| Arginine | 2.79 | 303.202 | Arginine:HCI (U-13C6, 99%) | 2.79 | 309.222 |
| Argininosuccinic acid | 4.62 | 443.249 | Argininosuccinic acid-13C6%99 - 15N7 %99 | 4.64 | 453.256 |
| Asparagine | 3.77 | 243.133 | Asparagine:H2O (15N2, 98%) | 3.77 | 262.154 |
| Aspartic acid | 7.66 | 304.174 | Aspartic acid (2,3,3-D3, 98%) | 7.59 | 307.194 |
| Beta alanine | 5.15 | 218.138 | Beta alanine-2,2,3,3-D4 (98%D) | 5.77 | 221.157 |
| 3-Aminoisobutyric acid | 6.01 | 232.154 | 2-aminobutyric-D6 acid (98%D) | 6.34 | 238.191 |
| Citrulline | 3.34 | 304.186 | Citrulline (Uredio-13C, 99%) | 3.34 | 305.189 |
| Cystathionine | 11.49 | 479.242 | Cystathionine (3,3,4,4-D4 98%D) | 11.45 | 483.266 |
| 4-Aminobutyric acid | 5.50 | 232.154 | 4-aminobutyric-2,2,3,3,4,4-D6 acid, 98%D | 5.44 | 238.191 |
| Glutamic acid | 9.36 | 318.190 | Glutamic acid (2,3,3,4,4-D5, 98%) | 8.11 | 323.211 |
| Glycine | 4.18 | 204.123 | Glycine (2,2-D2, 98%) | 4.27 | 206.135 |

**Table A4.** (Continued) LC-MS Parameters

| Analyte | RT (min) | MH+- m/z | Internal Standard | RT (min) | MH+- m/z |
|---|---|---|---|---|---|
| Histamine | 5.60 | 284.159 | Histamine:2HCl (A,A,B,B-D4, 98%) | 5.56 | 288.185 |
| Histidine | 7.78 | 370.196 | Histidine:HCl:H2O (<5% D)(RING-2-13C) | 7.80 | 373.204 |
| Hydroxyproline | 3.98 | 260.149 | 4-hydroxyproline-2,5,5-D3(99%D) | 3.96 | 263.167 |
| Isoleucine | 10.29 | 260.185 | Isoleucine (15N, 98%) | 10.29 | 261.184 |
| Leucine | 9.81 | 260.185 | Leucine (1-13C, 99%) | 9.81 | 261.18790 |
| Lysine | 7.64 | 361.231 | Lysine:2HCl (alpha-15N, 95-99%) | 7.64 | 362.231 |
| Methionine | 6.87 | 278.142 | Methionine (1-13C, 99%) | 6.87 | 279.145 |
| *N*-Methylhistidine | 4.10 | 298.175 | TAU-methyl-histidine (Methyl-D3) | 4.45 | 301.194 |
| Ornithine | 6.59 | 347.217 | Ornithine:HCl (3,3,4,4,5,5-D6. 98%) | 6.55 | 353.254 |
| Phenylalanine | 9.90 | 294.169 | Phenylalanine (Ring-D5, 98%) | 9.77 | 299.201 |
| Proline | 7.02 | 244.153 | Proline (15N, 98%) | 7.00 | 245.151 |
| Serine | 3.71 | 234.133 | Serine (2,3,3-D3, 98%) | 3.69 | 237.152 |
| Threonine | 4.21 | 248.149 | Threonine (4-13C, 97%; 2,3-D2, 98%) | 4.21 | 251.164 |
| Tryptophan | 8.36 | 333.180 | Histidine:HCl:H2O (<5% D)(RING-2-13C) | 7.80 | 373.204 |
| Tyrosine | 12.80 | 396.201 | Tyrosine (ring-3,5-D2, 98%) | 12.78 | 398.214 |
| Valine | 8.09 | 246.169 | Valine (1-13C, 99%) | 8.09 | 247.172 |
| Ethanolamine | 2.68 | 148.097 | Ethanolamine (D4, 98%) | 2.68 | 152.122 |
| Creatinine | 4.27 | 260.160 | Creatinine (N-methyl-D3, 98%) | 4.25 | 263.179 |
| *N*-Acetyltyrosine | 7.29 | 352.175 | Tyrosine (ring-3,5-D2, 98%) | 12.78 | 398.214 |
| Sarcosine | 5.15 | 218.139 | Sarcosine (N-methyl-D3-glycine HCl) | 5.77 | 221.157 |

**Table A5.** NIST SRM 1950 Measurement Results

| Analyte | Measurement Result (mg/kg, *n* = 11) | Certified / *Reference Value (mg/kg) | Recovery (%) |
|---|---|---|---|
| Alanine | 24.98 | 26.2 | 95 |
| Glycine | 17.86 | 18.0 | 99 |
| Histidine | 10.74 | 11.0 | 97 |
| Isoleucine | 7.063 | 7.1 | 99 |
| Leucine | 13.88 | 12.9 | 108 |
| Lycine | 21.23 | 20.0 | 106 |
| Methionine | 3.96 | 3.3 | 121 |
| Proline | 20.03 | 19.9 | 101 |
| Serine | 8.46 | 9.9 | 86 |
| Threonine | 13.82 | 13.9 | 99 |
| Tyrosine | 4.93 | 10.2 | 48 |
| Valine | 21.45 | 20.9 | 103 |
| *Arginine | 13.32 | 13.9 | 96 |
| *Phenylalanine | 8.2 | 8.2 | 100 |

