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The Effects of a Single Freeze-Thaw Cycle on Concentrations of Nutritional, Noncommunicable Disease, and Inflammatory Biomarkers in Serum Samples

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Abstract

Background The stability of biological samples is vital for reliable measurements of biomarkers in large-scale survey settings, which may be affected by freeze-thaw procedures. We examined the effect of a single freeze-thaw cycle on 13 nutritional, noncommunicable diseases (NCD), and inflammatory bioanalytes in serum samples.

Method Blood samples were collected from 70 subjects centrifuged after 30 minutes and aliquoted immediately. After a baseline analysis of the analytes, the samples were stored at − 70°C for 1 month and reanalyzed for all the parameters. Mean percentage differences between baseline (fresh blood) and freeze-thaw concentrations were calculated using paired sample *t*-tests and evaluated according to total allowable error (TEa) limits (desirable bias).

Results Freeze-thaw concentrations differed significantly ($p < 0.05$) from baseline concentrations for soluble transferrin receptor (sTfR) (− 5.49%), vitamin D (− 12.51%), vitamin B12 (− 3.74%), plasma glucose (1.93%), C-reactive protein (CRP) (3.45%),

Keywords
- nutrition and noncommunicable diseases biomarkers
- serum sample
- bioanalytes
- freeze-thaw
- stability
- analysis
- storage

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high-density lipoprotein (HDL) (7.98%), and cholesterol (9.76%), but they were within respective TEa limits. Low-density lipoprotein (LDL) (− 0.67%), creatinine (0.94%), albumin (0.87%), total protein (1.00%), ferritin (− 0.58%), and triglycerides (TAG) (2.82%) concentrations remained stable following the freeze-thaw cycle. In conclusion, single freeze-thaw cycle of the biomarkers in serum/plasma samples after storage at − 70°C for 1 month had minimal effect on stability of the studied analytes, and the changes in concentration were within acceptable limit for all analytes.

Introduction

The stability of biological samples collected in large-scale field surveys is critical for accurate biochemical measurements. Following collection and transport to laboratories for processing, biological samples are analyzed immediately or frozen for a period of time and thawed prior to analysis. Freeze-thaw cycles have been shown to adversely affect the integrity of specimens, thereby reducing the reliability of biochemical