## Annex 2. Graphs for Homogeneity Studies

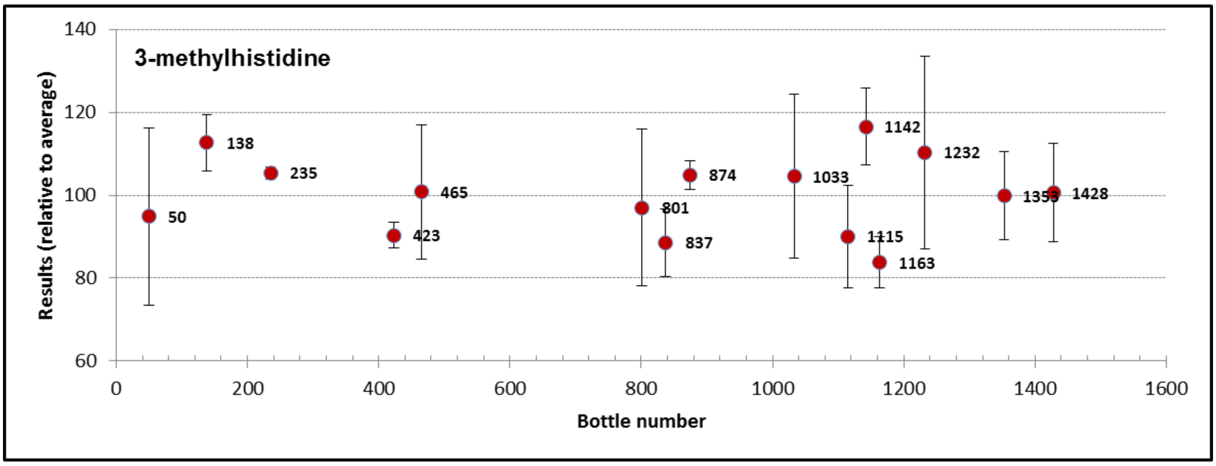


**Figure A1.** Homogeneity plot for 3-methylhistidine

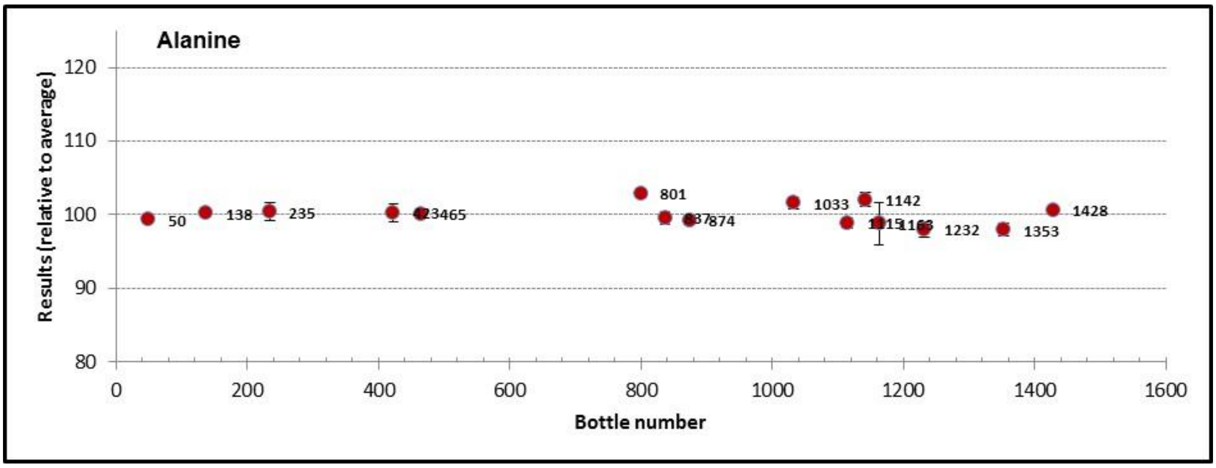


**Figure A2.** Homogeneity plot for Alanine

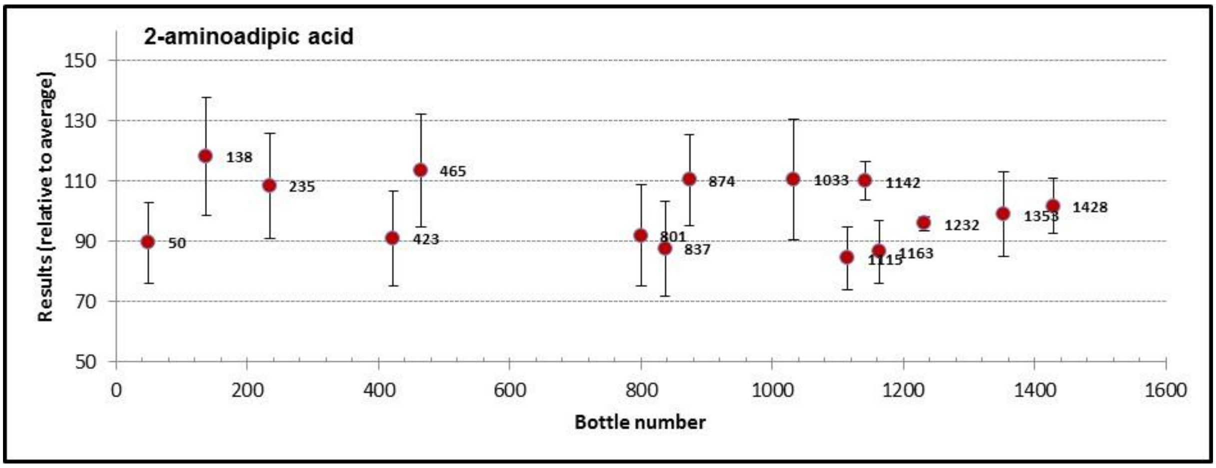


**Figure A3.** Homogeneity plot for 2-aminoadipic acid

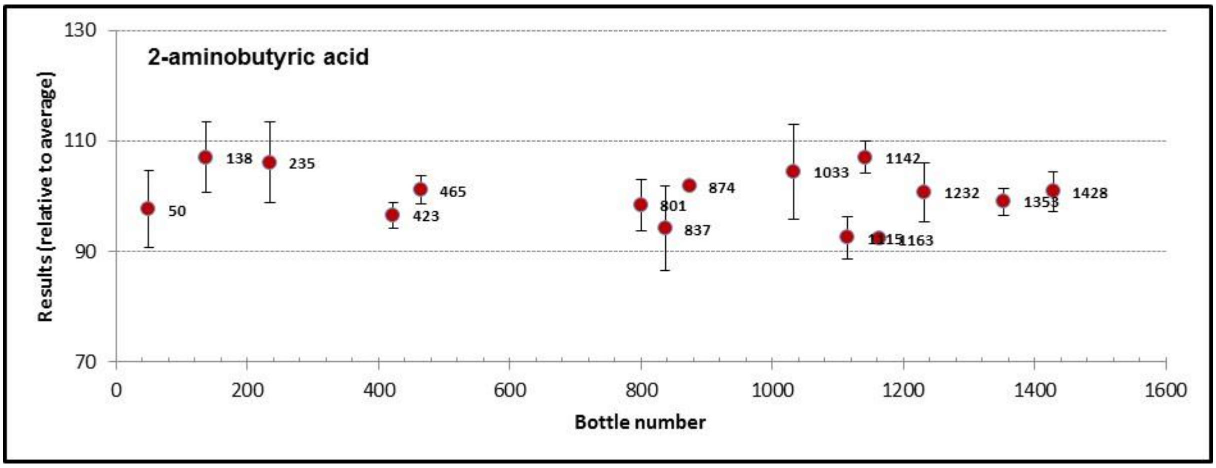


**Figure A4.** Homogeneity plot for 2-aminobutyric acid

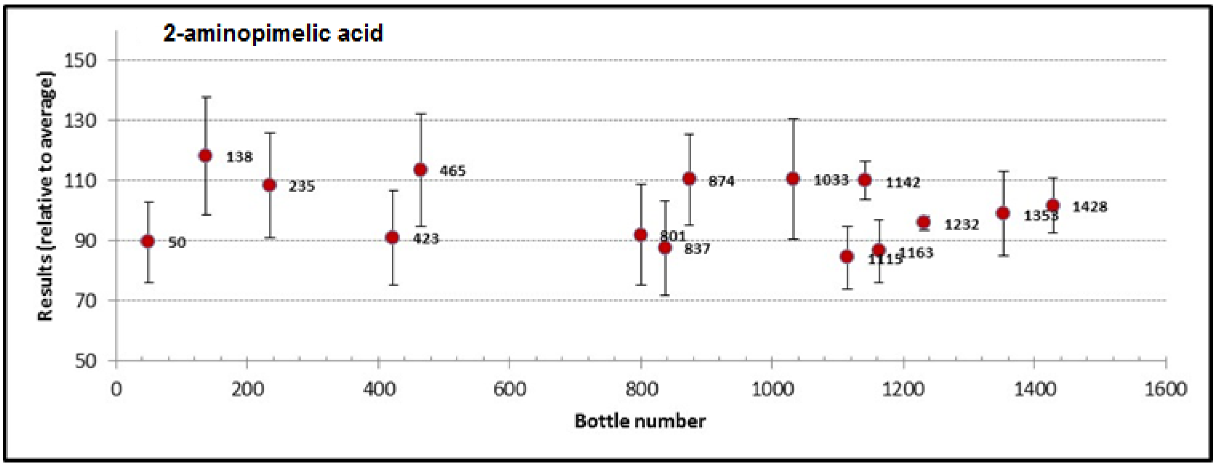


**Figure A5.** Homogeneity plot for 2-aminopimelic acid

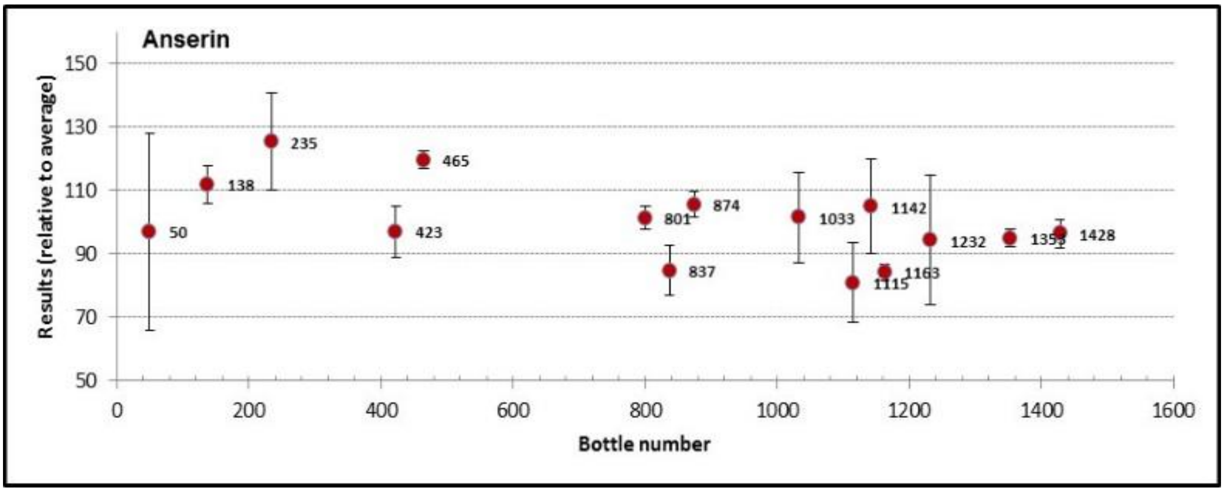


**Figure A6.** Homogeneity plot for Anserin

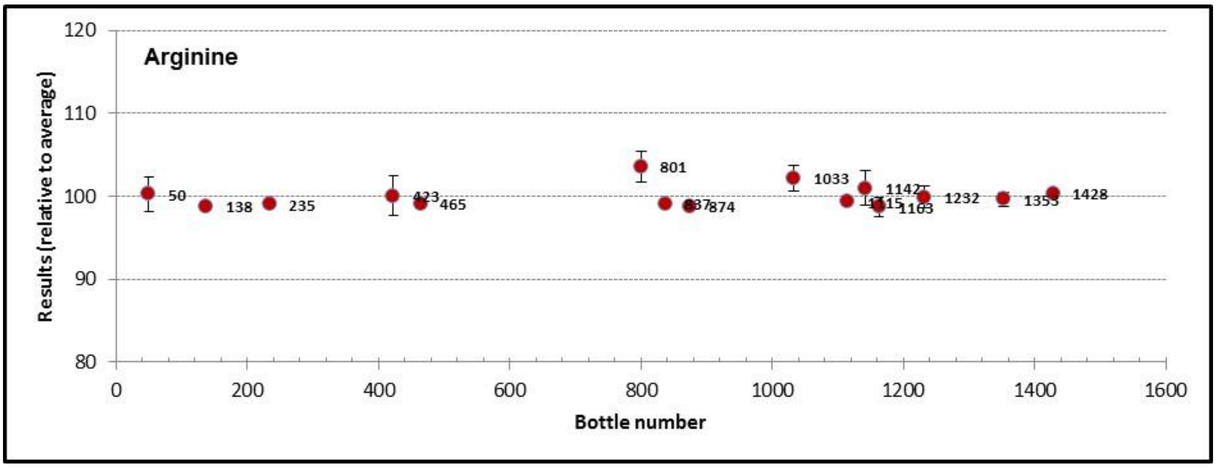


**Figure A7.** Homogeneity plot for Arginine

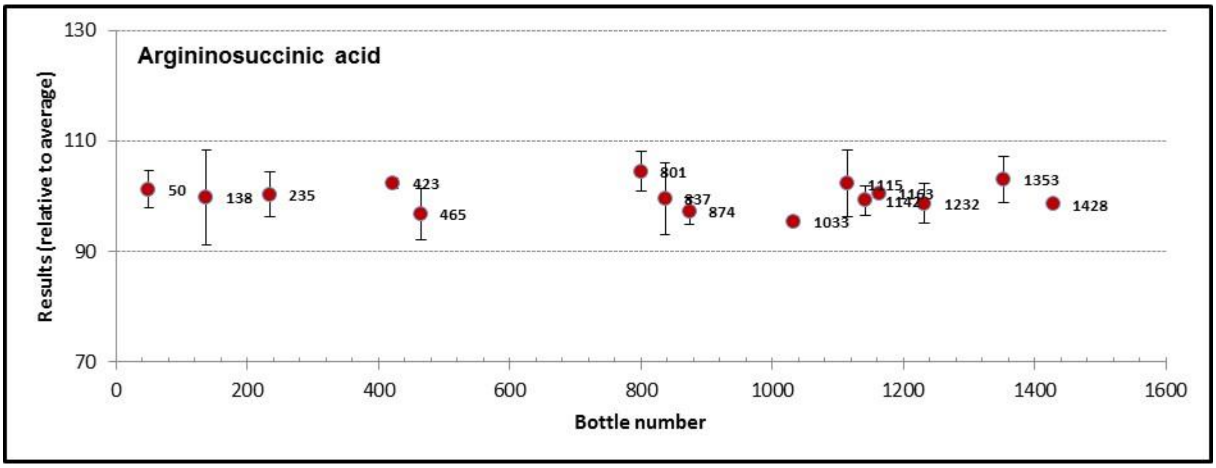


**Figure A8.** Homogeneity plot for Argininosuccinic acid

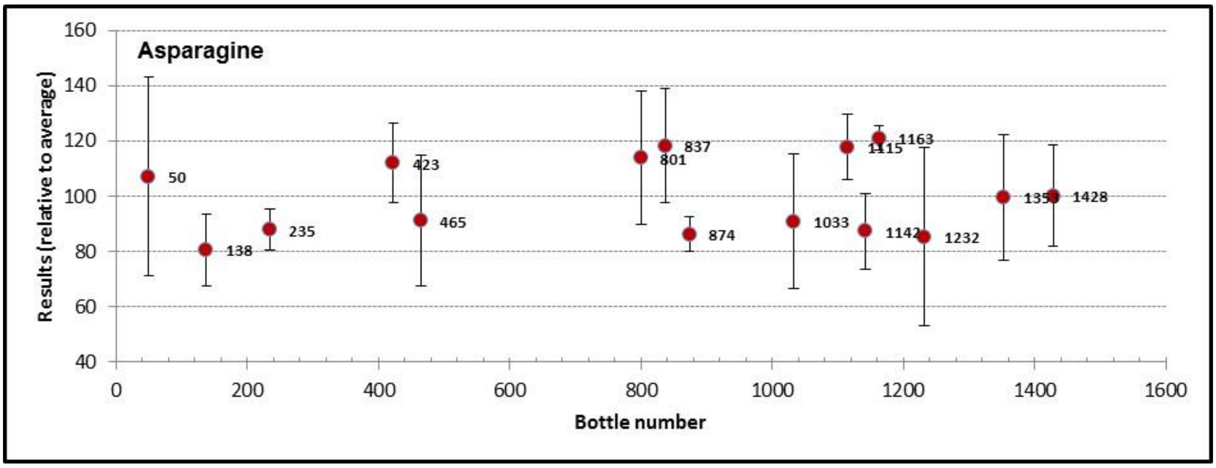


**Figure A9.** Homogeneity plot for Asparagine

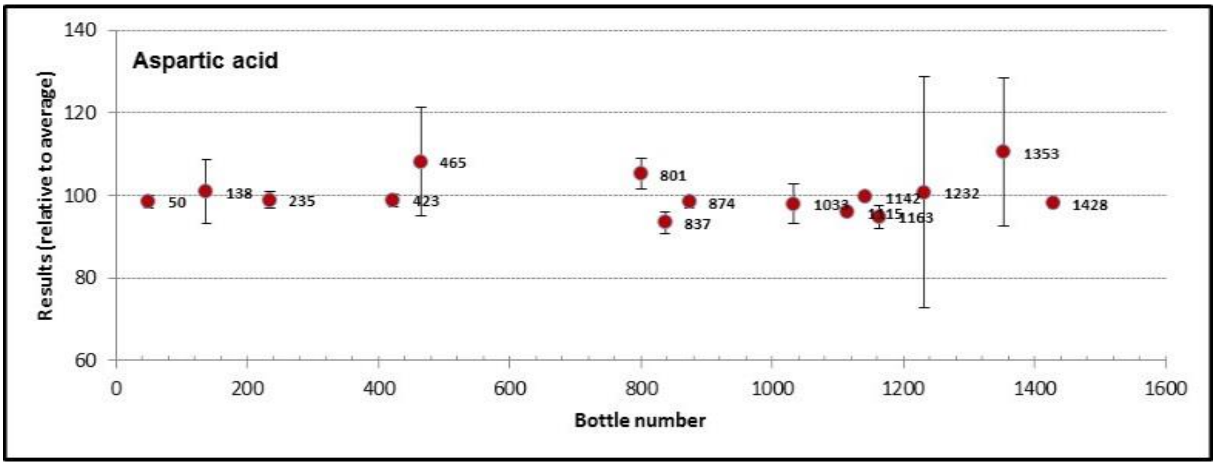


**Figure A10.** Homogeneity plot for Aspartic acid

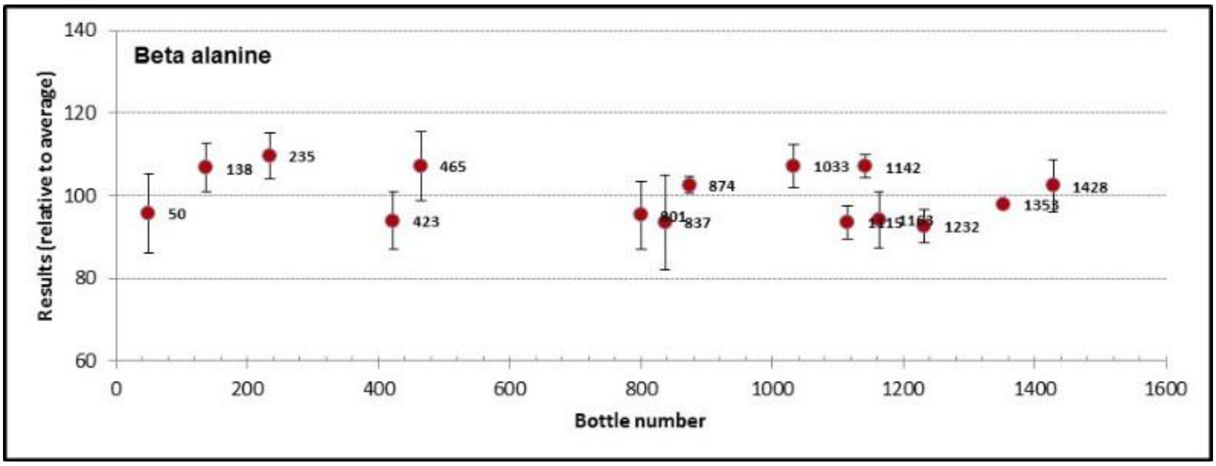


**Figure A11.** Homogeneity plot for Beta alanine

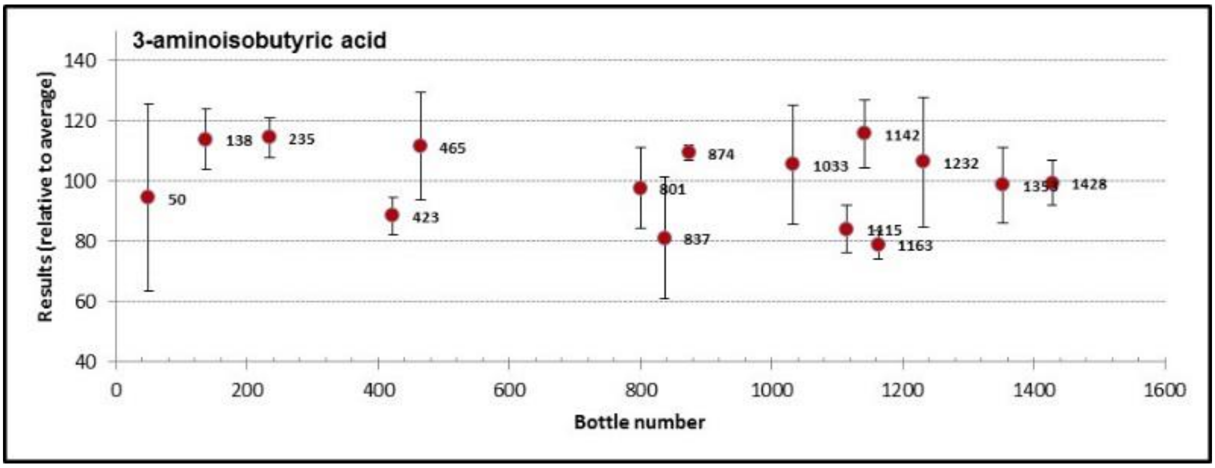


**Figure A12.** Homogeneity plot for 3-aminoisobutyric acid

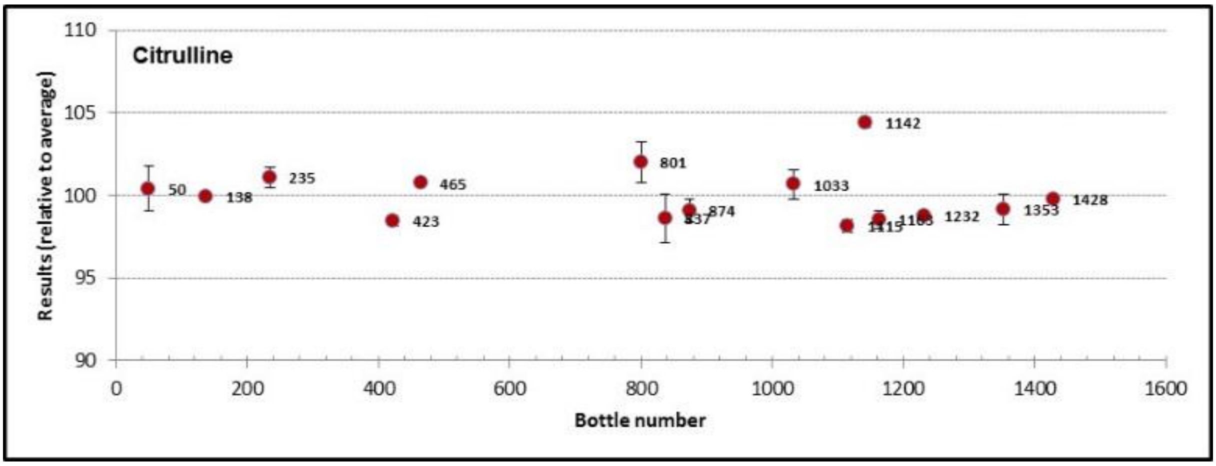


**Figure A13.** Homogeneity plot for Citrulline

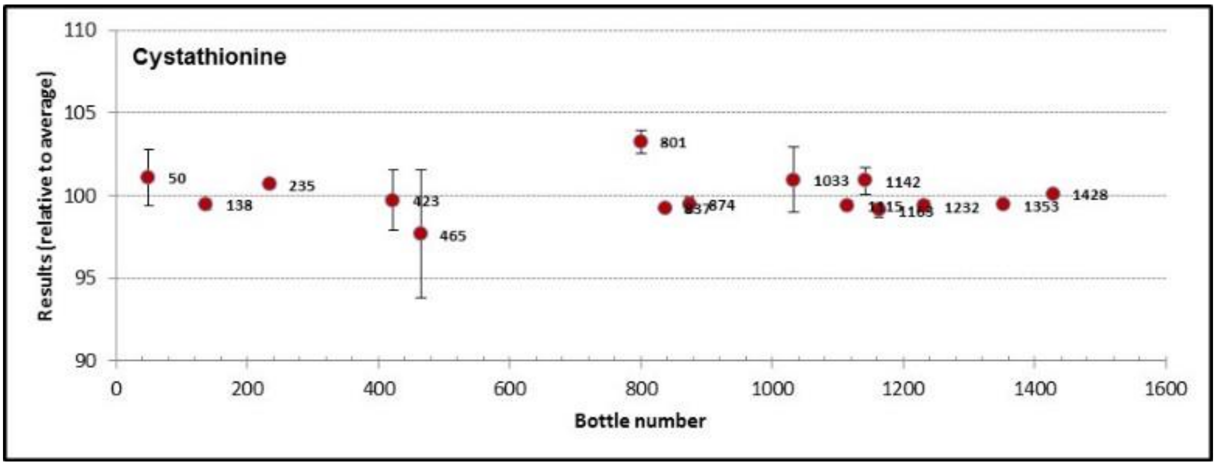


**Figure A14.** Homogeneity plot for Cystathionine

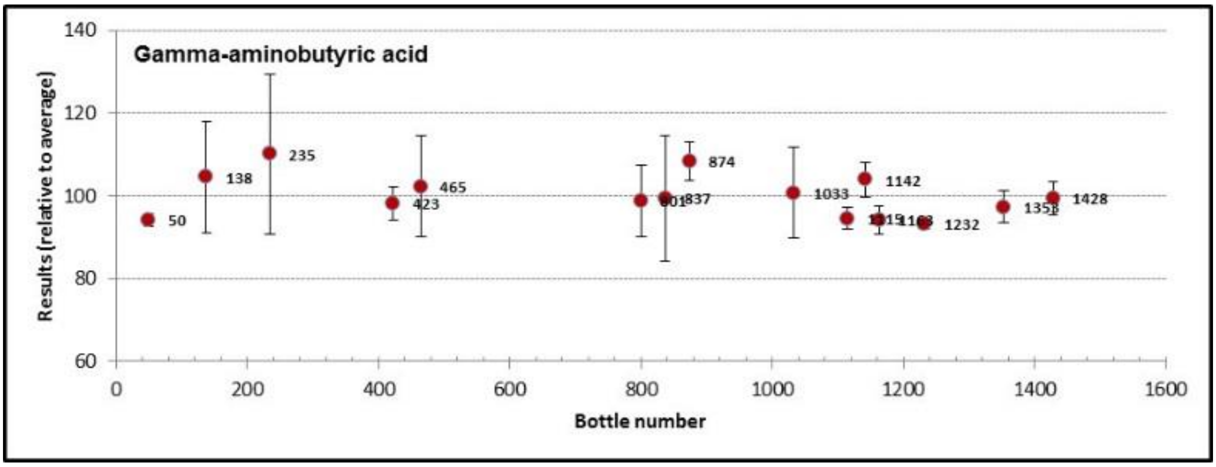


**Figure A15.** Homogeneity plot for Gamma-Aminobutyric acid

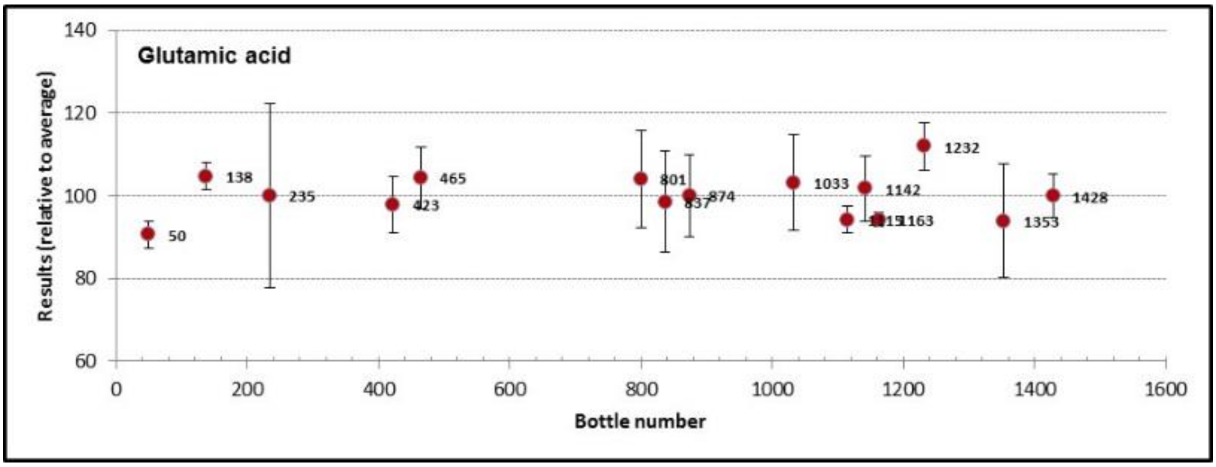


**Figure A16.** Homogeneity plot for Glutamic acid

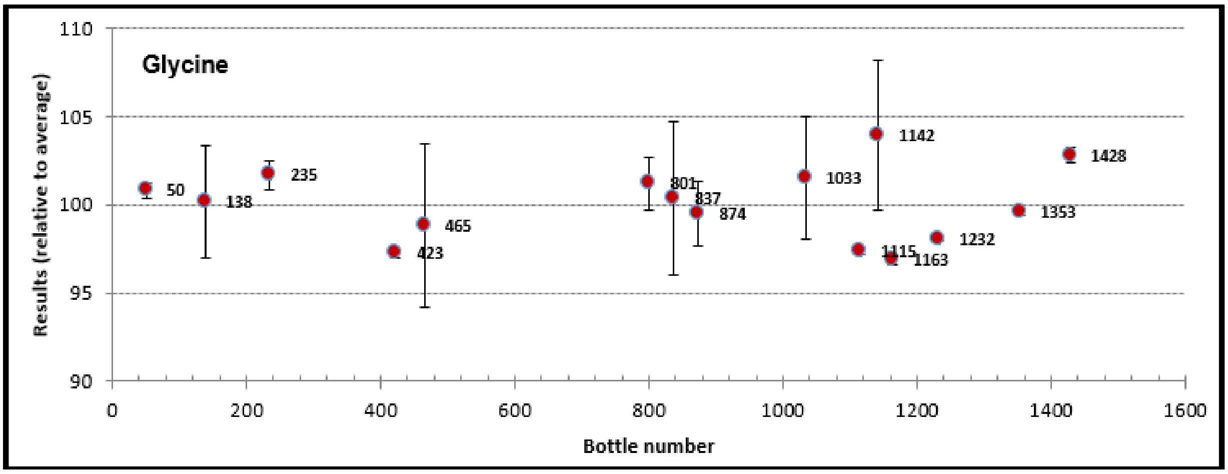


**Figure A17.** Homogeneity plot for Glycine

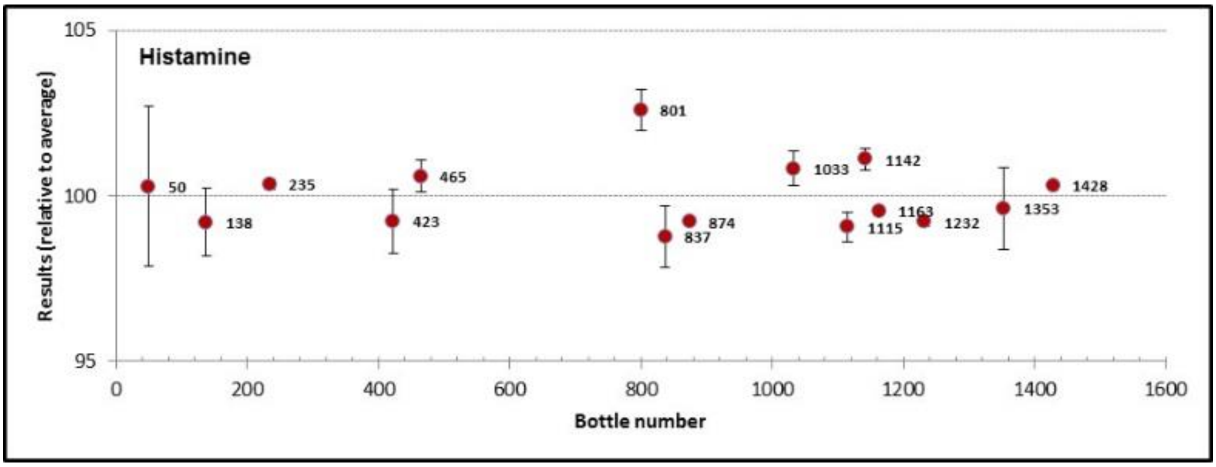


**Figure A18.** Homogeneity plot for Histamine

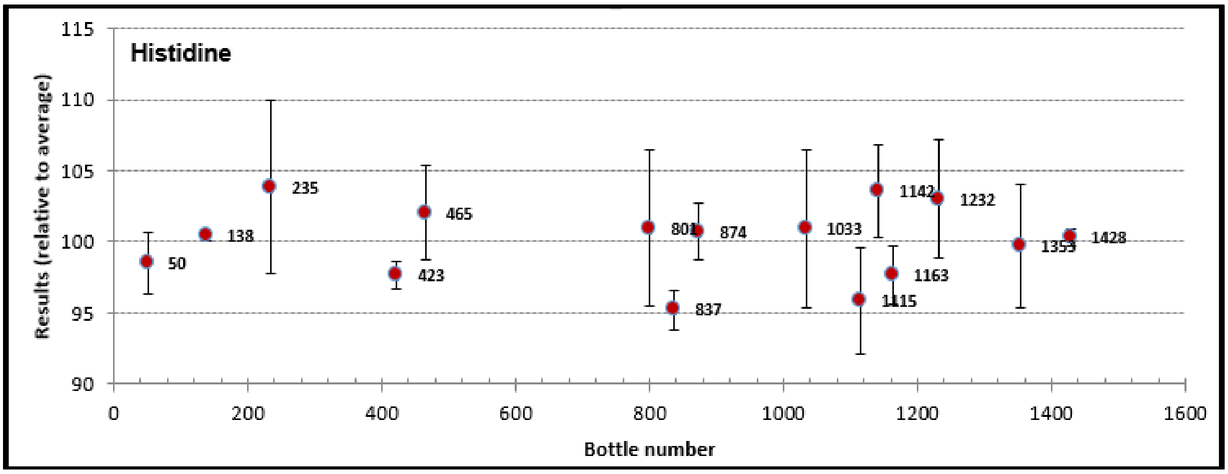


**Figure A19.** Homogeneity plot for Histidine

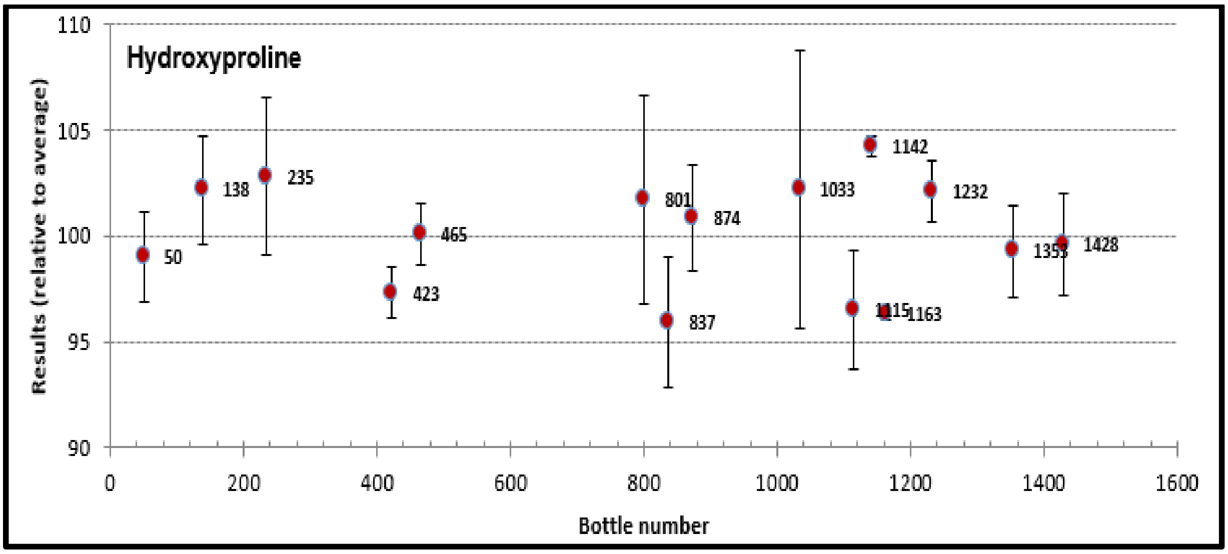


**Figure A20.** Homogeneity plot for Hydroxyproline

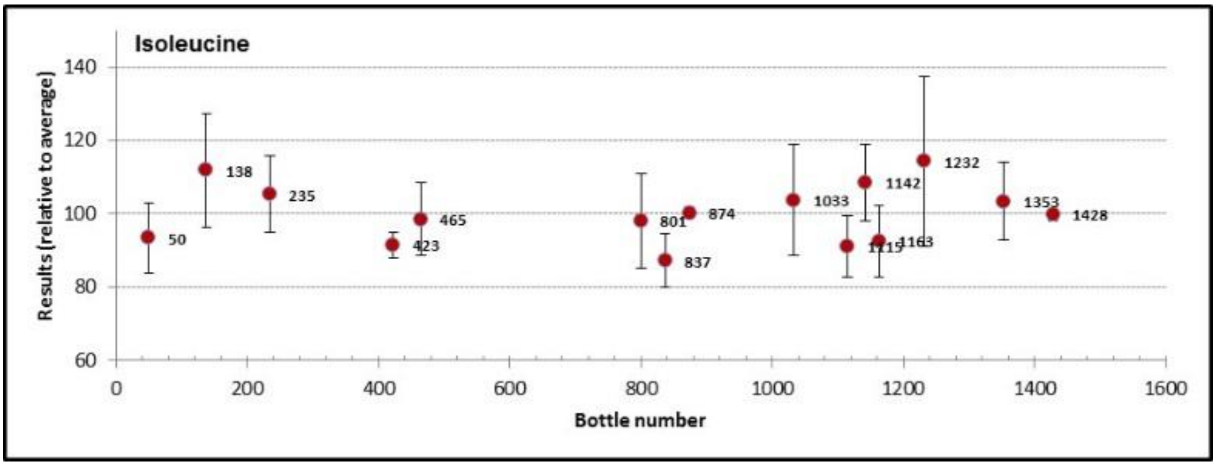


**Figure A21.** Homogeneity plot for Isoleucine

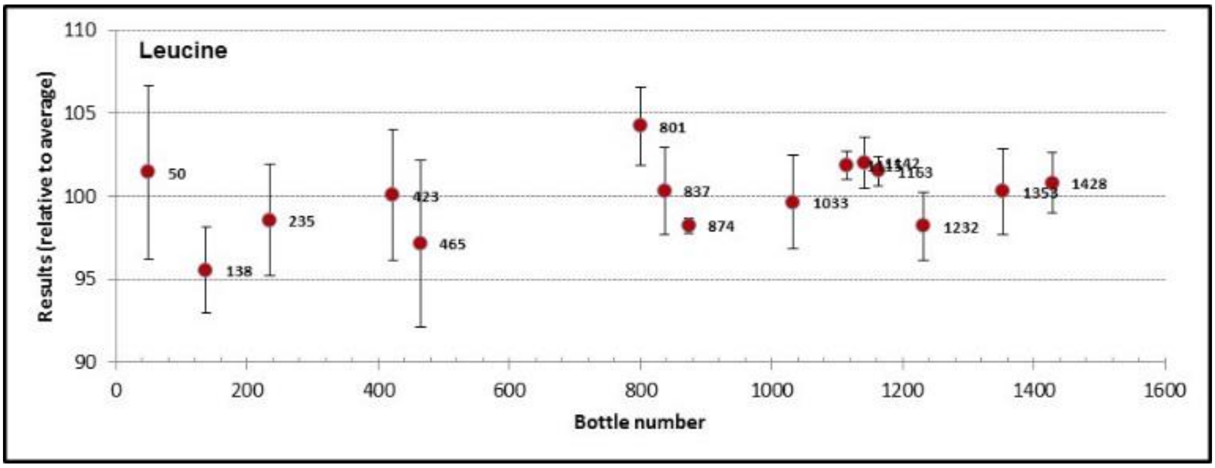


**Figure A22.** Homogeneity plot for Leucine

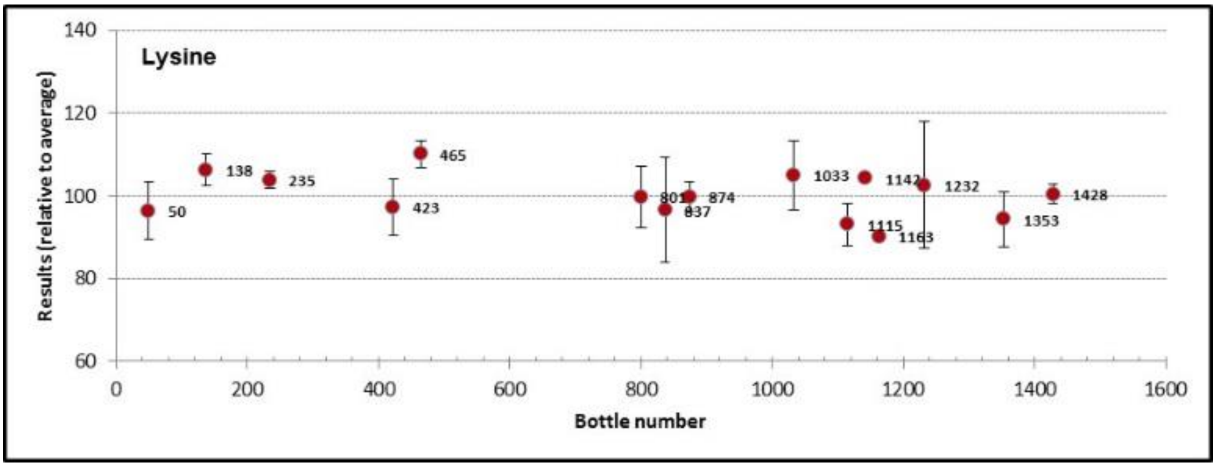


**Figure A23.** Homogeneity plot for Lysine

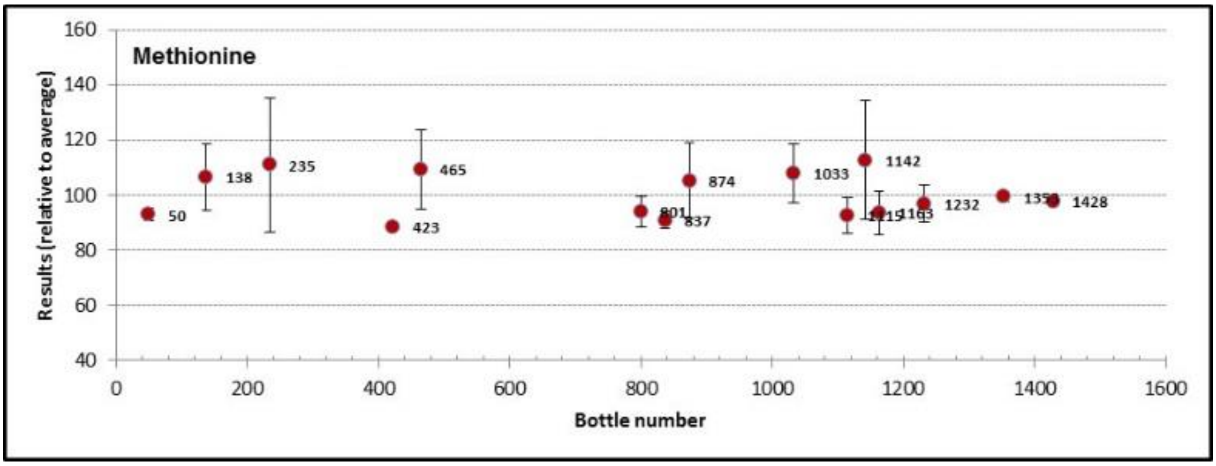


**Figure A24.** Homogeneity plot for Methionine

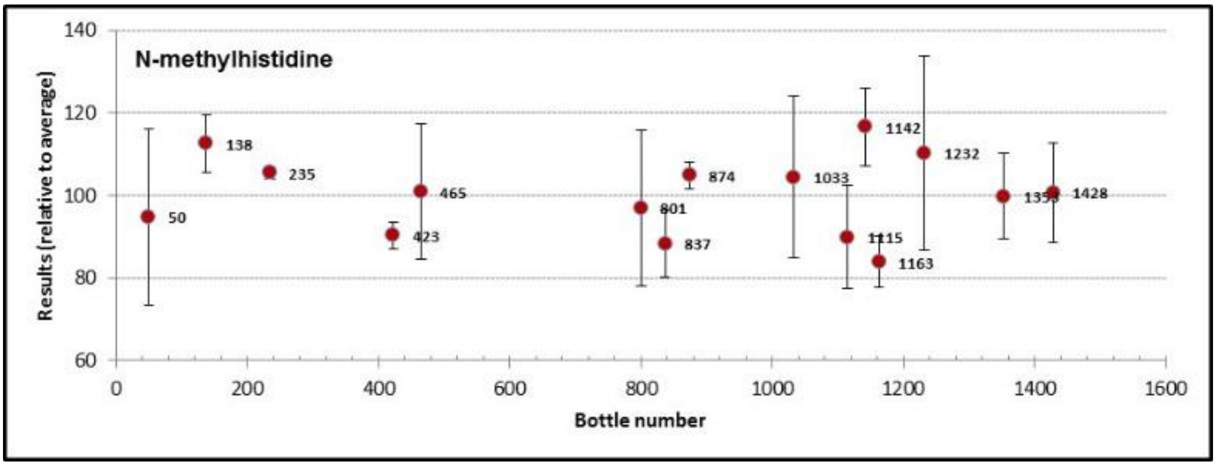


**Figure A25.** Homogeneity plot for N-methylhistidine

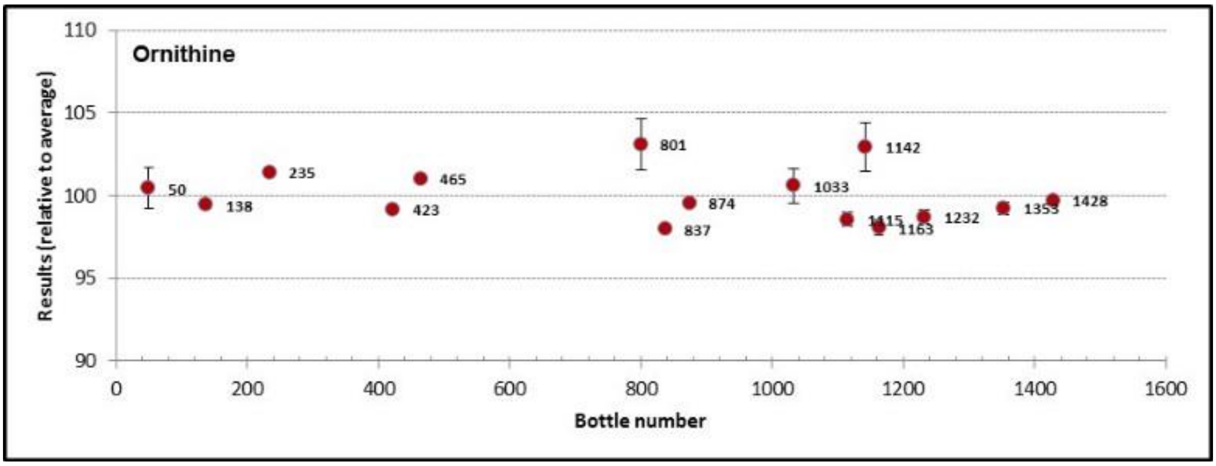


**Figure A26.** Homogeneity plot for Ornithine

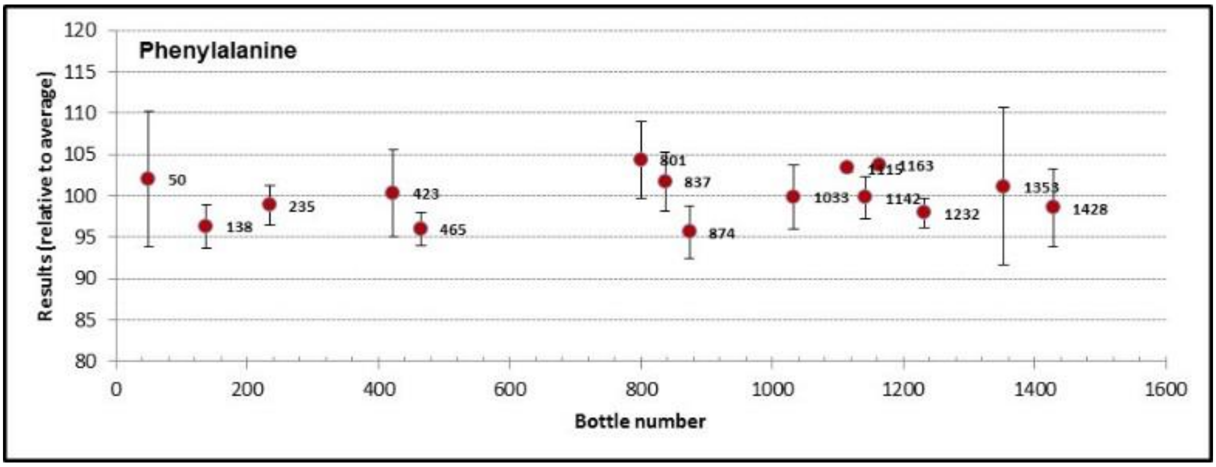


**Figure A27.** Homogeneity plot for Phenylalanine

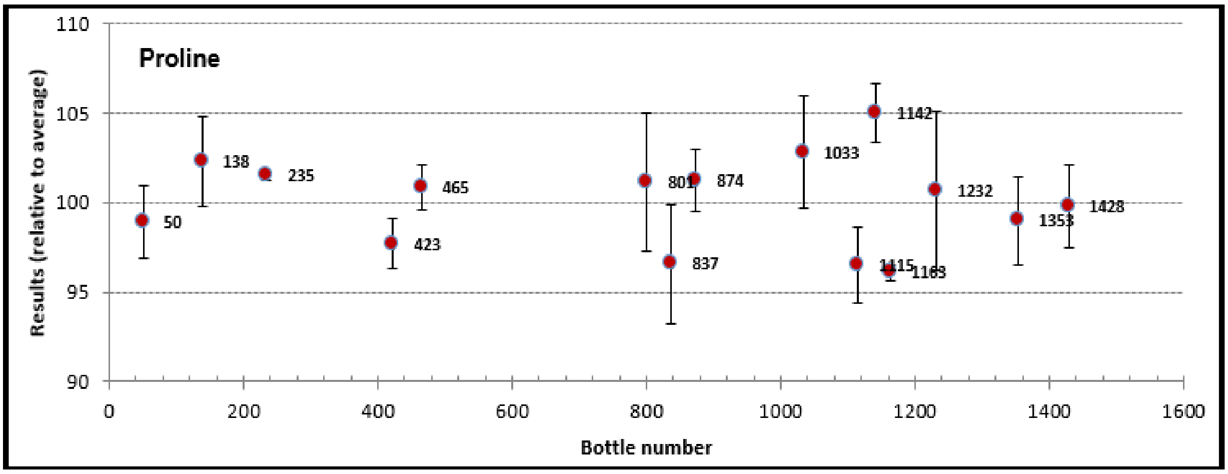


**Figure A28.** Homogeneity plot for Proline

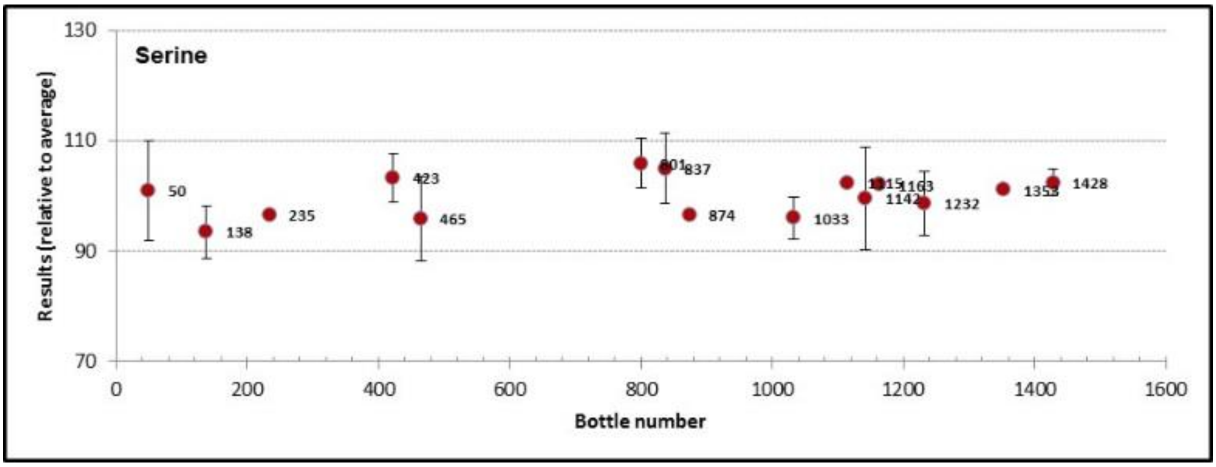


**Figure A29.** Homogeneity plot for Serine

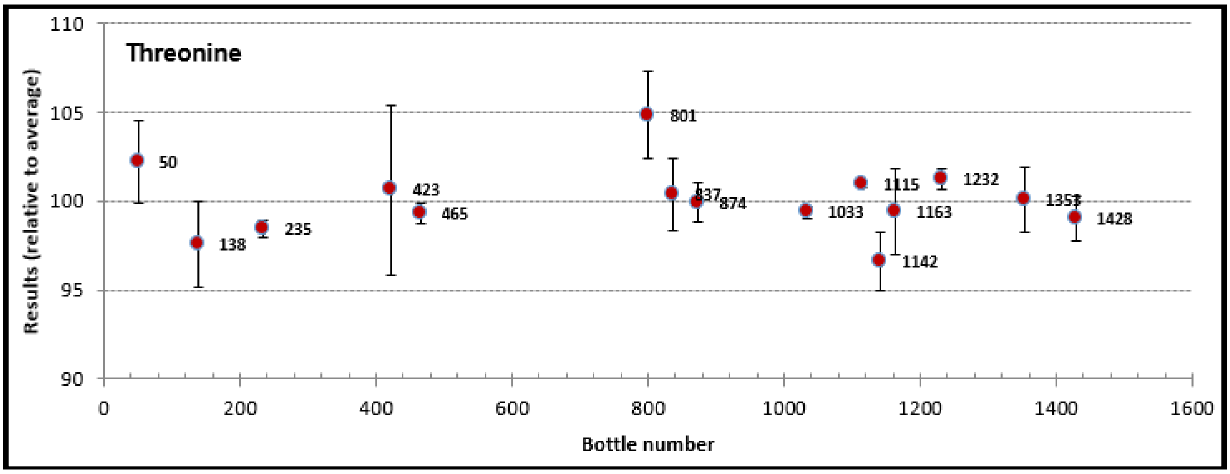


**Figure A30.** Homogeneity plot for Threonine

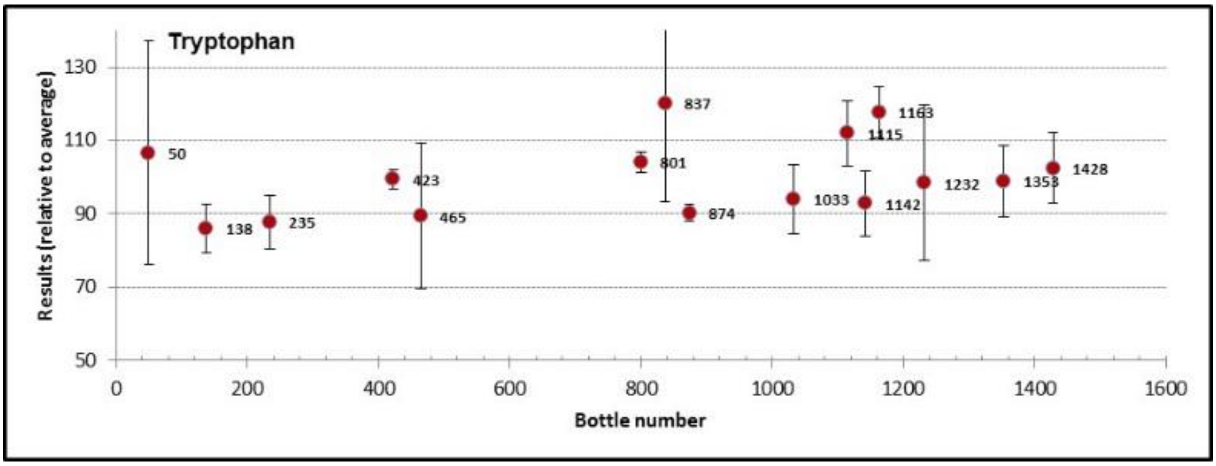


**Figure A31.** Homogeneity plot for Tryptophan

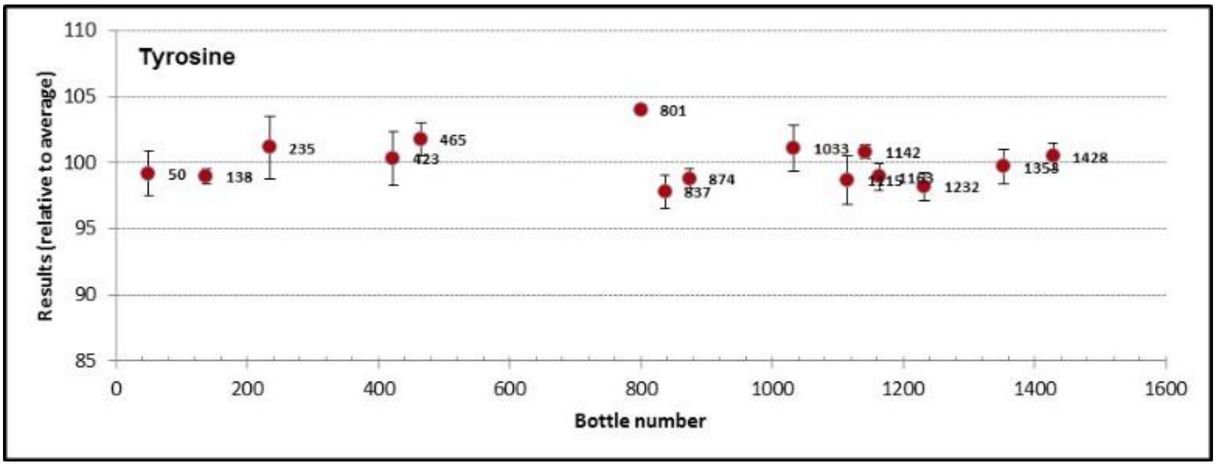


**Figure A32.** Homogeneity plot for Tyrosine

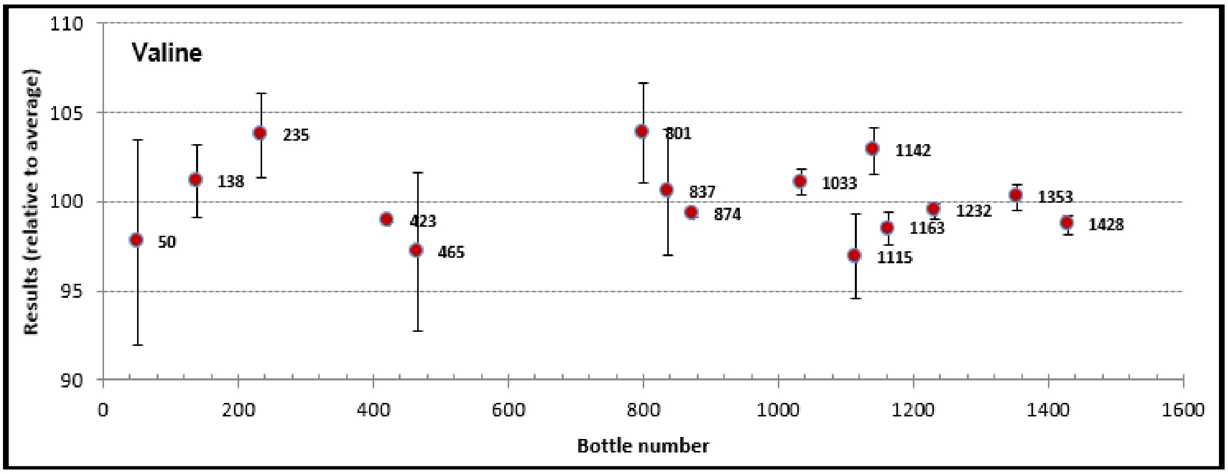


**Figure A33.** Homogeneity plot for Valine

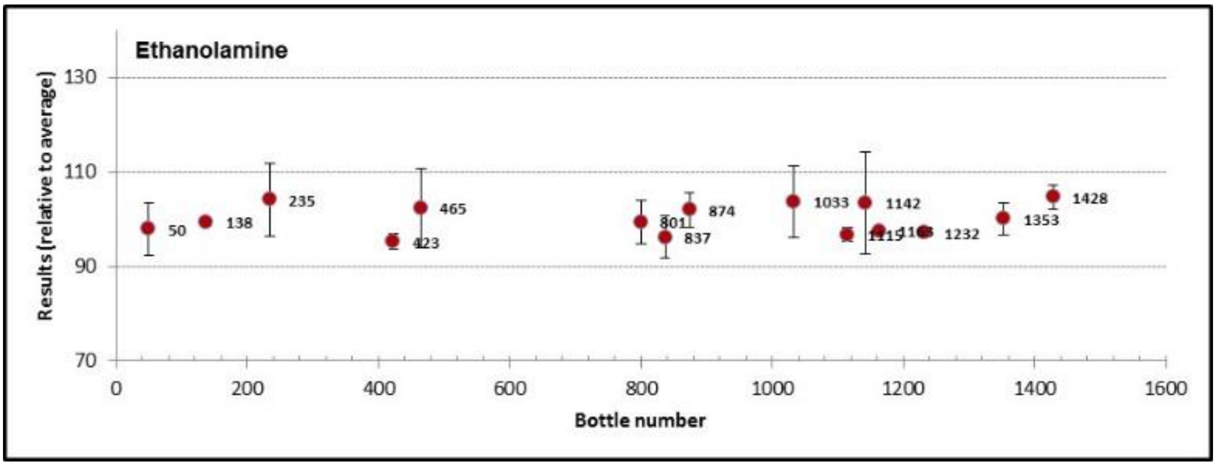


**Figure A34.** Homogeneity plot for Ethanolamine

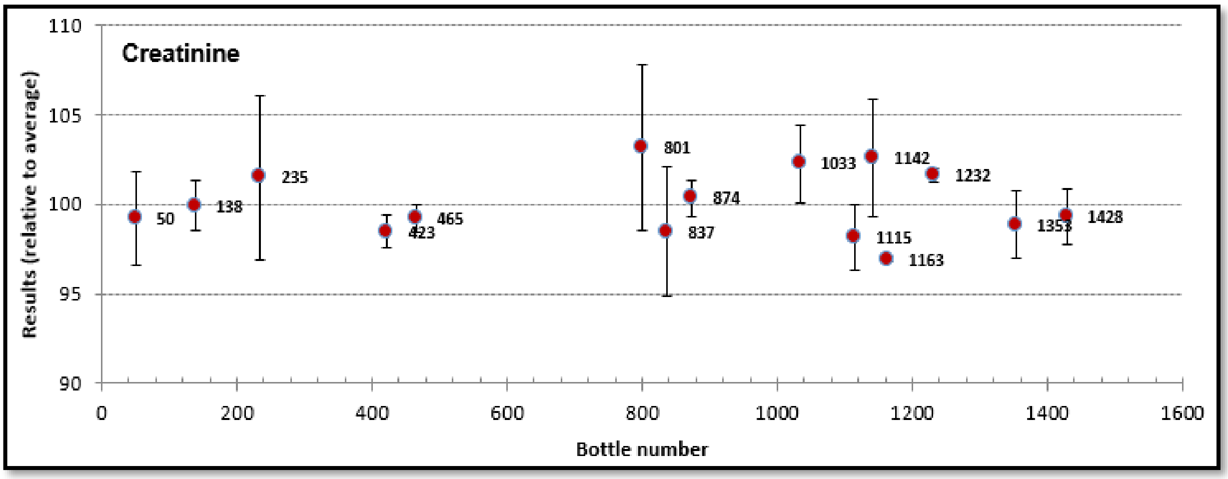


**Figure A35.** Homogeneity plot for Creatinine

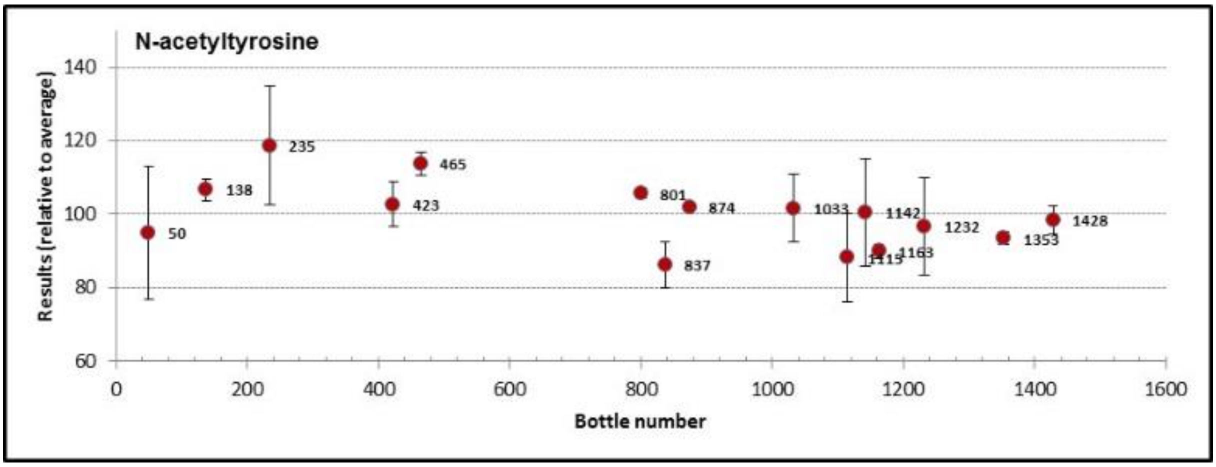


**Figure A36.** Homogeneity plot for N-acetyltyrosine

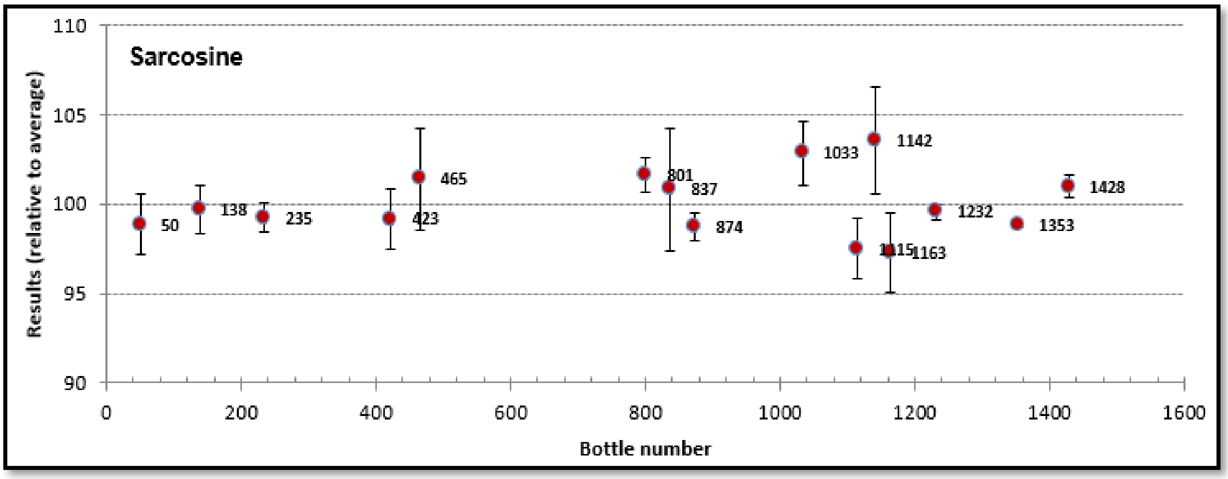


**Figure A37.** Homogeneity plot for Sarcosine

## Annex 3. Graphs for Short Term Stability Studies

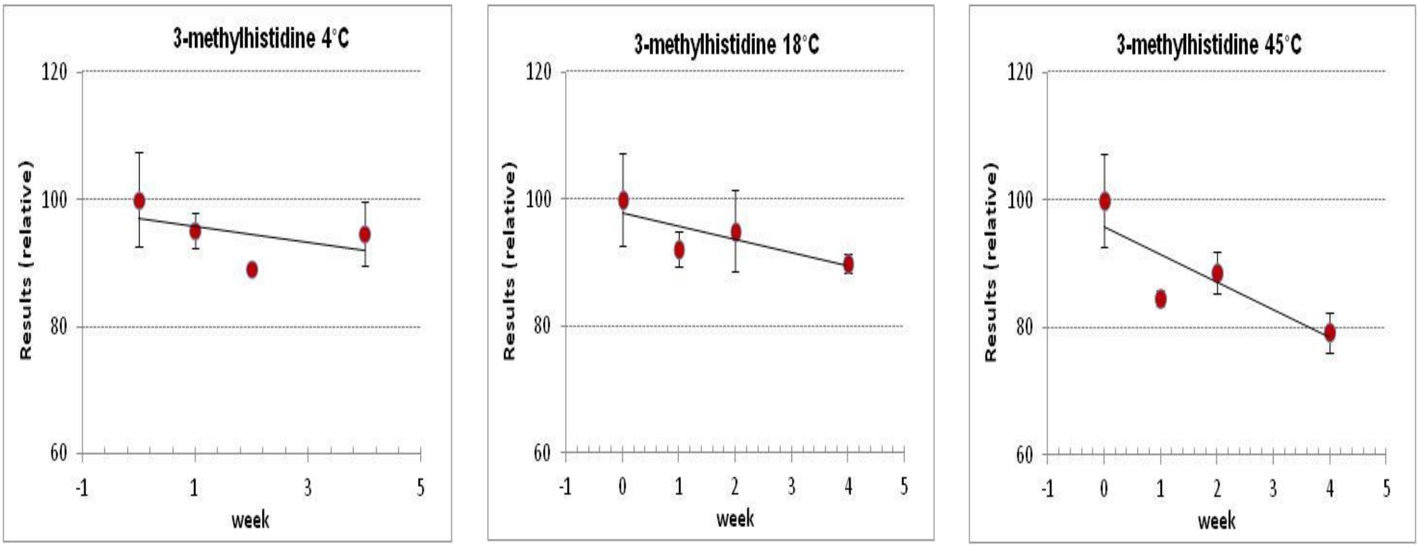


**Figure A38.** Short Term Stability Plot for 3-methylhistidine at 4 °C, 18 °C and 45 °C

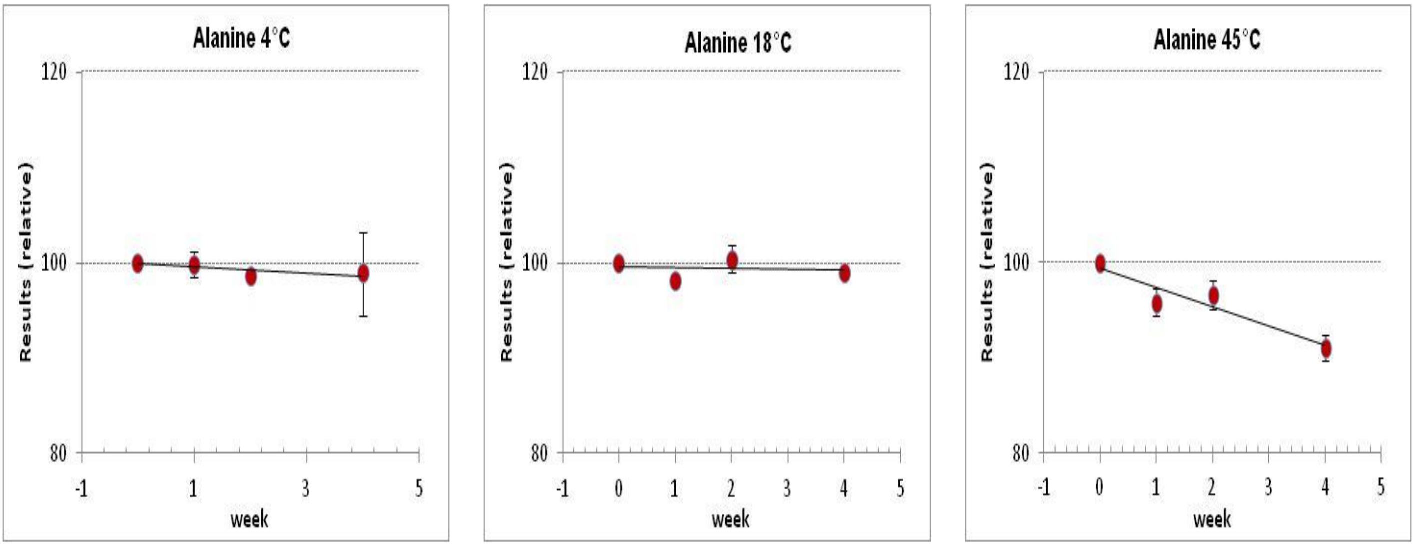


**Figure A39.** Short Term Stability Plot for Alanine at 4 °C, 18 °C and 45 °C

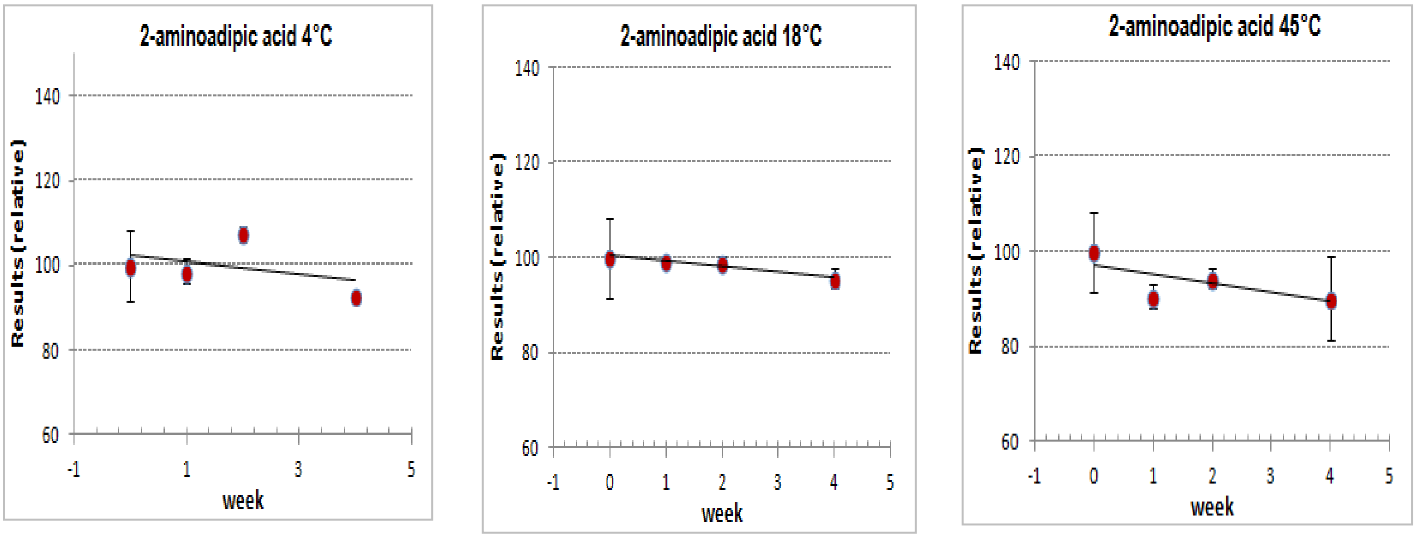


**Figure A40.** Short Term Stability Plot for 2-aminoadipic acid at 4 °C, 18 °C and 45 °C

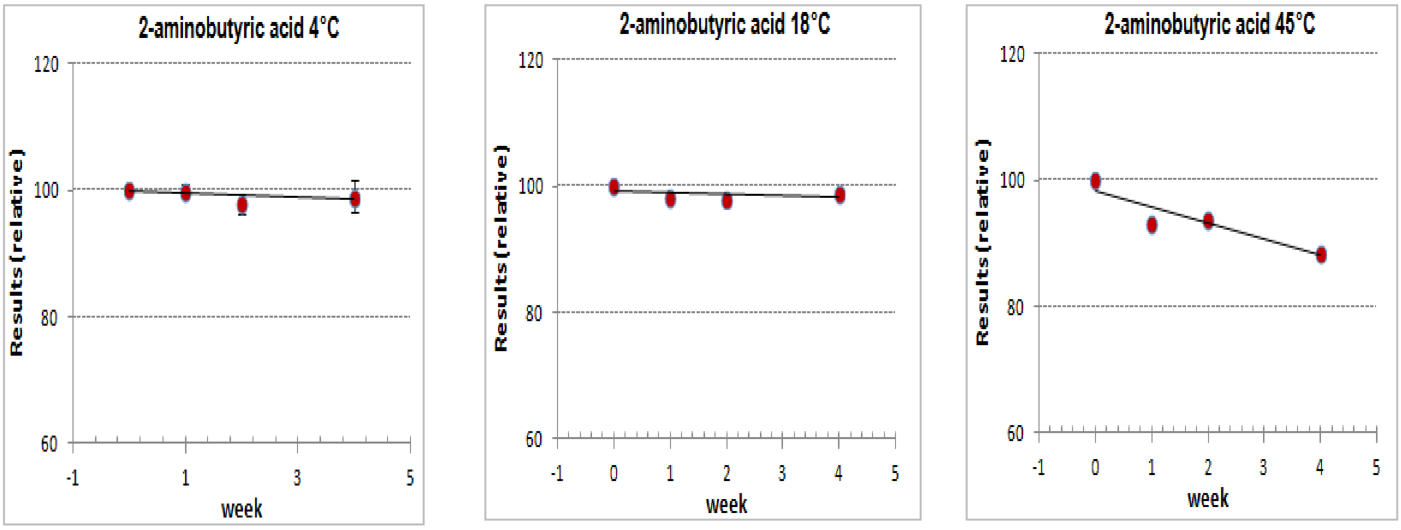


**Figure A41.** Short Term Stability Plot for 2-aminobutyric acid at 4 °C, 18 °C and 45 °C

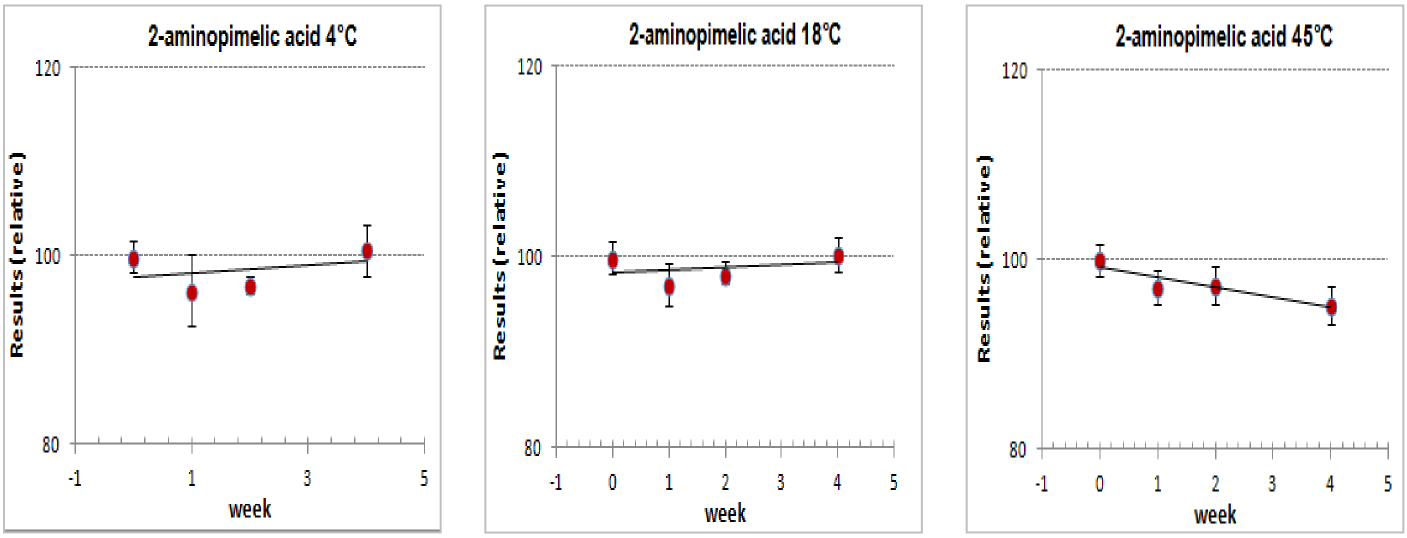


**Figure A42.** Short Term Stability Plot for 2-aminopimelic acid at 4 °C, 18 °C and 45 °C

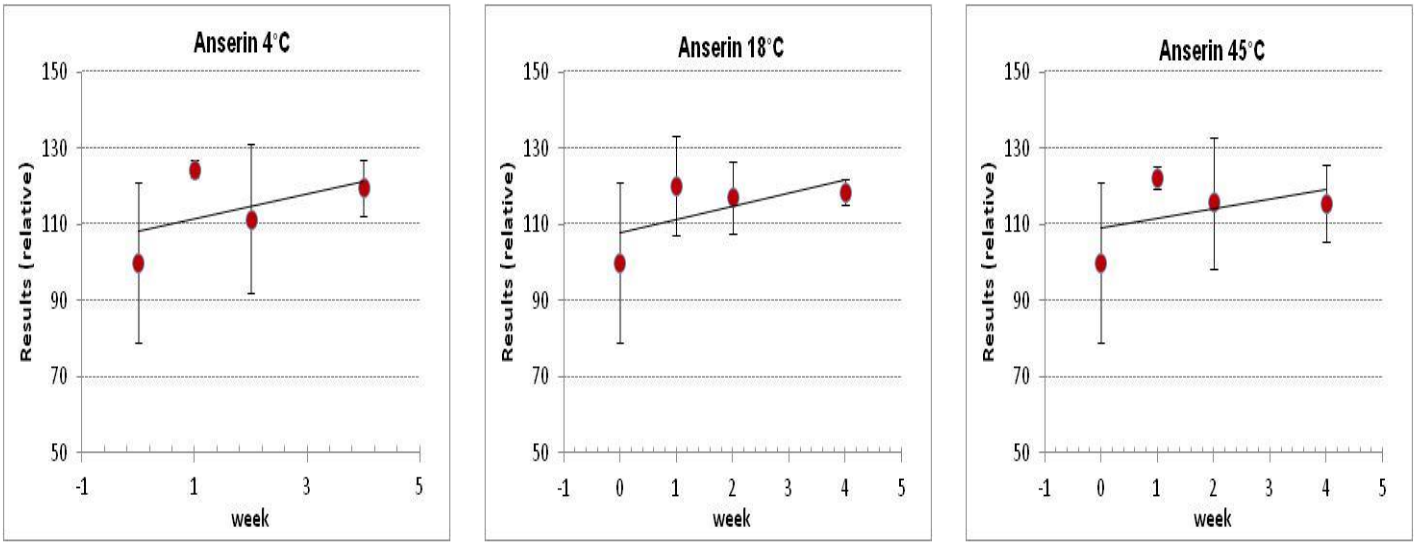


**Figure A43.** Short Term Stability Plot for Anserin at 4 °C, 18 °C and 45 °C

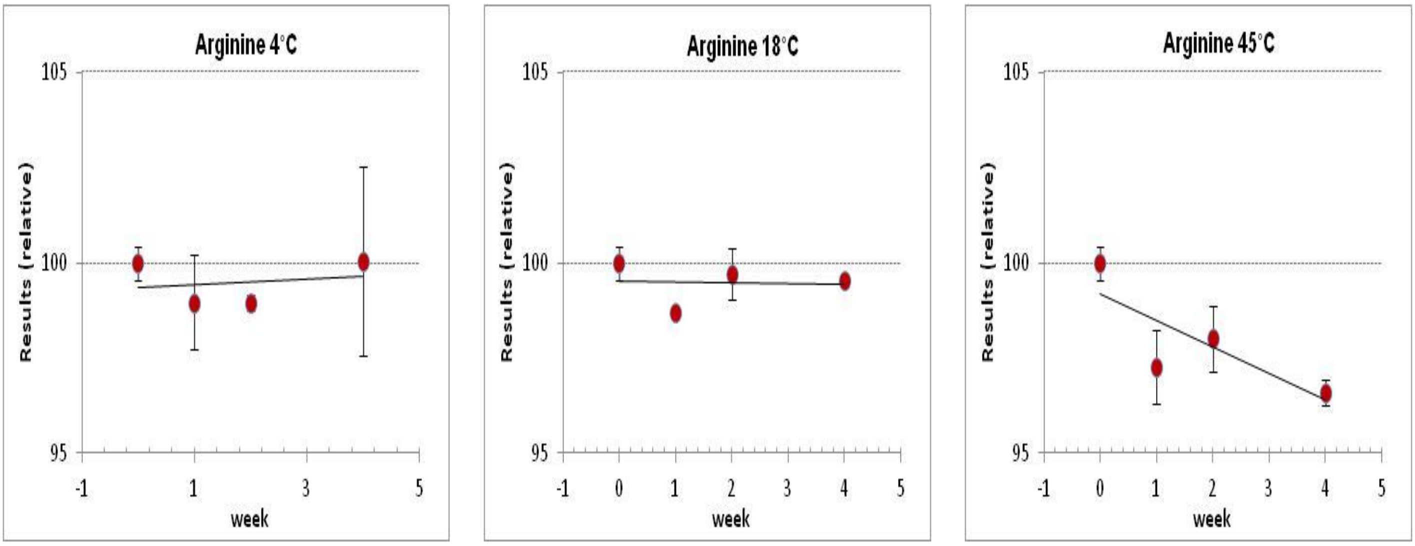


**Figure A44.** Short Term Stability Plot for Arginine at 4 °C, 18 °C and 45 °C

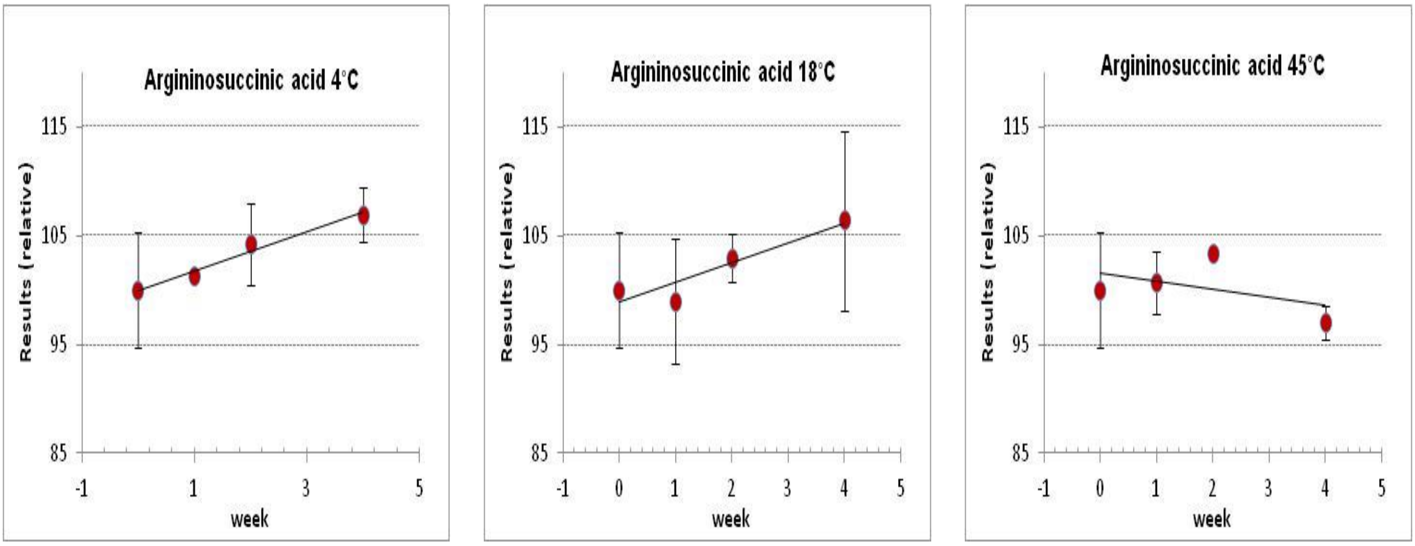


**Figure A45.** Short Term Stability Plot for Argininosuccinic acid at 4 °C, 18 °C and 45 °C

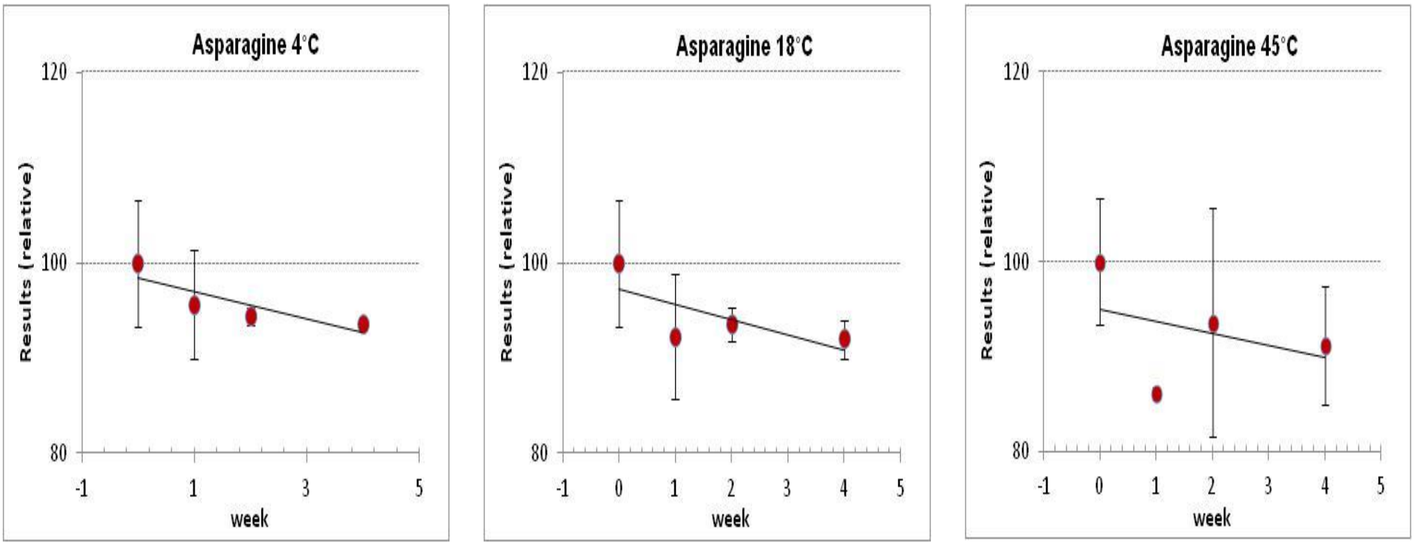


**Figure A46.** Short Term Stability Plot for Asparagine at 4 °C, 18 °C and 45 °C

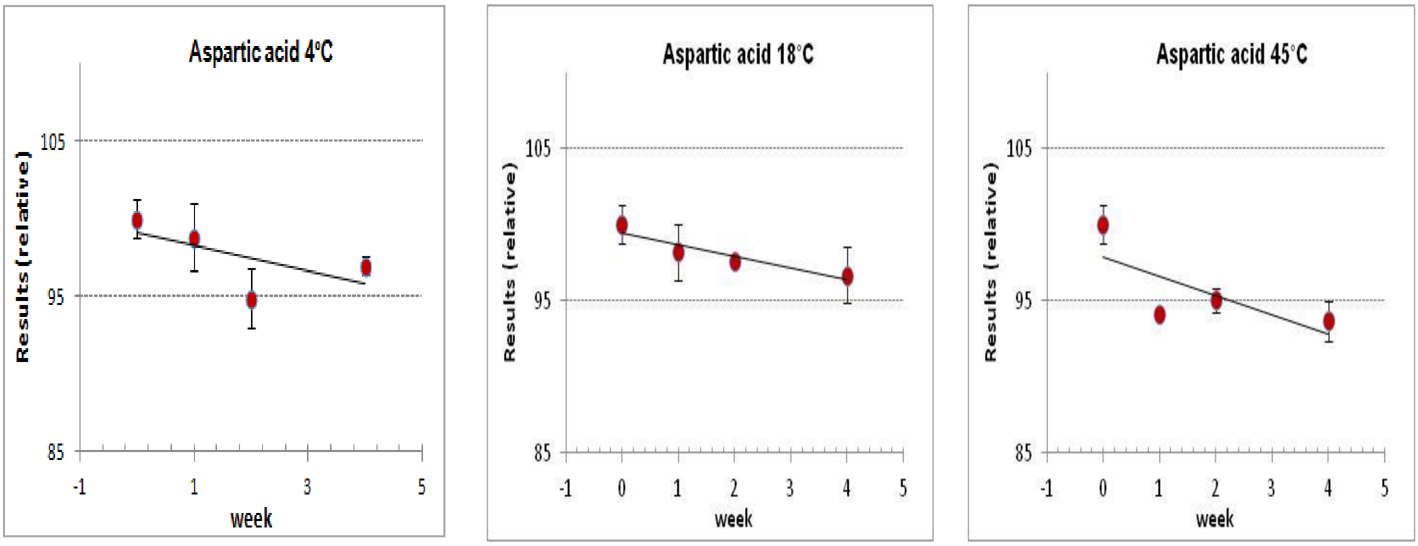


**Figure A47.** Short Term Stability Plot for Aspartic acid at 4 °C, 18 °C and 45 °C

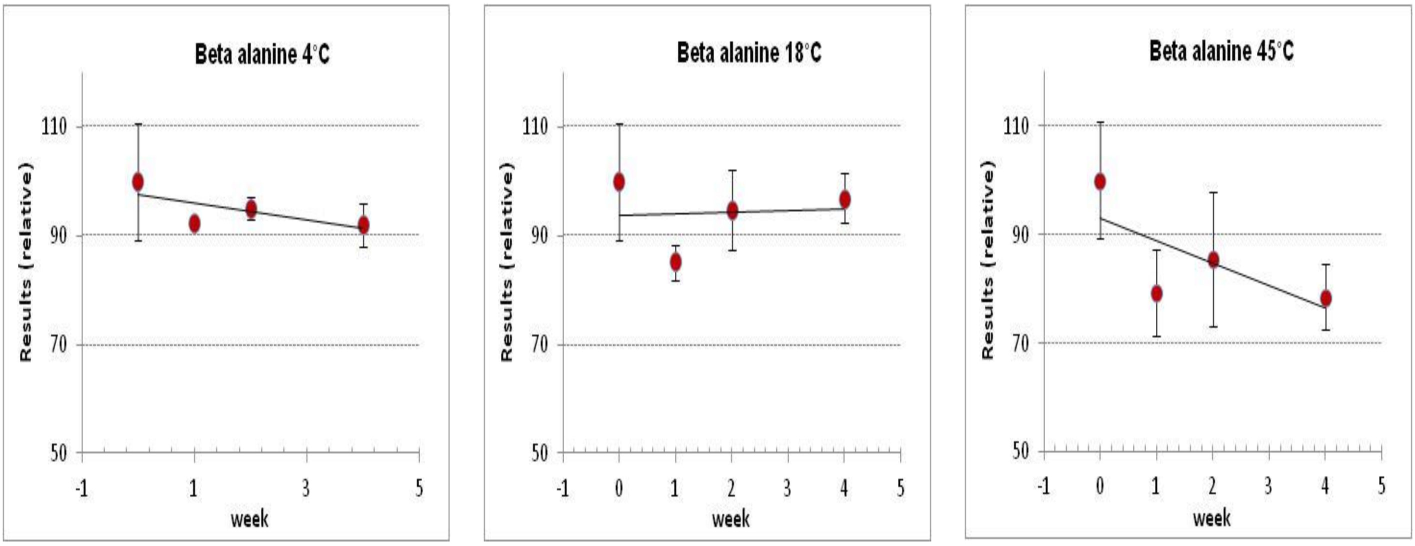


**Figure A48.** Short Term Stability Plot for Beta alanine at 4 °C, 18 °C and 45 °C

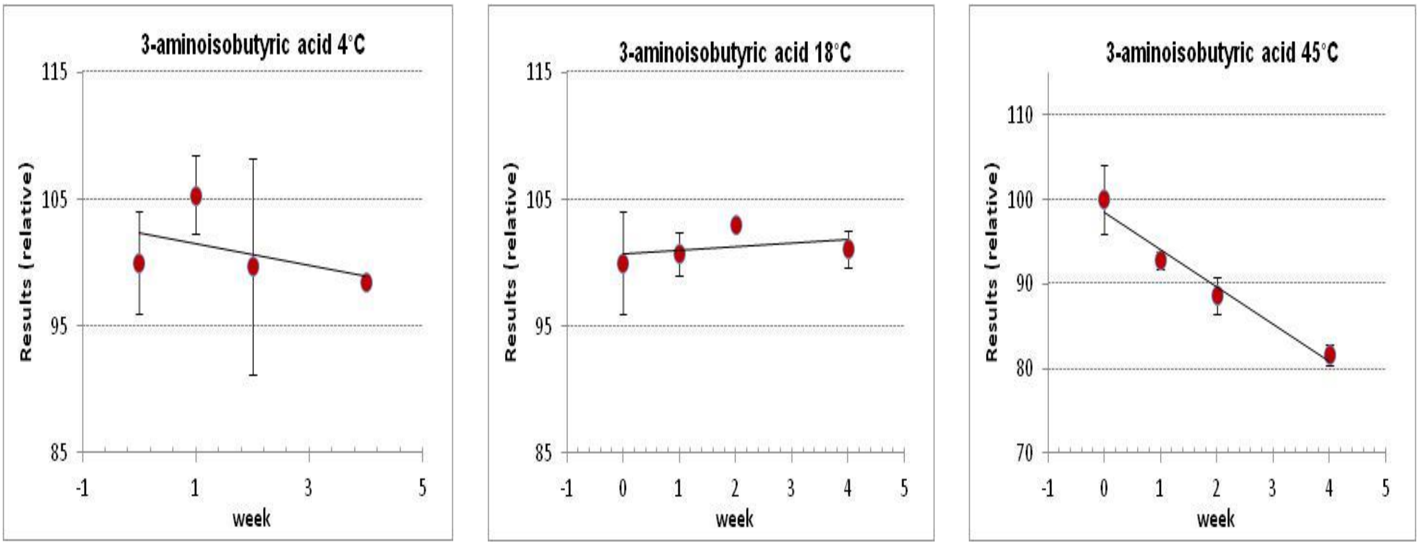


**Figure A49.** Short Term Stability Plot for 3-aminoisobutyric acid at 4 °C, 18 °C and 45 °C

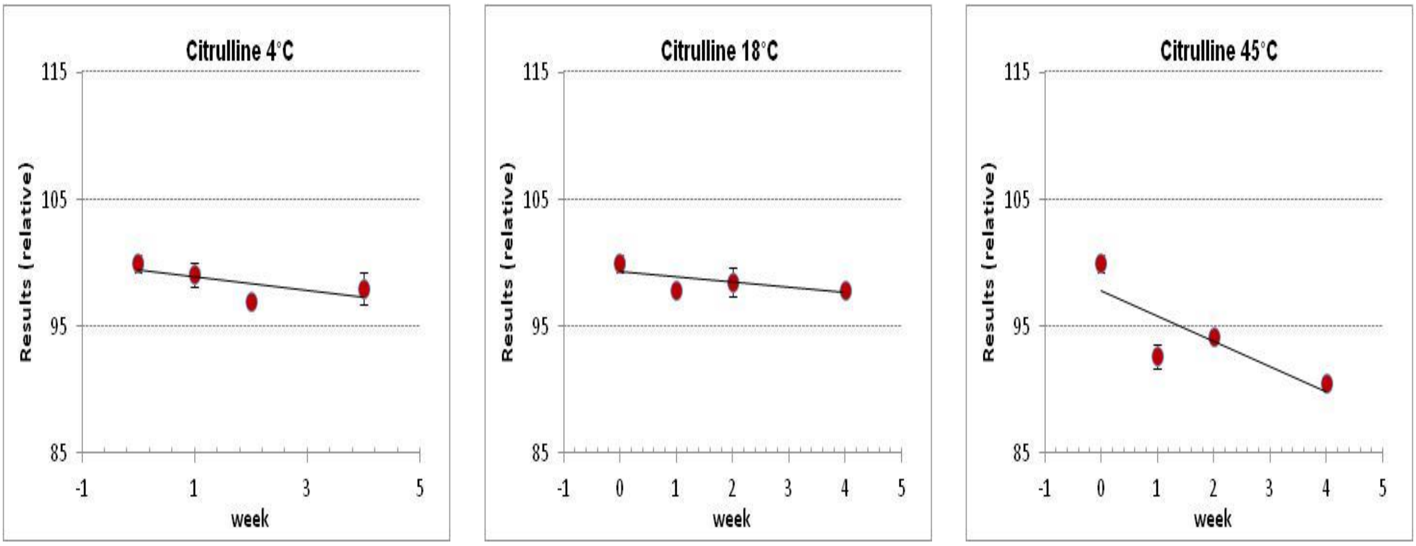


**Figure A50.** Short Term Stability Plot for Citrulline at 4 °C, 18 °C and 45 °C

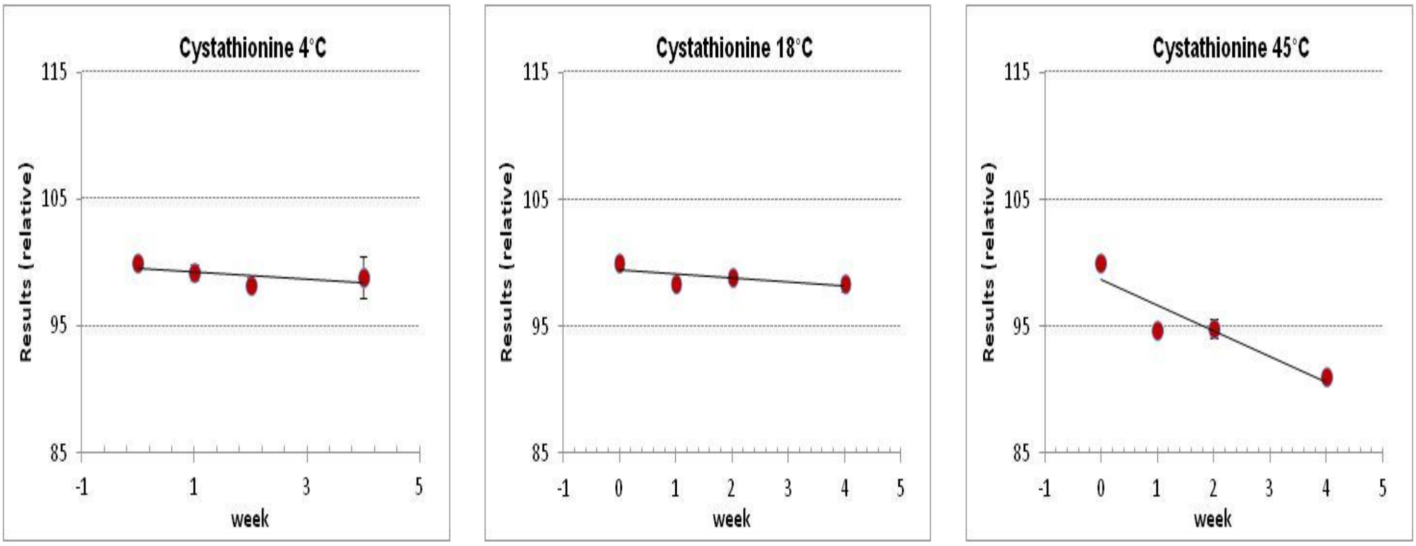


**Figure A51.** Short Term Stability Plot for Cystathionine at 4 °C, 18 °C and 45 °C

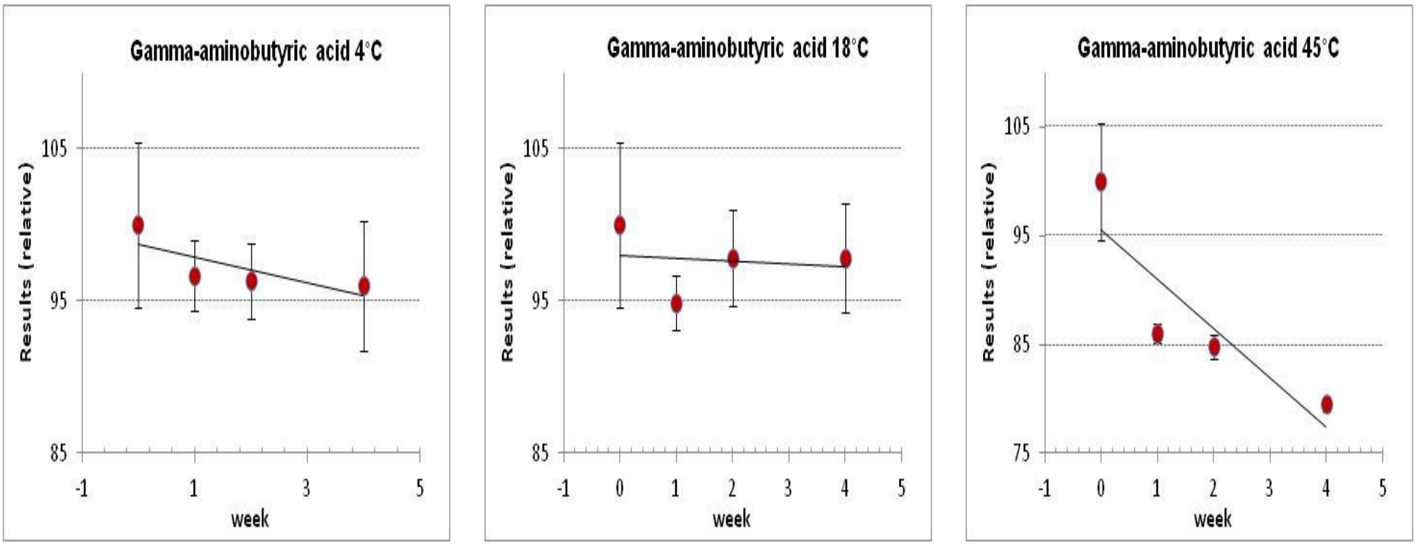


**Figure A52.** Short Term Stability Plot for 4-Aminobutyric acid at 4 °C, 18 °C and 45 °C

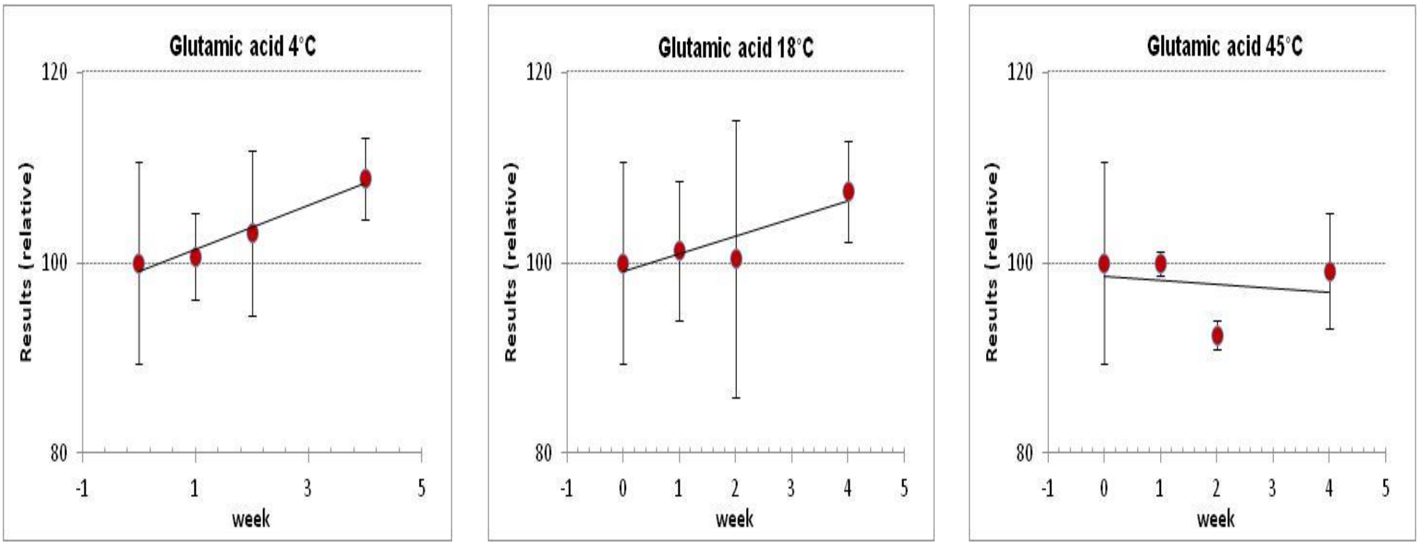


**Figure A53.** Short Term Stability Plot for Glutamic acid at 4 °C, 18 °C and 45 °C

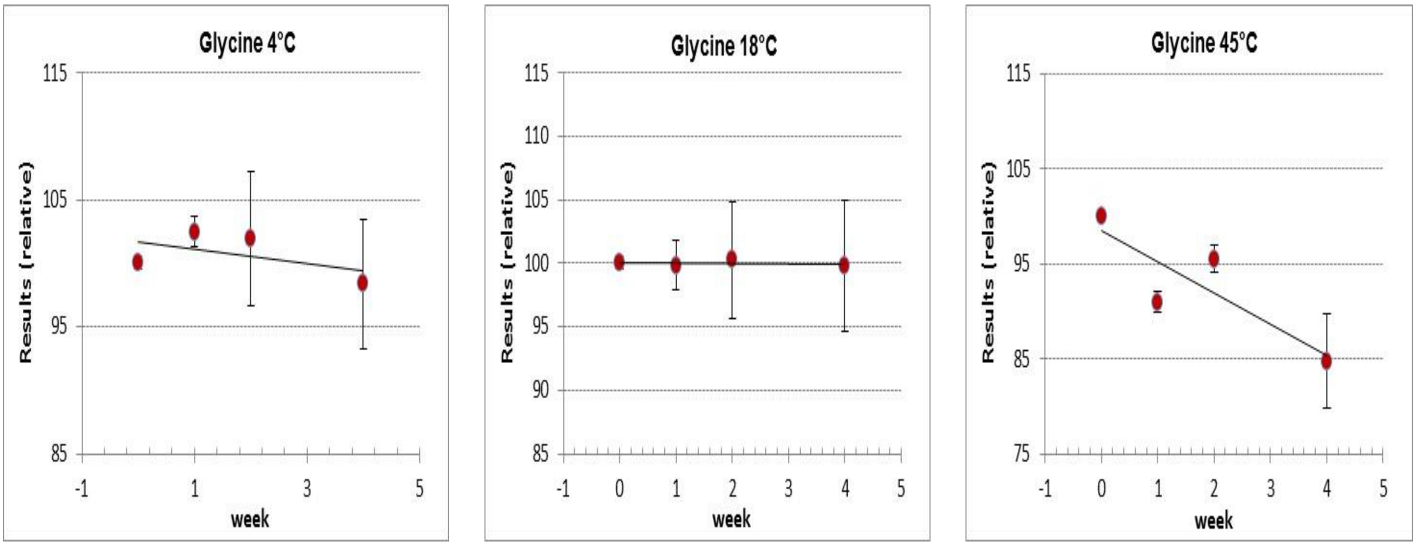


**Figure A54.** Short Term Stability Plot for Glycine at 4 °C, 18 °C and 45 °C

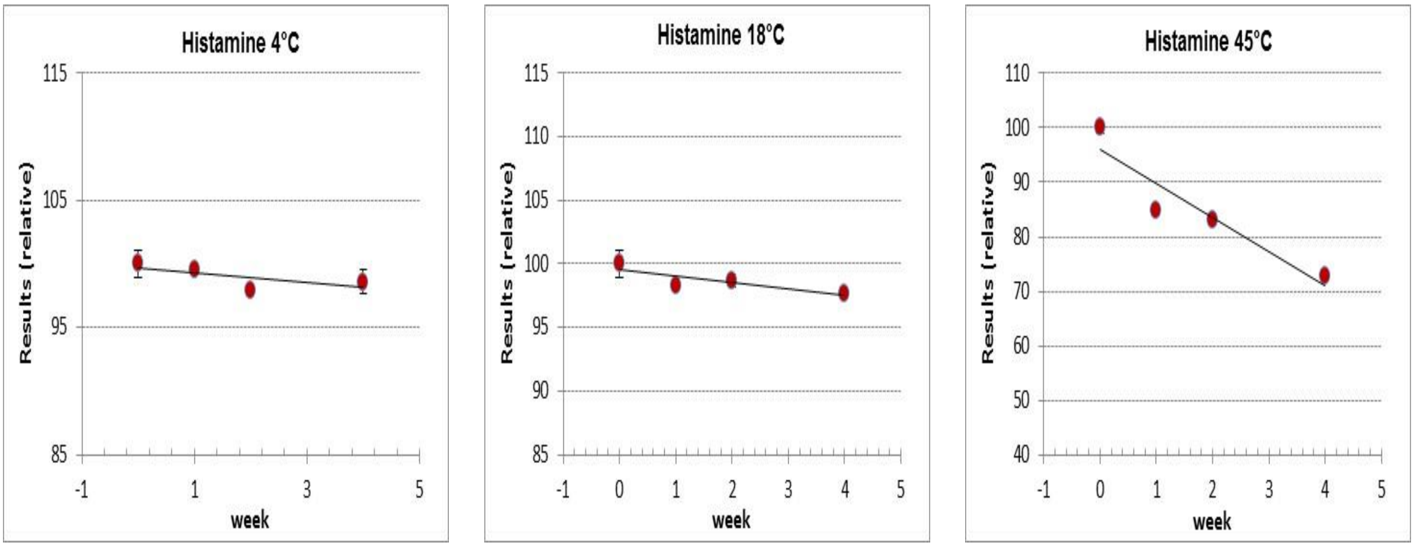


**Figure A55.** Short Term Stability Plot for Histamine at 4 °C, 18 °C and 45 °C

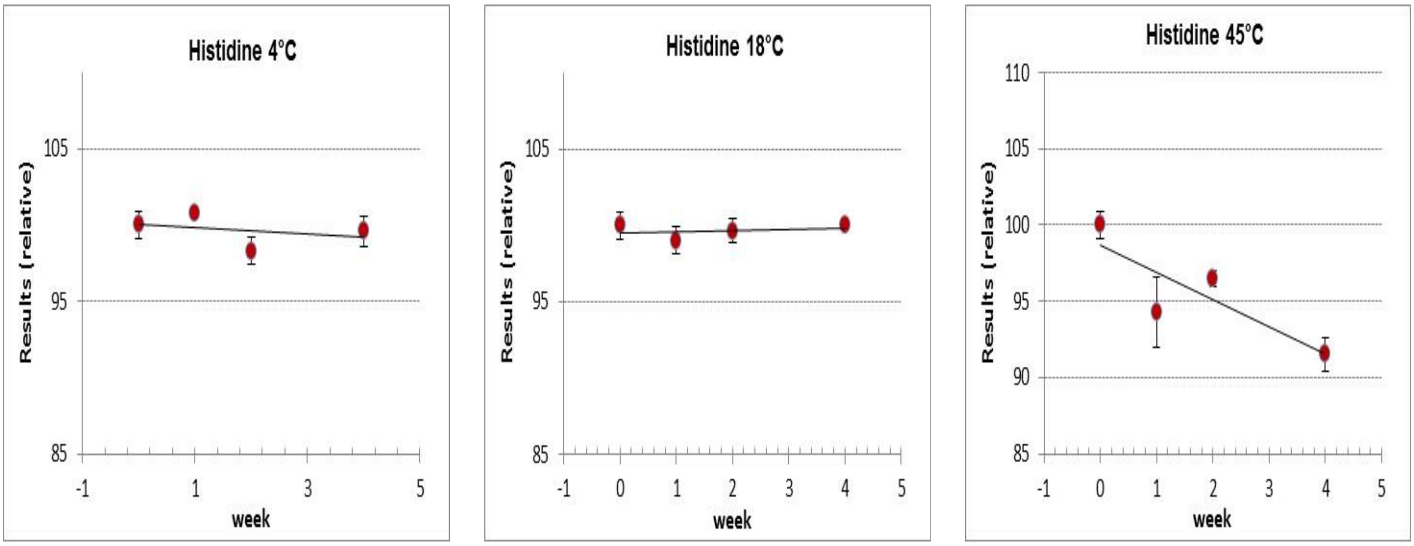


**Figure A56.** Short Term Stability Plot for Histidine at 4 °C, 18 °C and 45 °C

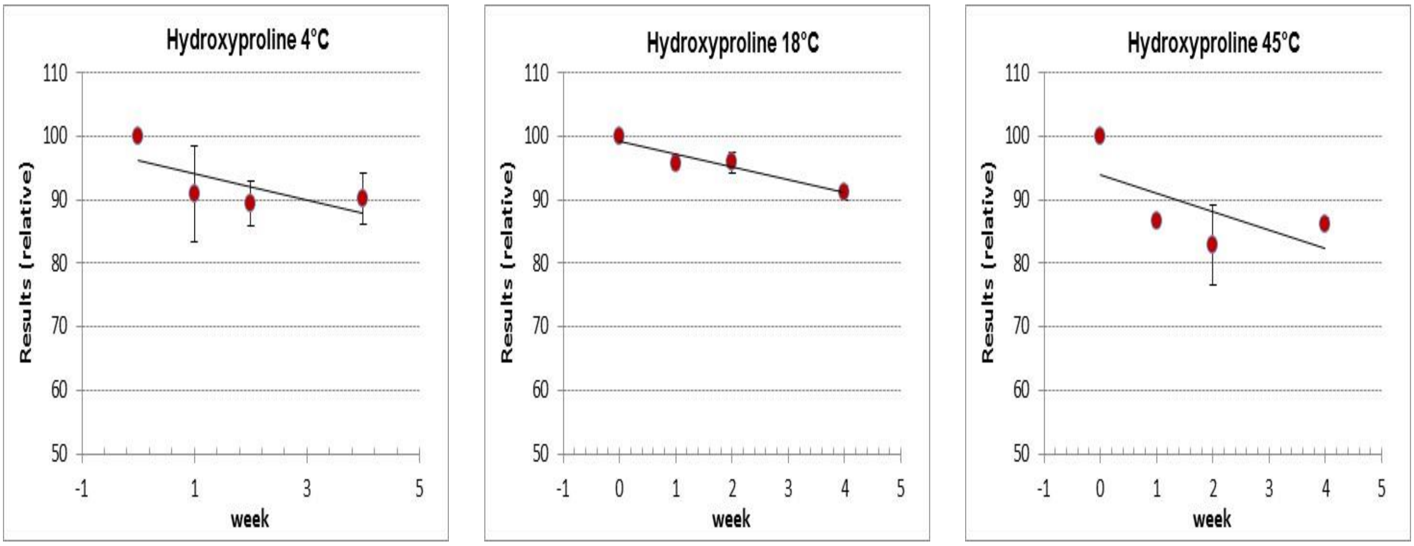


**Figure A57.** Short Term Stability Plot for Hydroxyproline at 4 °C, 18 °C and 45 °C

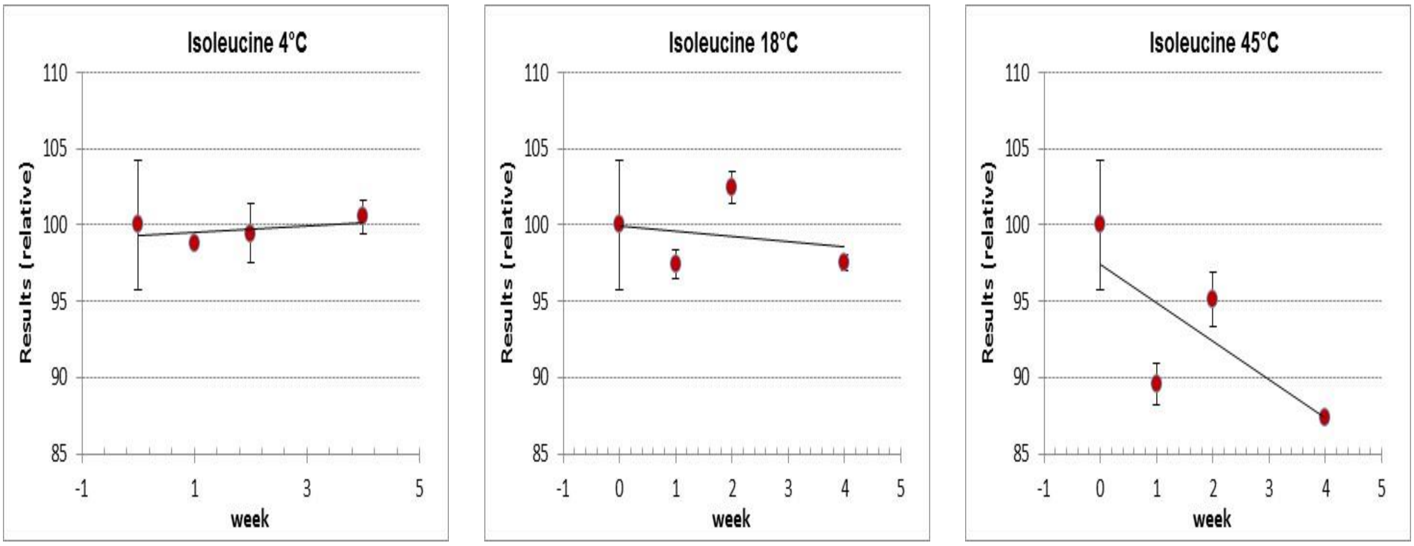


**Figure A58.** Short Term Stability Plot for Isoleucine at 4 °C, 18 °C and 45 °C

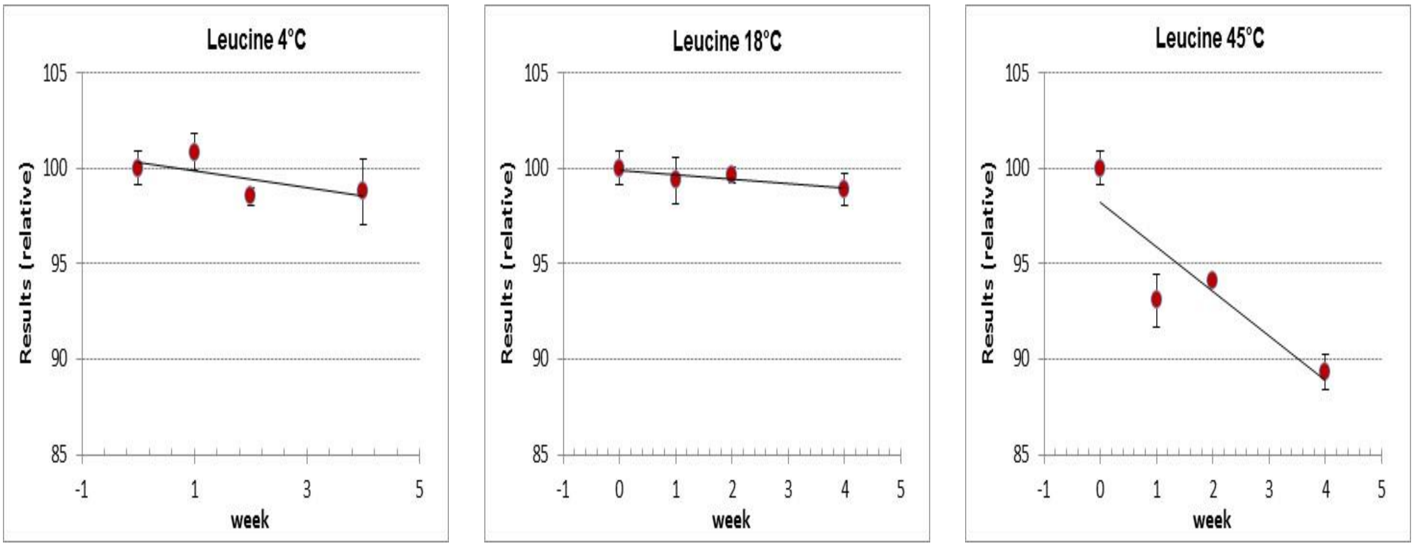


**Figure A59.** Short Term Stability Plot for Leucine at 4 °C, 18 °C and 45 °C

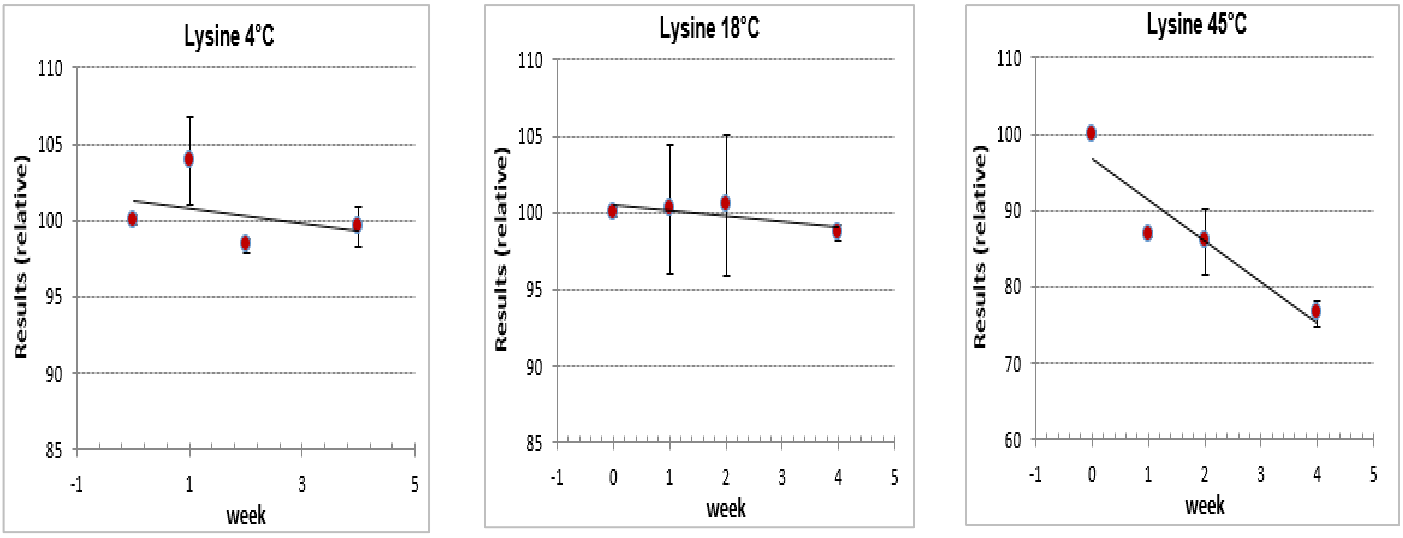


**Figure A60.** Short Term Stability Plot for Lysine at 4 °C, 18 °C and 45 °C

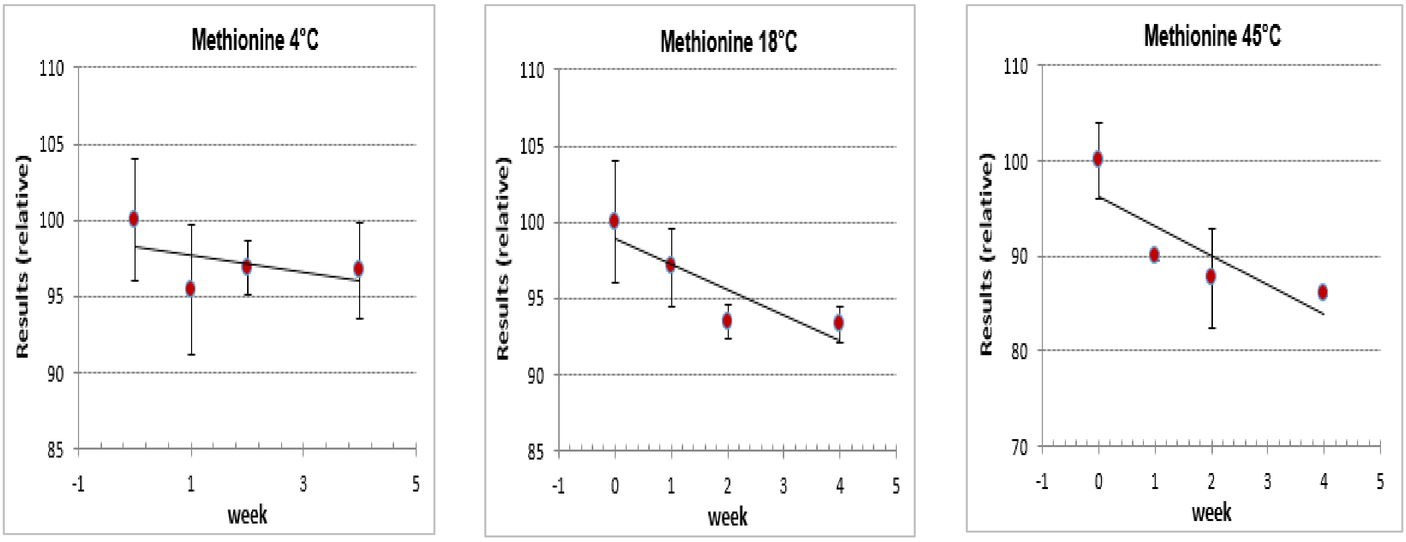


**Figure A61.** Short Term Stability Plot for Methionine at 4 °C, 18 °C and 45 °C

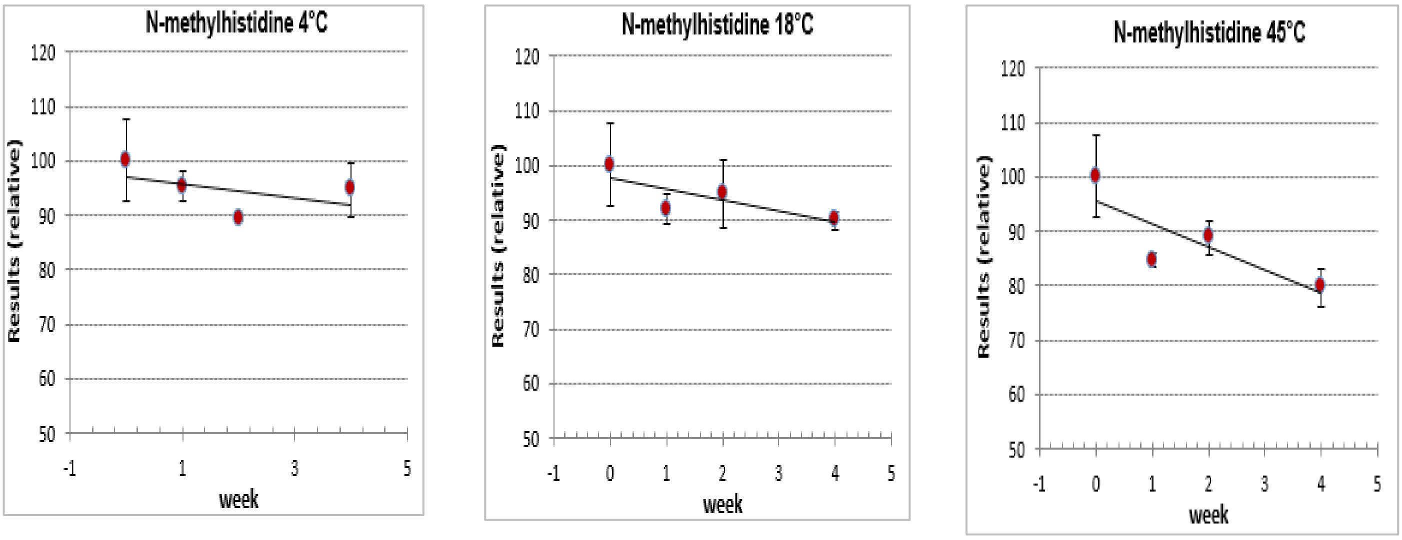


**Figure A62.** Short Term Stability Plot for N-methylhistidine at 4 °C, 18 °C and 45 °C

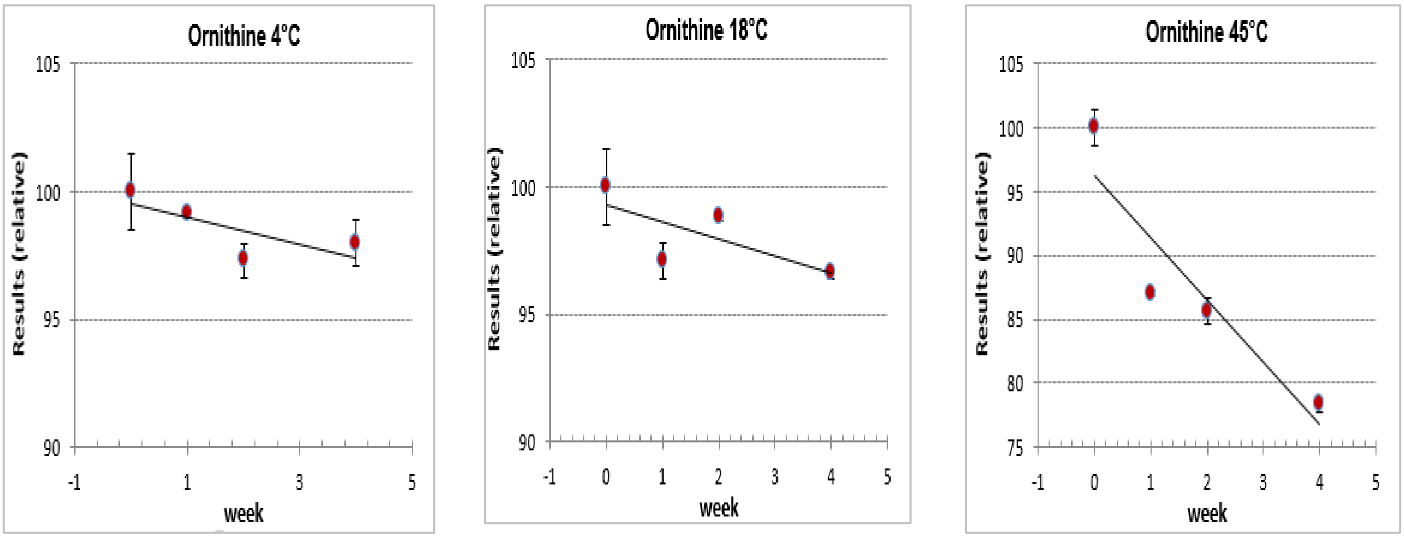


**Figure A63.** Short Term Stability Plot for Ornithine at 4 °C, 18 °C and 45 °C

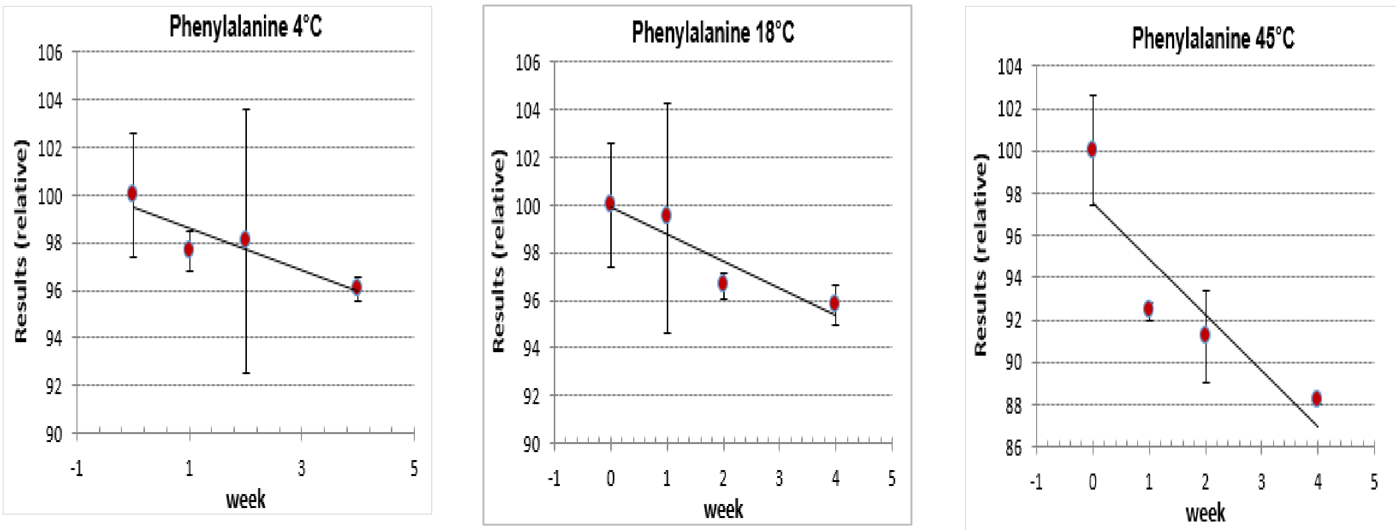


**Figure A64.** Short Term Stability Plot for Phenylalanine at 4 °C, 18 °C and 45 °C

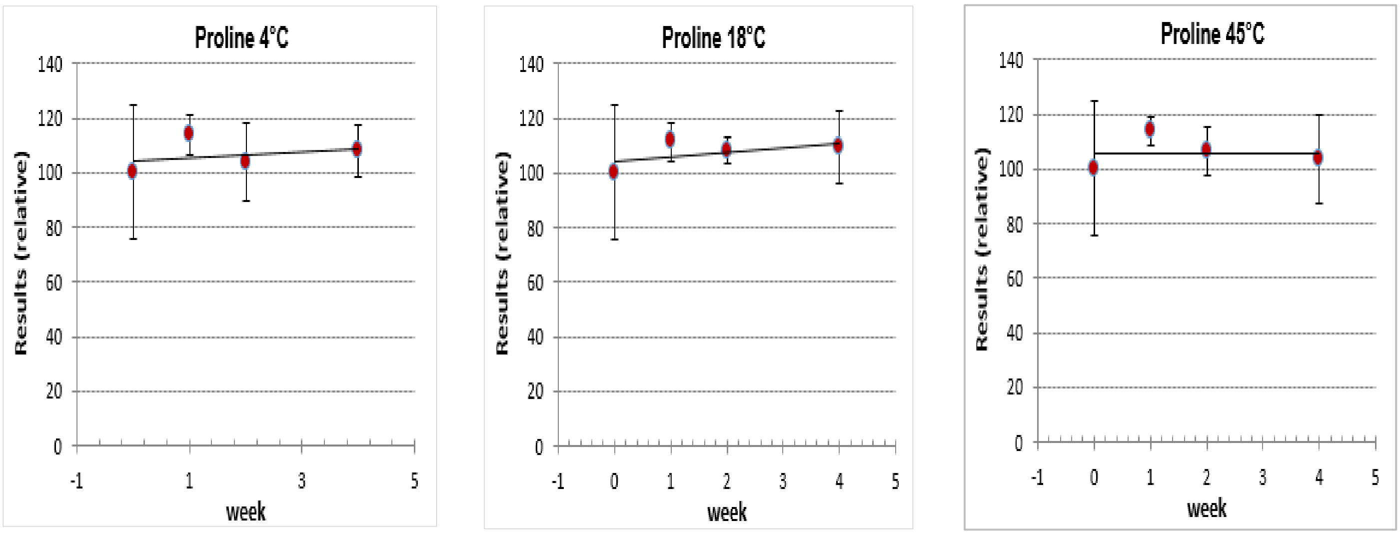


**Figure A65.** Short Term Stability Plot for Proline at 4 °C, 18 °C and 45 °C

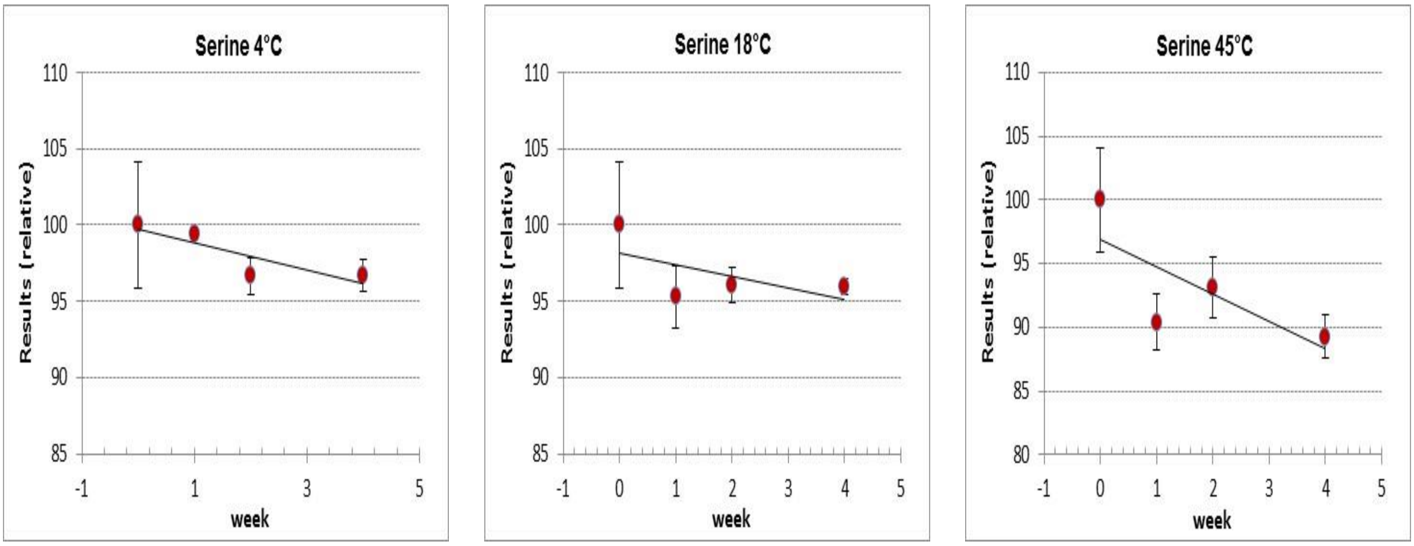


**Figure A66.** Short Term Stability Plot for Serine at 4 °C, 18 °C and 45 °C

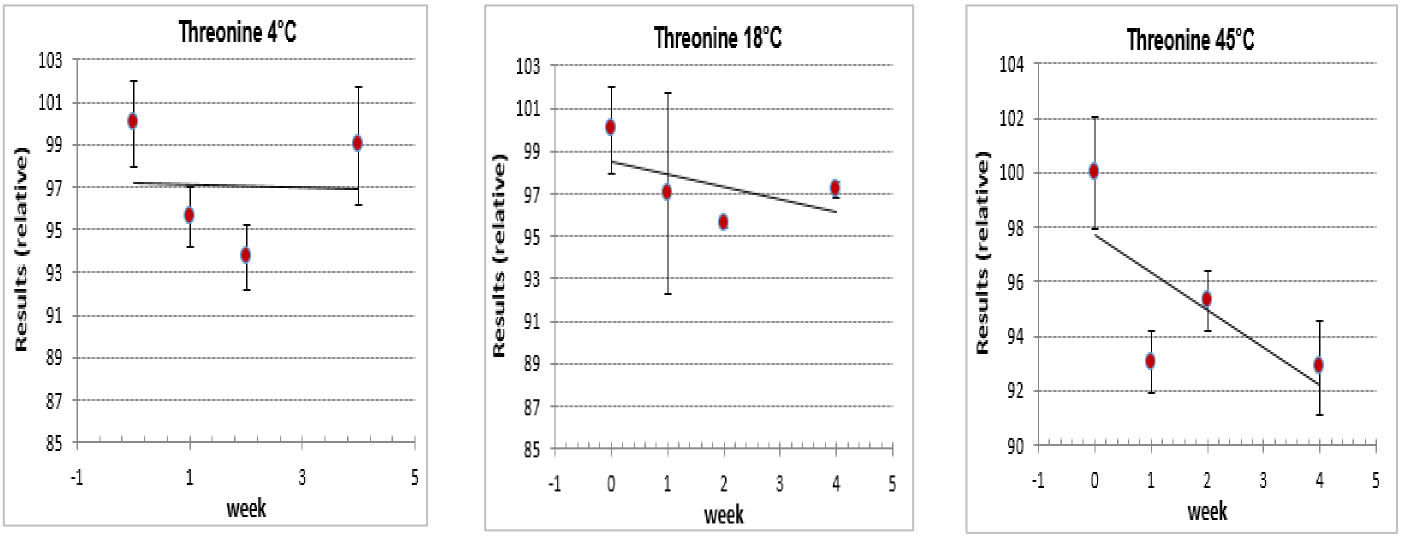


**Figure A67.** Short Term Stability Plot for Threonine at 4 °C, 18 °C and 45 °C

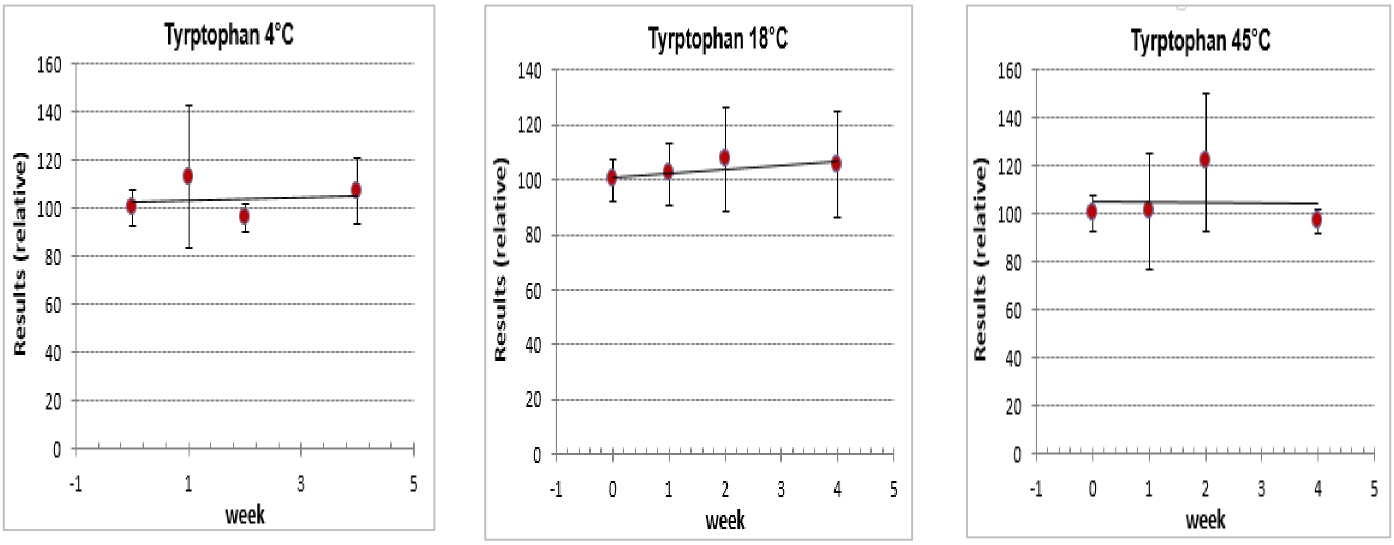


**Figure A68.** Short Term Stability Plot for Tryptophan at 4 °C, 18 °C and 45 °C

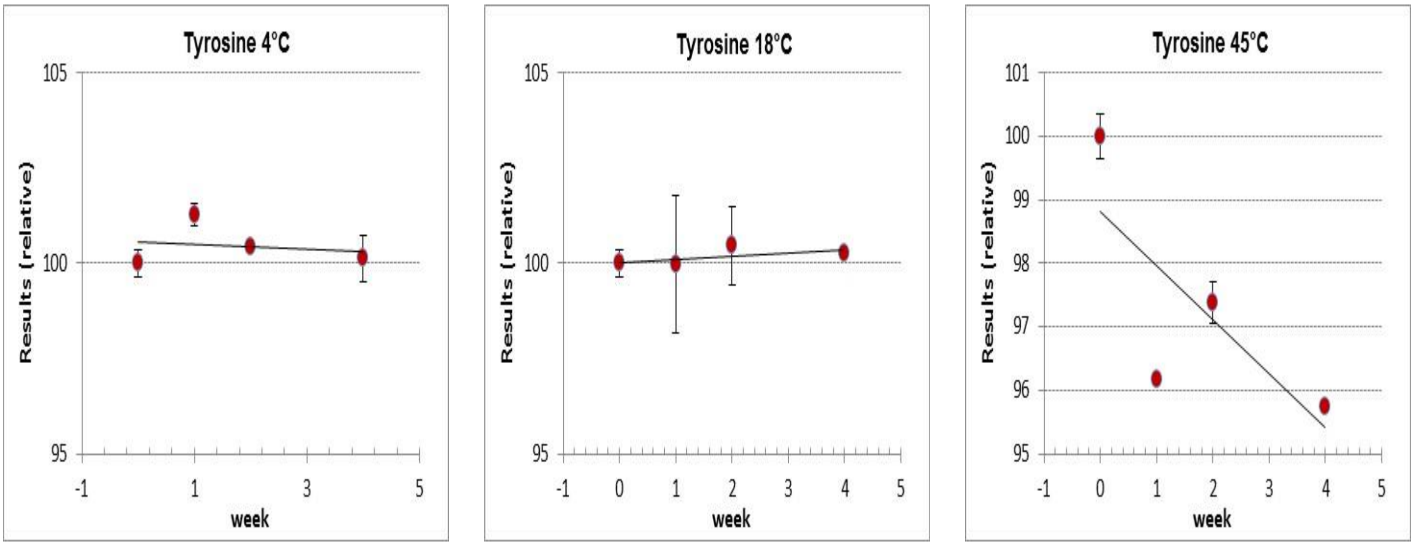


**Figure A69.** Short Term Stability Plot for Tyrosine at 4 °C, 18 °C and 45 °C

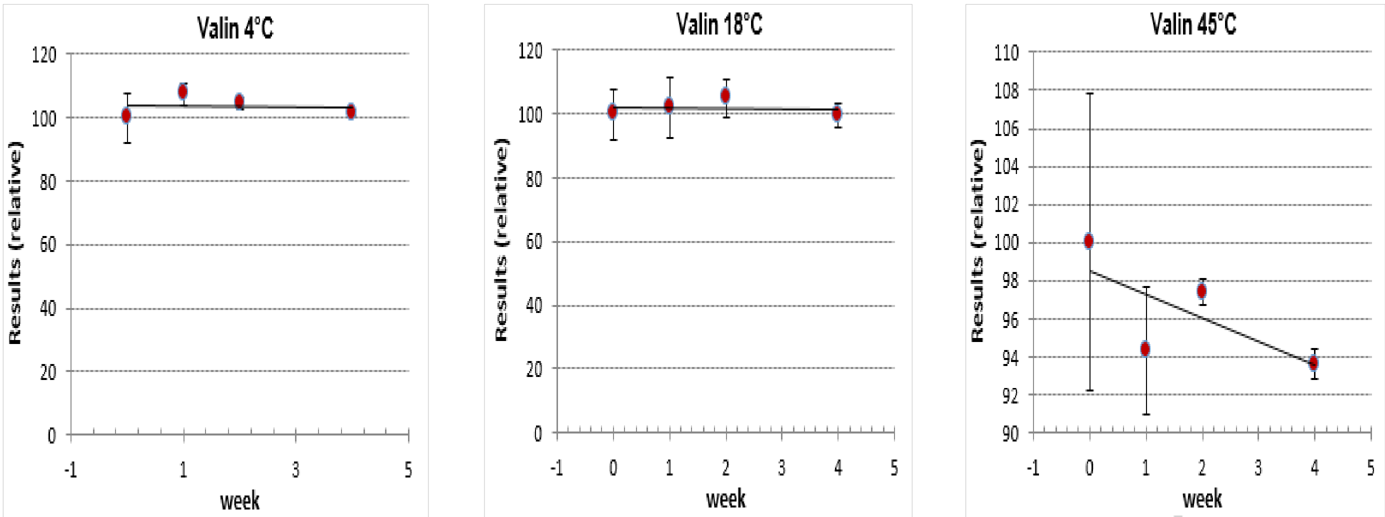


**Figure A70.** Short Term Stability Plot for Valine at 4 °C, 18 °C and 45 °C

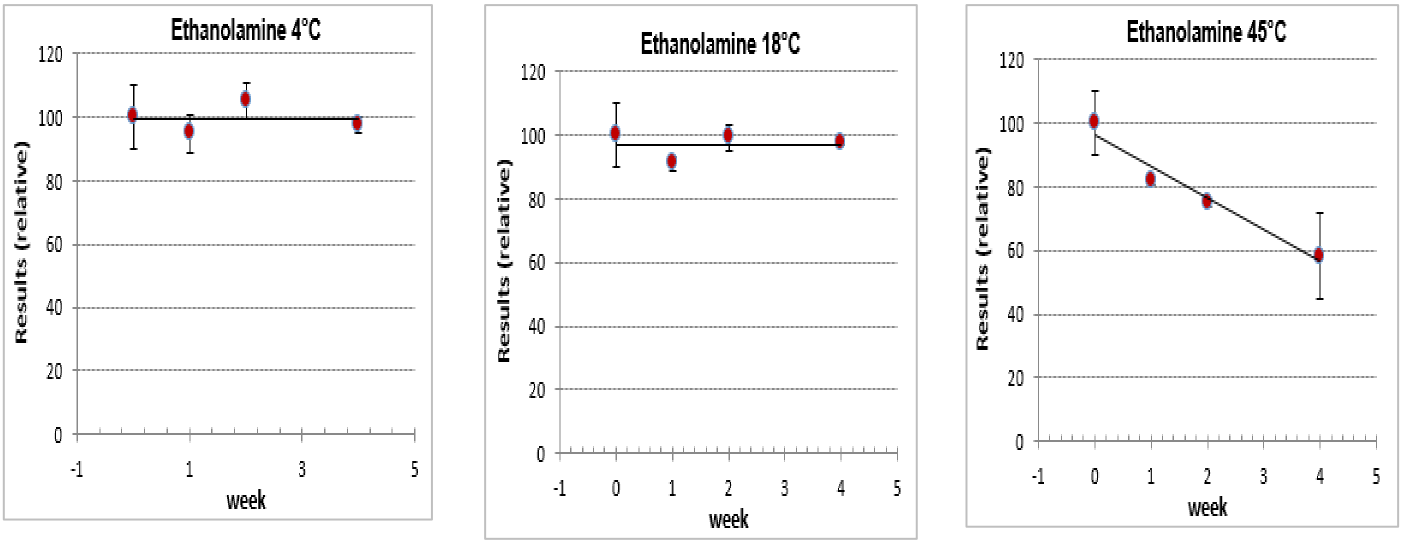


**Figure A71.** Short Term Stability Plot for Ethanolamine at 4 °C, 18 °C and 45 °C

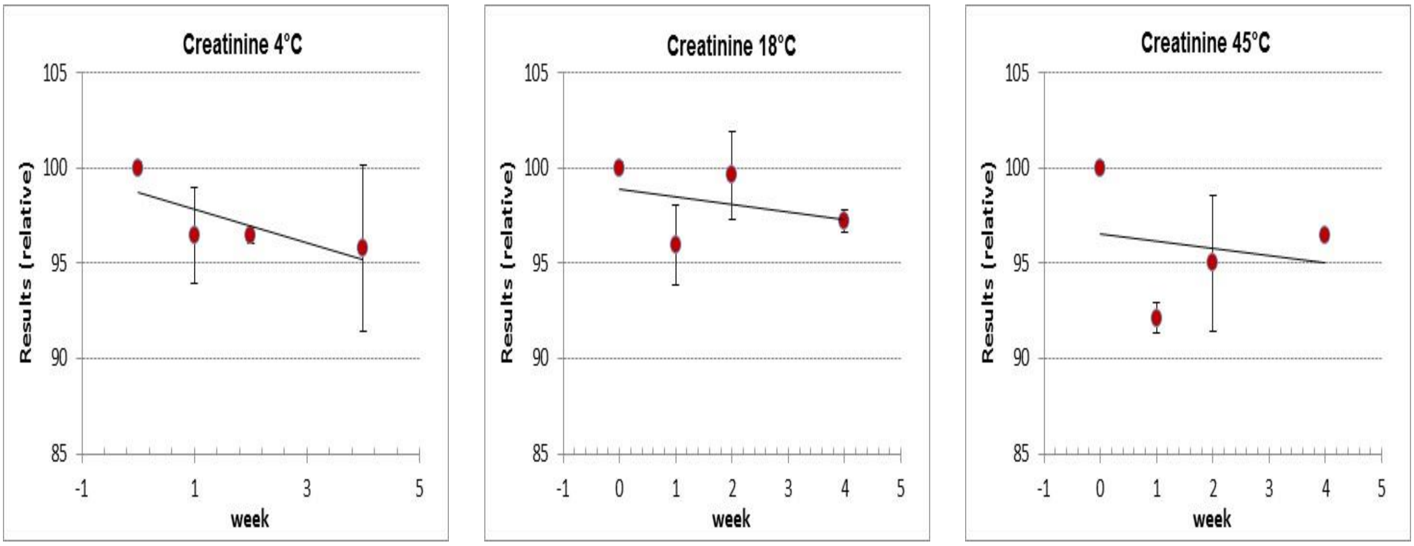


**Figure A72.** Short Term Stability Plot for Creatinine at 4 °C, 18 °C and 45 °C

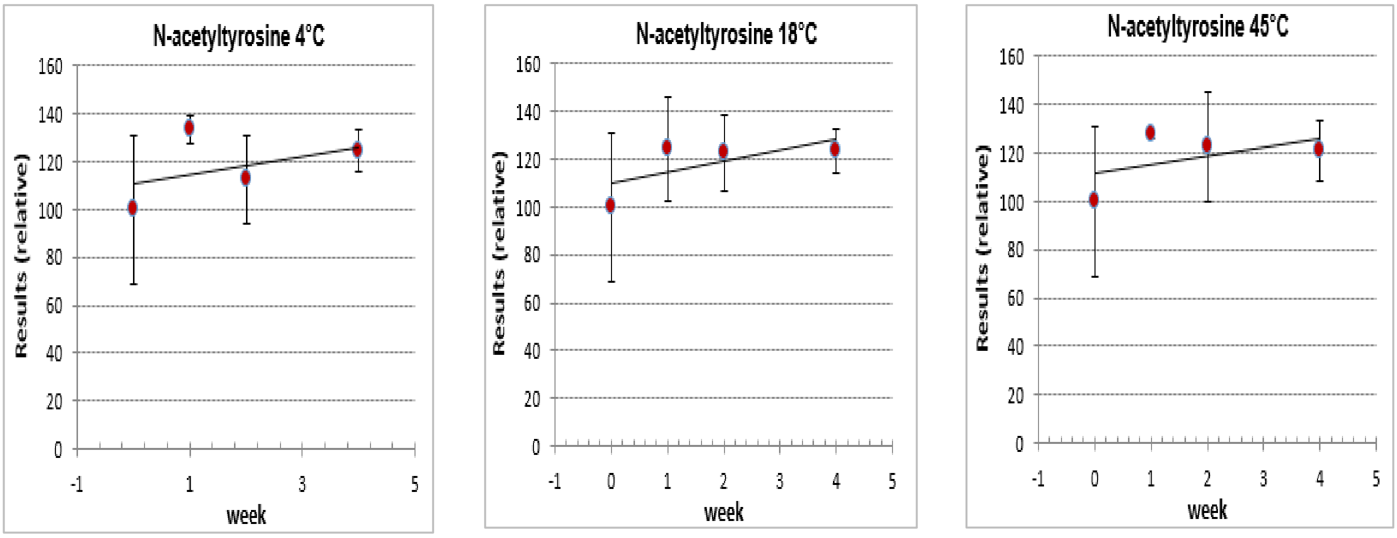


**Figure A73.** Short Term Stability Plot for N-acetyltyrosine at 4 °C, 18 °C and 45 °C

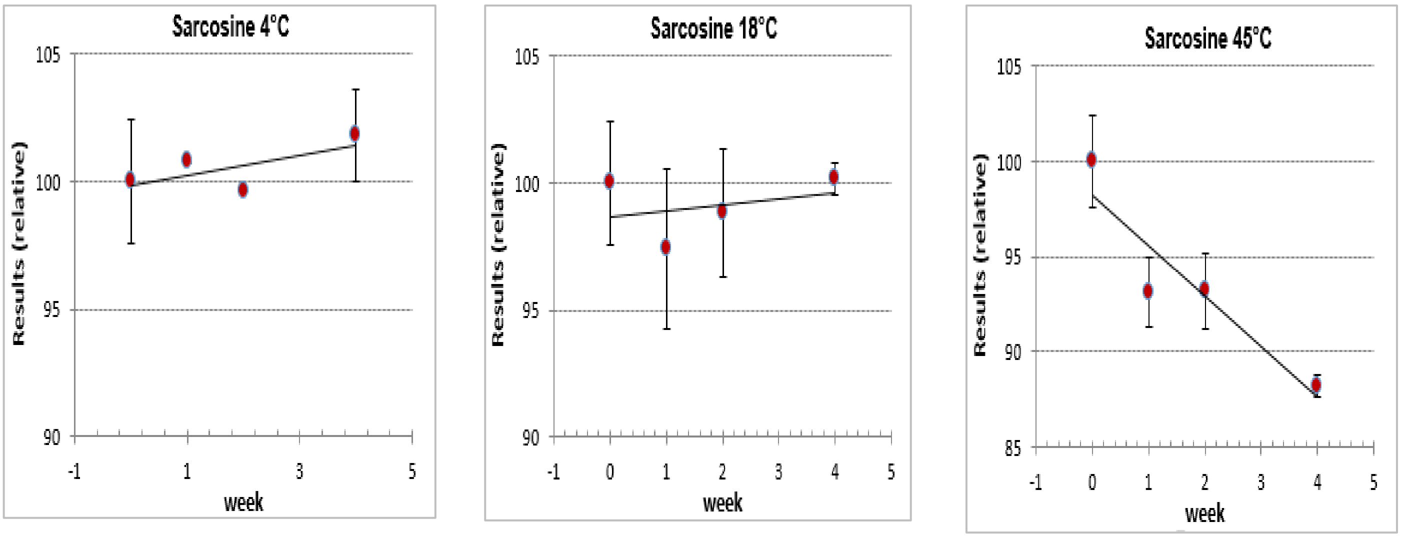


**Figure A74.** Short Term Stability Plot for Sarcosine at 4 °C, 18 °C and 45 °C

## Annex 4. Graphs for Long Term Stability Studies

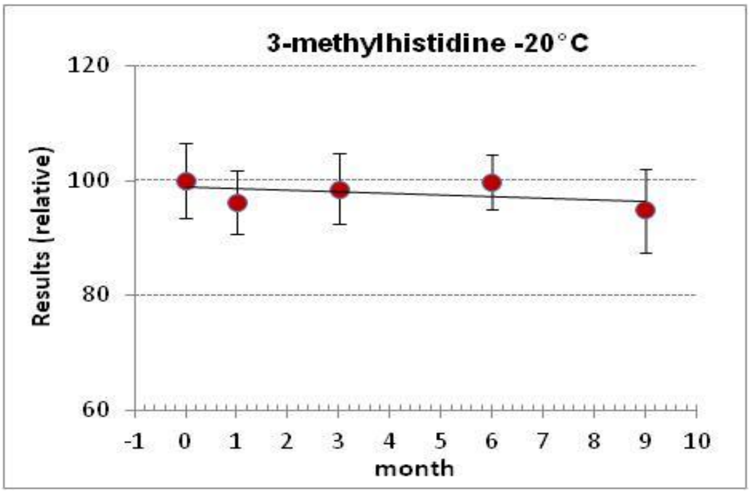


**Figure A75.** Long Term Stability Plot for 3-methylhistidine at -20 °C

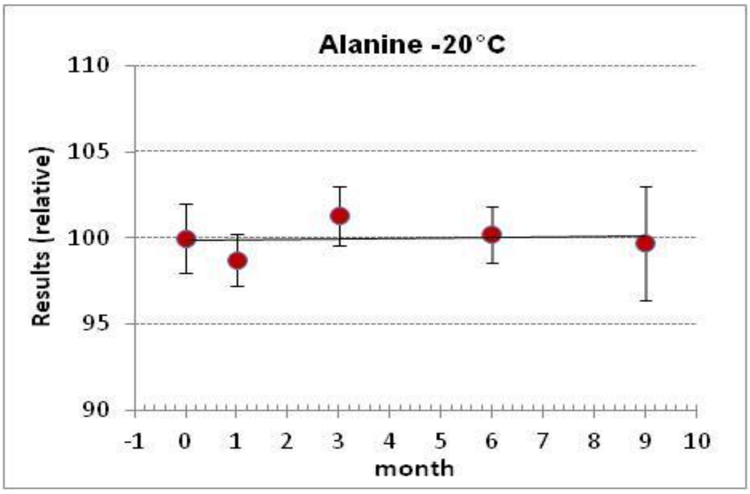


**Figure A76.** Long Term Stability Plot for Alanine at -20 °C

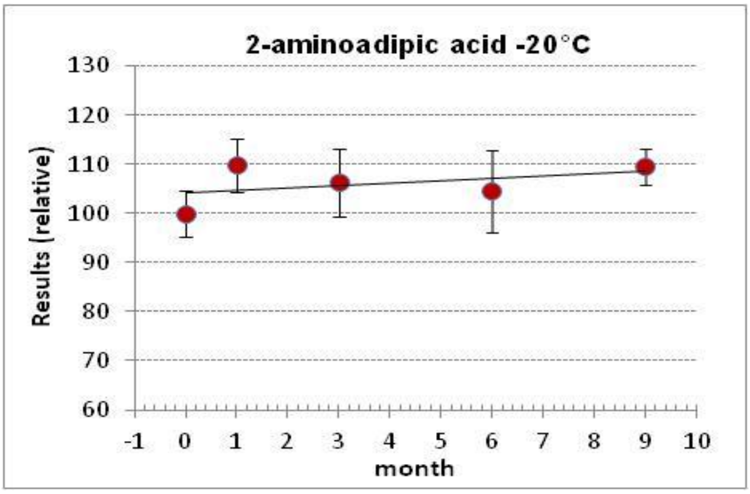


**Figure A77.** Long Term Stability Plot for 2-aminoadipic acid at -20 °C

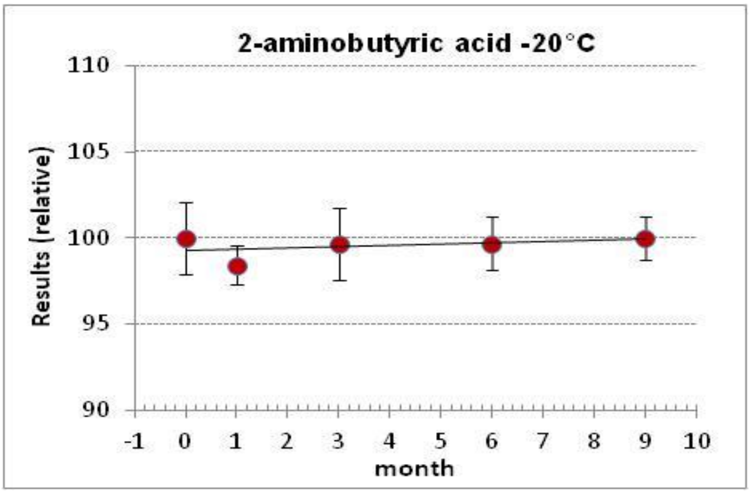


**Figure A78.** Long Term Stability Plot for 2-aminobutyric acid at -20 °C

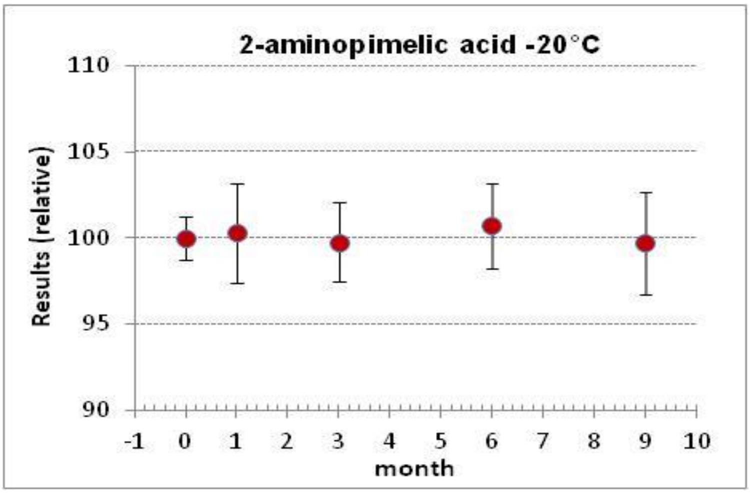


**Figure A79.** Long Term Stability Plot for 2-aminopimelic acid at -20 °C

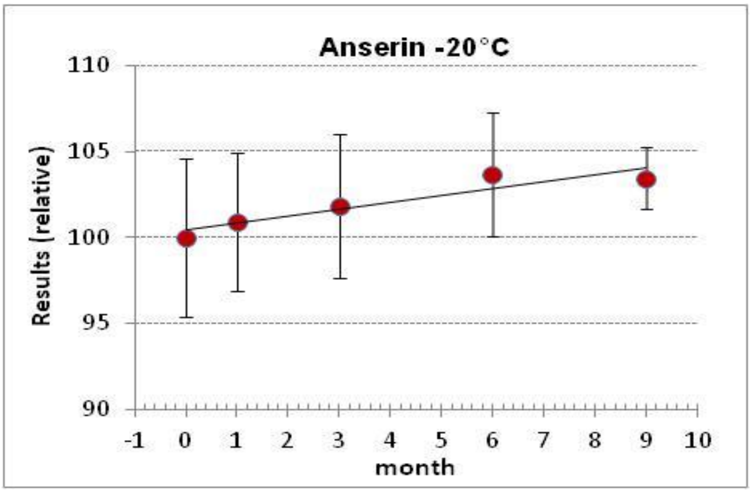


**Figure A80.** Long Term Stability Plot for Anserin at -20 °C

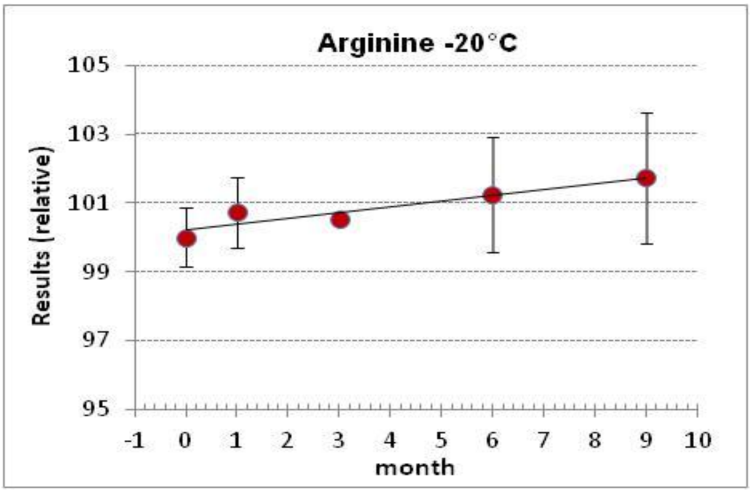


**Figure A81.** Long Term Stability Plot for Arginine at -20 °C

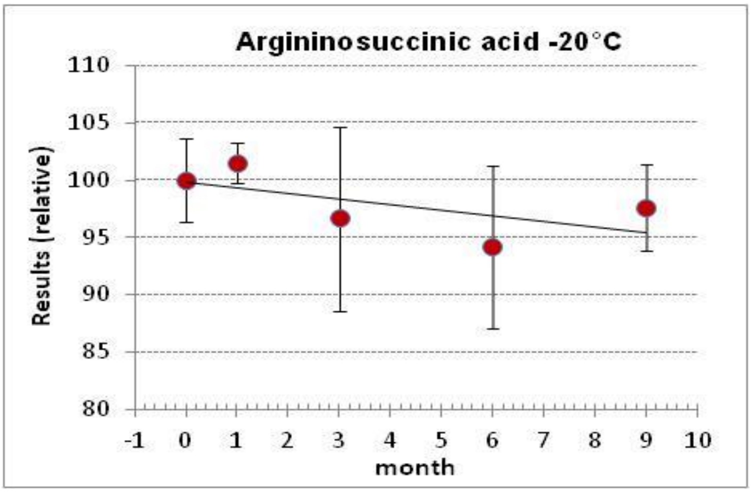


**Figure A82.** Long Term Stability Plot for Argininosuccinic acid at -20 °C

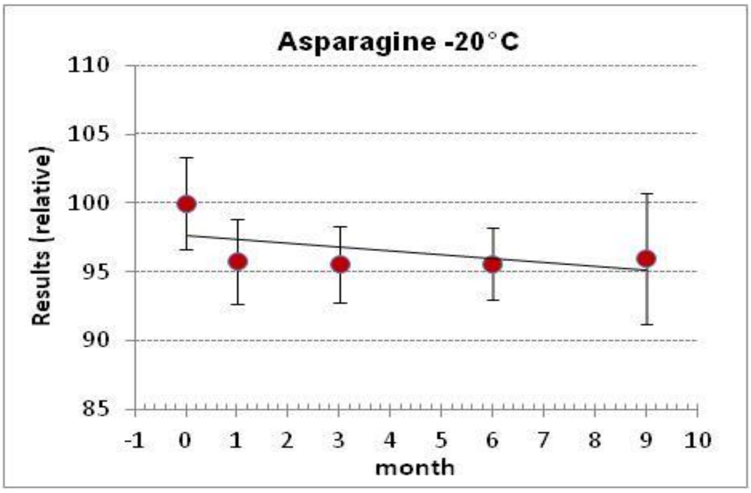


**Figure A83.** Long Term Stability Plot for Asparagine acid at -20 °C

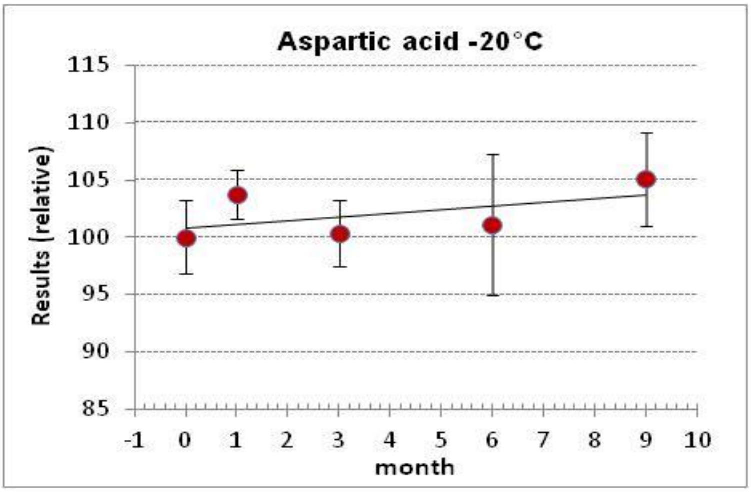


**Figure A84.** Long Term Stability Plot for Aspartic acid at -20 °C

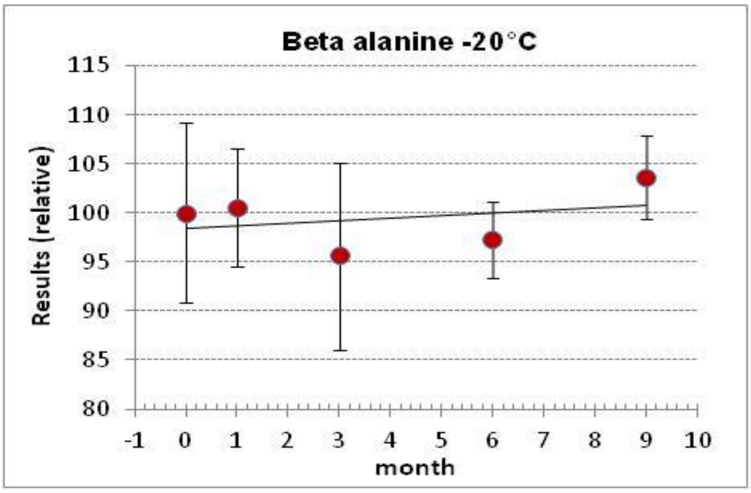


**Figure A85.** Long Term Stability Plot for Beta alanine at -20 °C

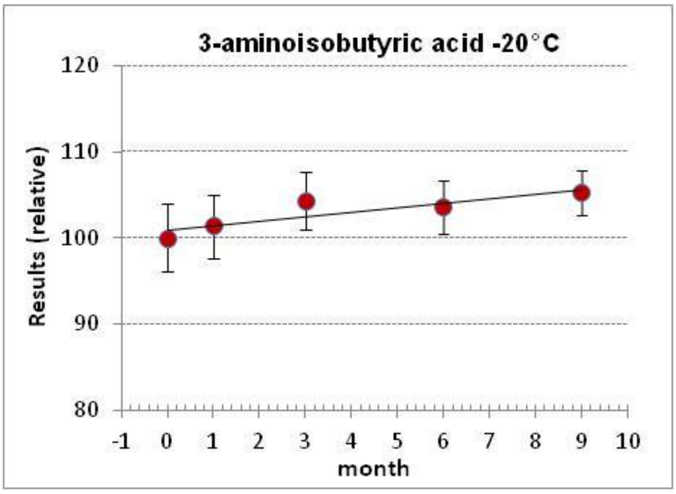


**Figure A86.** Long Term Stability Plot for 3-aminoisobutyric acid at -20 °C

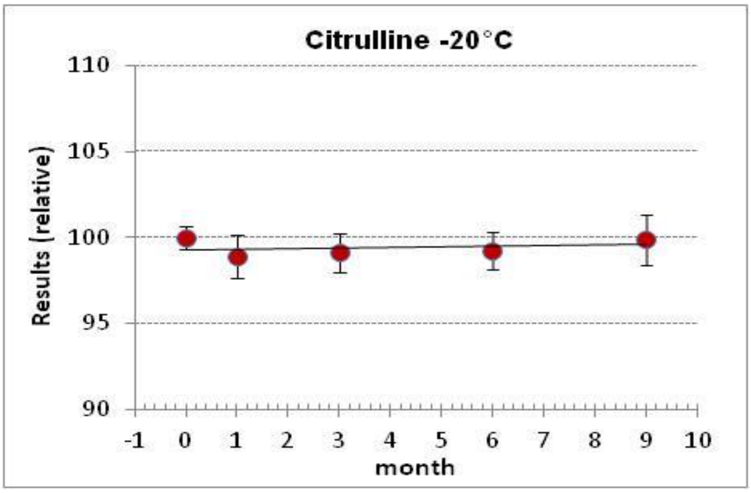


**Figure A87.** Long Term Stability Plot for Citrulline at -20 °C

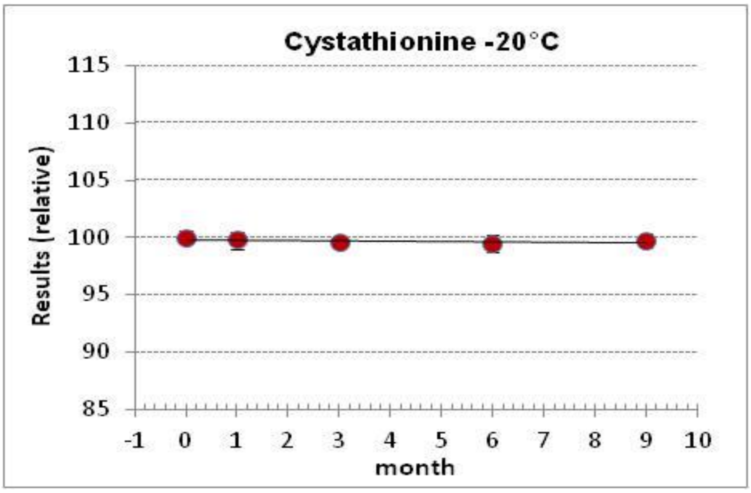


**Figure A88.** Long Term Stability Plot for Cystathionine at -20 °C

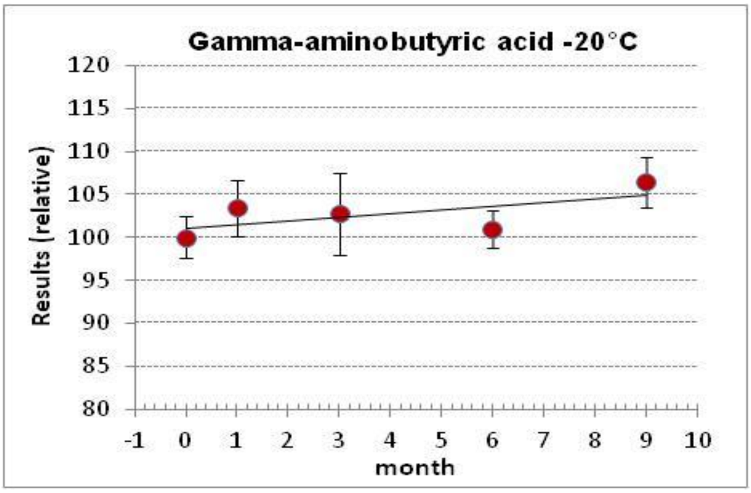


**Figure A89.** Long Term Stability Plot for 4-aminobutyric acid at -20 °C

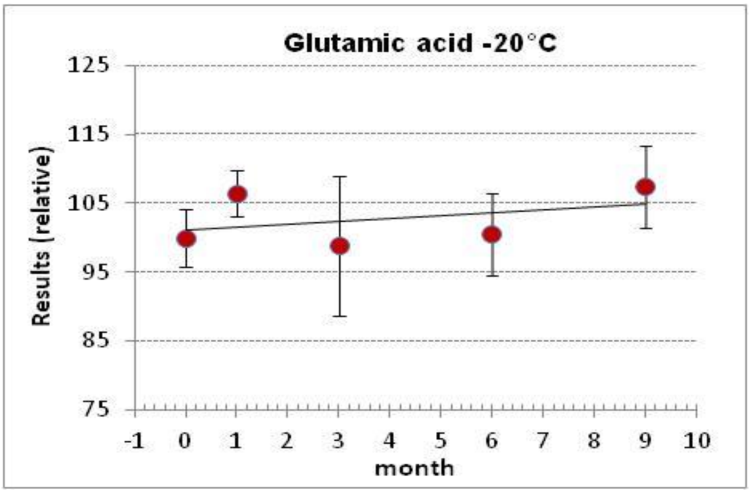


**Figure A90.** Long Term Stability Plot for Glutamic acid at -20 °C

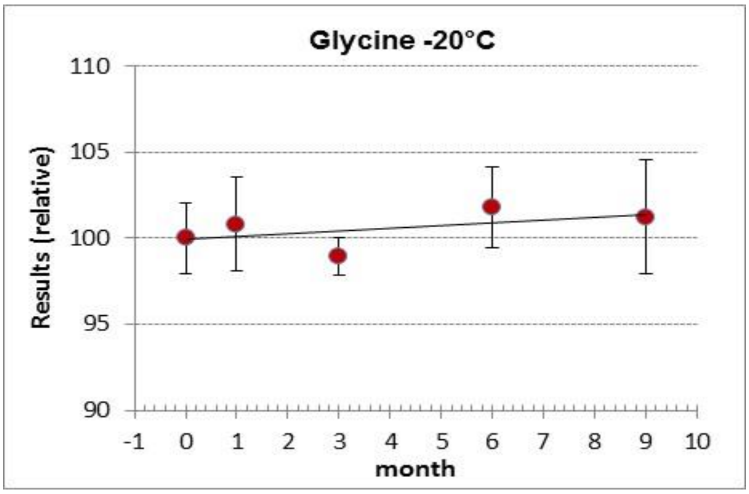


**Figure A91.** Long Term Stability Plot for Glycine acid at -20 °C

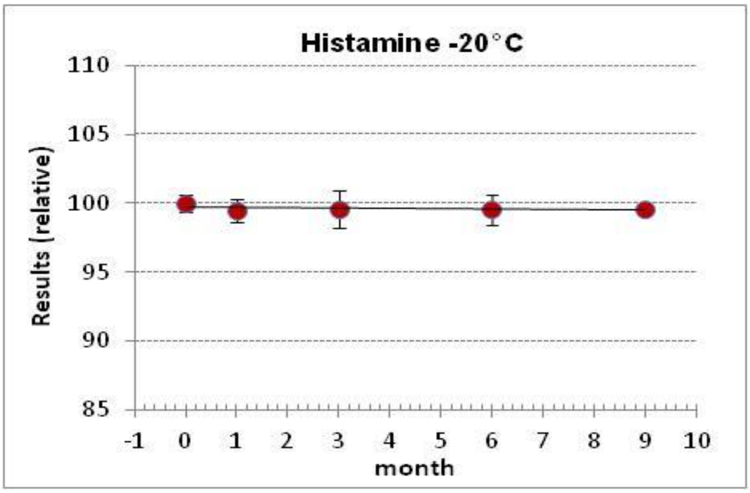


**Figure A92.** Long Term Stability Plot for Histamine at -20 °C

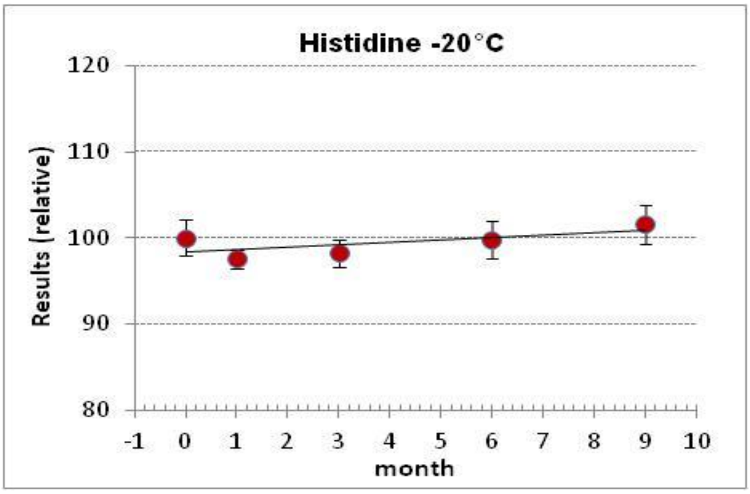


**Figure A93.** Long Term Stability Plot for Histidine at -20 °C

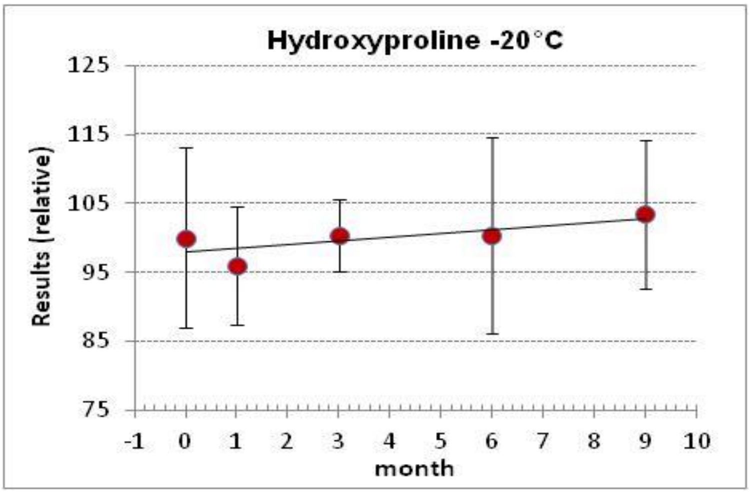


**Figure A94.** Long Term Stability Plot for Hydroxyproline at -20 °C

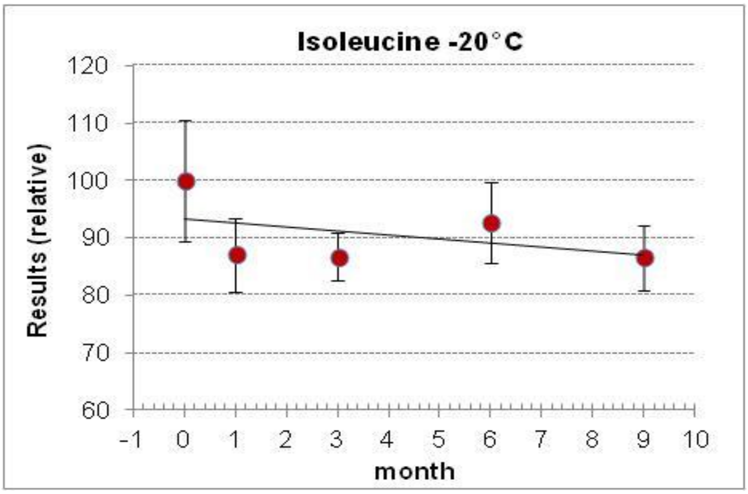


**Figure A95.** Long Term Stability Plot for Isoleucine at -20 °C

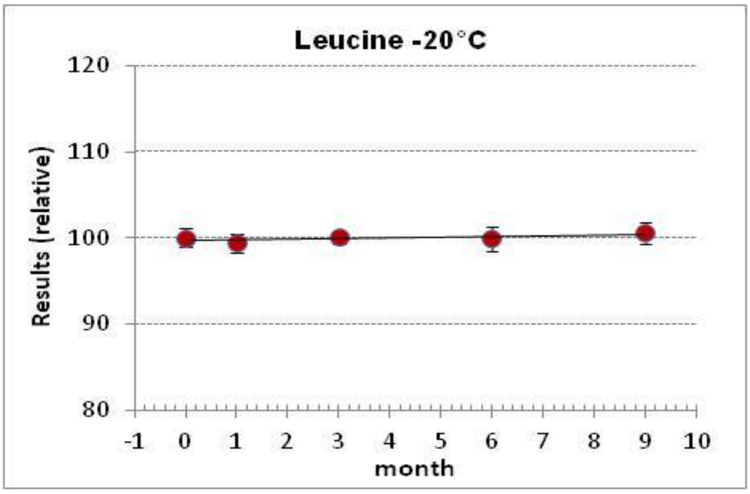


**Figure A96.** Long Term Stability Plot for Leucine at -20 °C

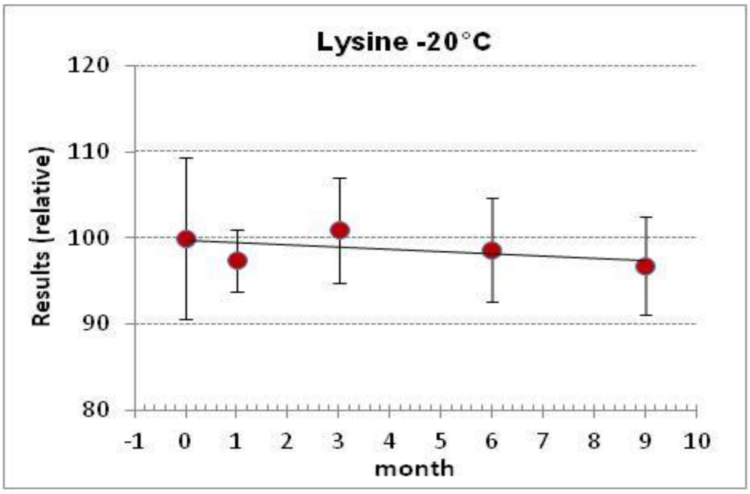


**Figure A97.** Long Term Stability Plot for Lysine at -20 °C

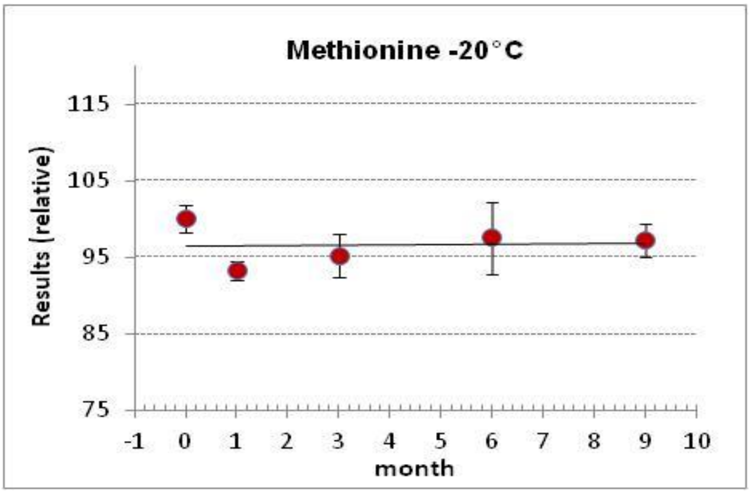


**Figure A98.** Long Term Stability Plot for Methionine at -20 °C

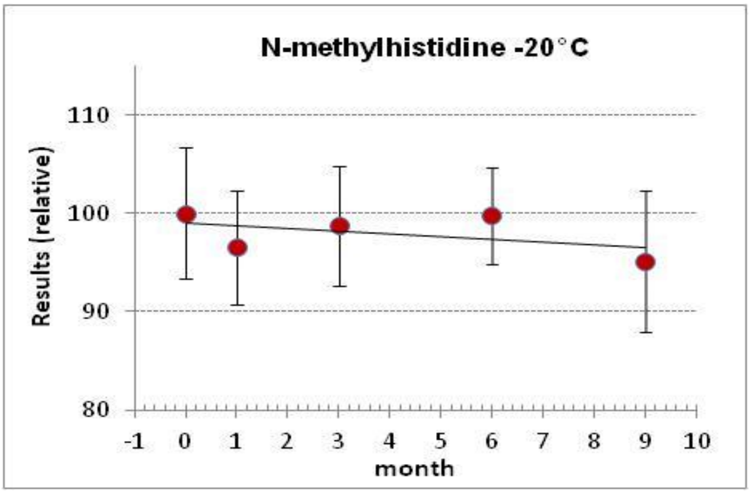


**Figure A99.** Long Term Stability Plot for N-methylhistidine at -20 °C

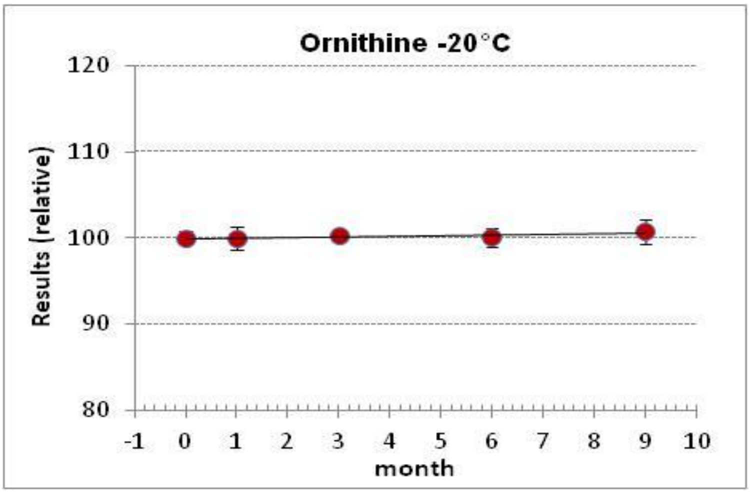


**Figure A100.** Long Term Stability Plot for Ornithine at -20 °C

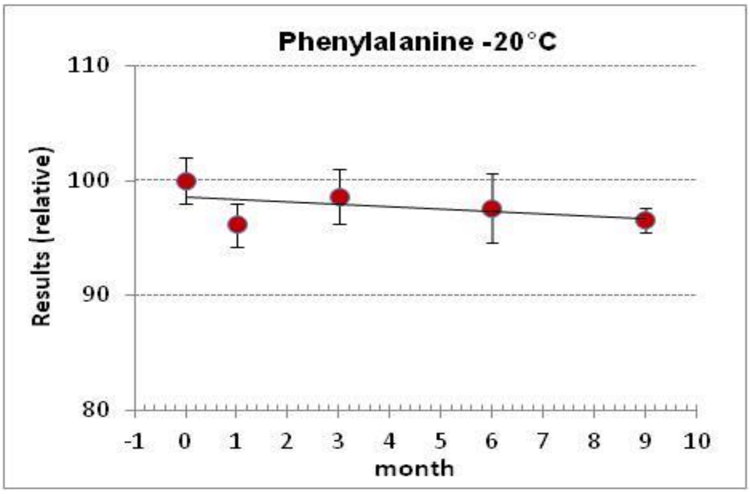


**Figure A101.** Long Term Stability Plot for Phenylalanine at -20 °C

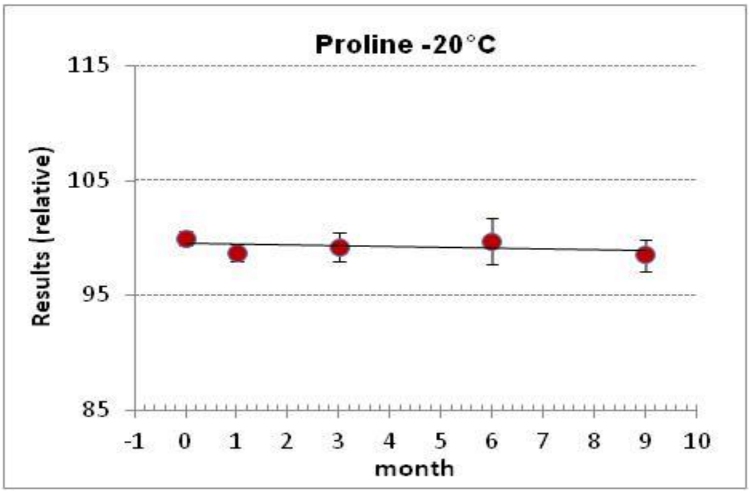


**Figure A102.** Long Term Stability Plot for Proline at -20 °C

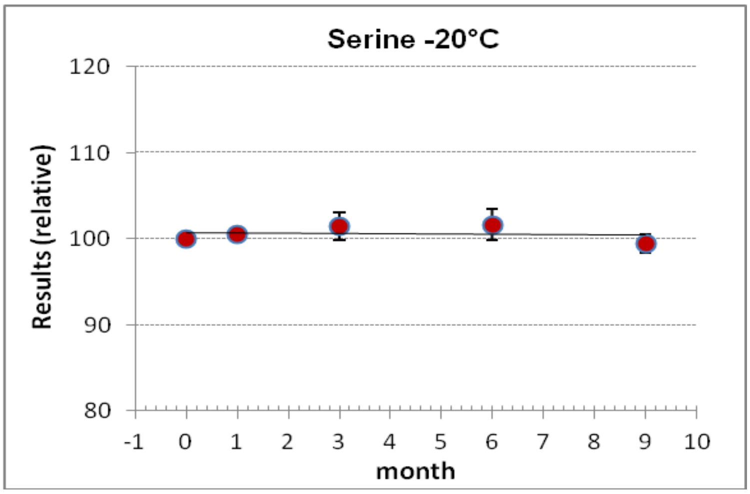


**Figure A103.** Long Term Stability Plot for Serine at -20 °C

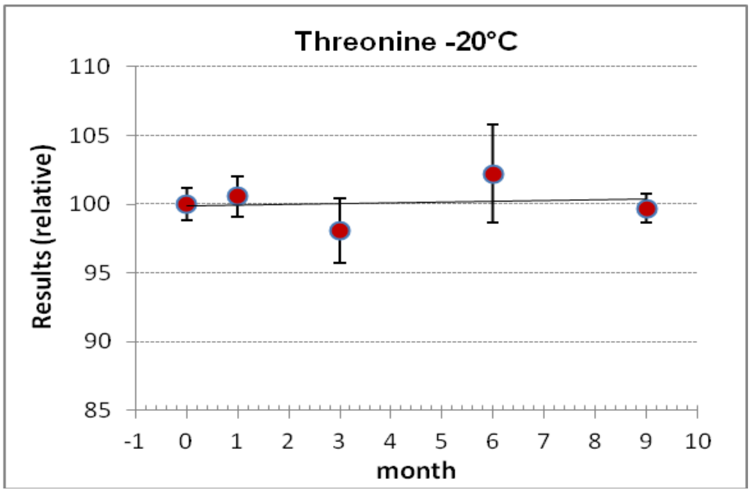


**Figure A104.** Long Term Stability Plot for Threonine at -20 °C

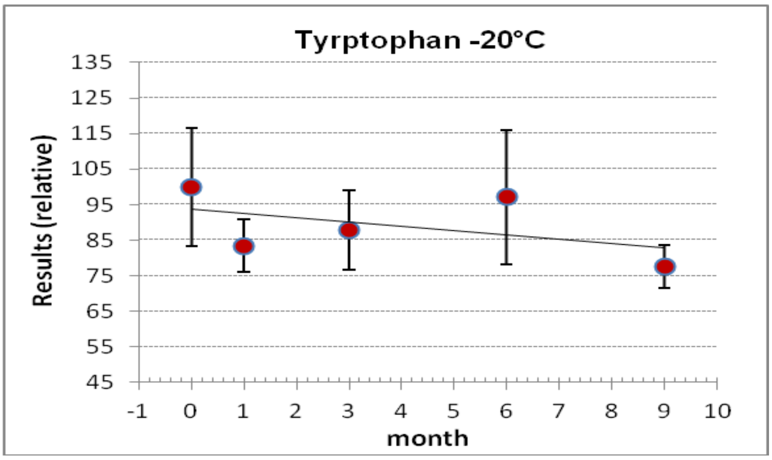


**Figure A105.** Long Term Stability Plot for Tryptophan at -20 °C

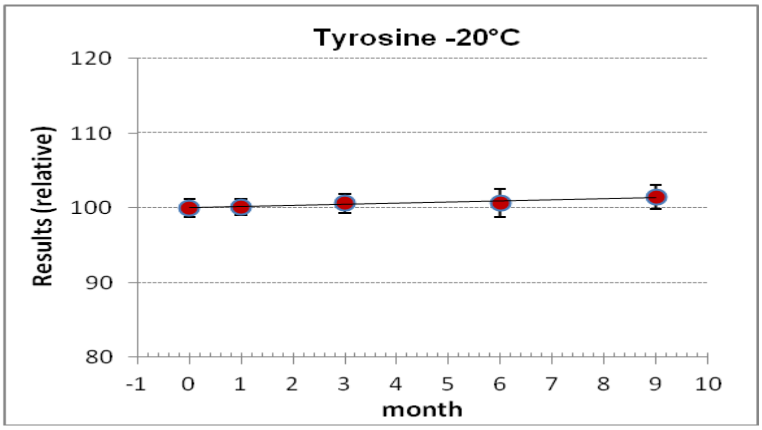


**Figure A106.** Long Term Stability Plot for Tyrosine at -20 °C

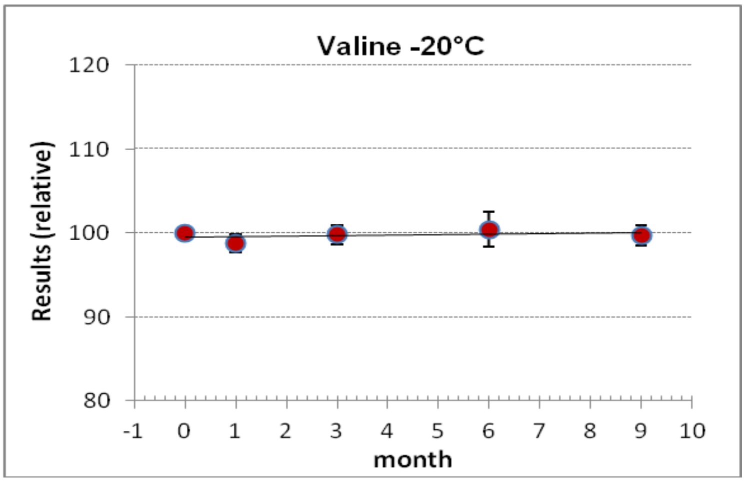


**Figure A107.** Long Term Stability Plot for Valine at -20 °C

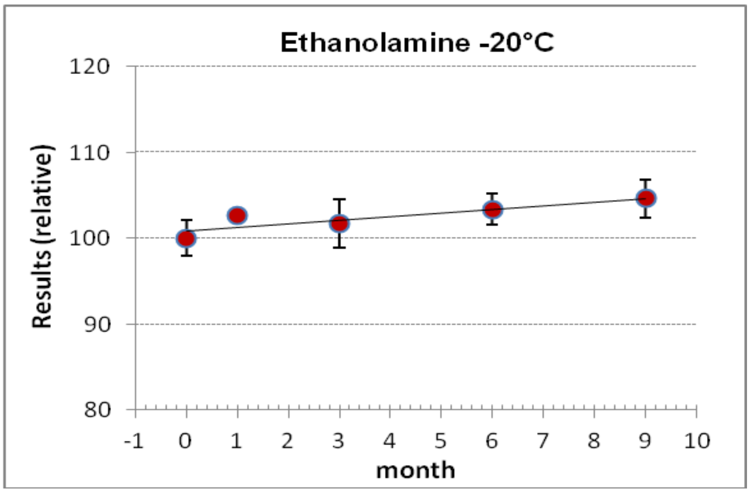


**Figure A108.** Long Term Stability Plot for Ethanolamine at -20 °C

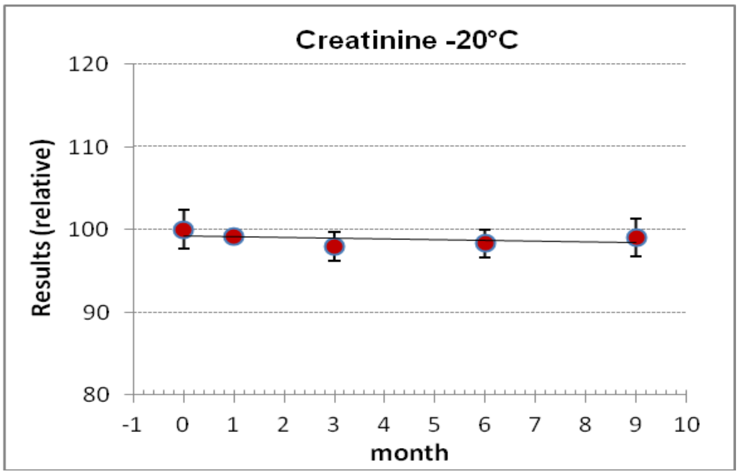


**Figure A109.** Long Term Stability Plot for Creatinine at -20 °C

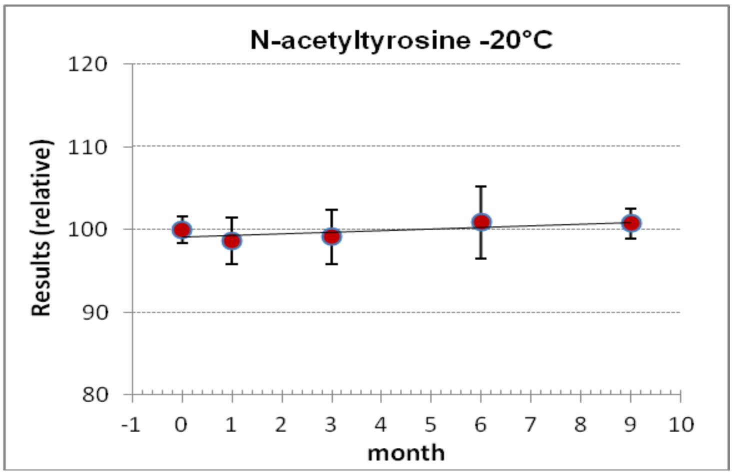


**Figure A110.** Long Term Stability Plot for N-acetyltyrosine at -20 °C

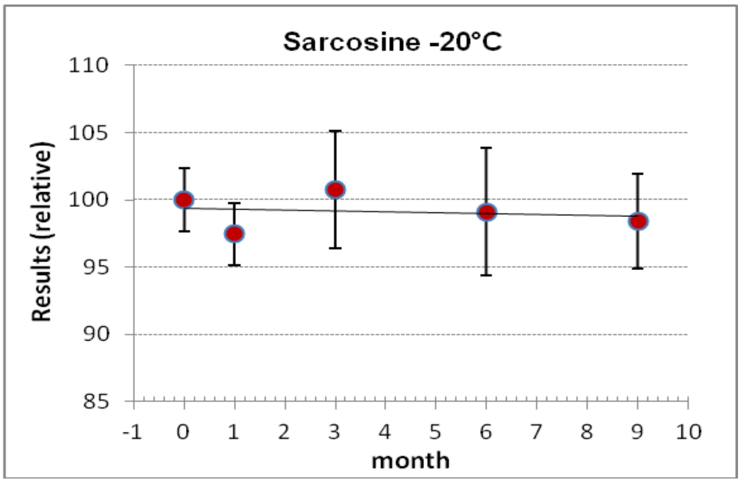


**Figure A111.** Long Term Stability Plot for Sarcosine at -20 °C

## Annex 5. Graph for CCQM K-159 Comparison Study

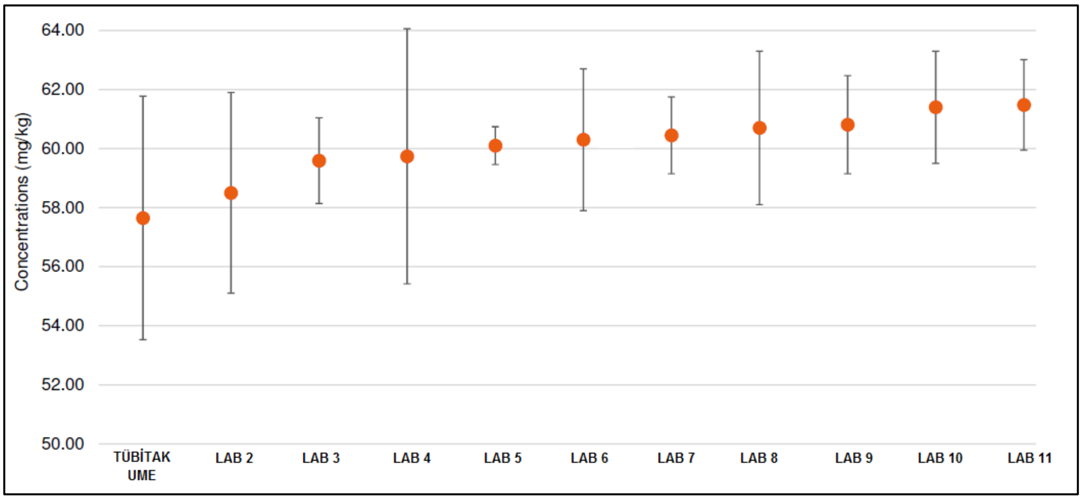


**Figure A112.** CCQM K-159 Comparison Study Plot for Phenylalanine in human plasma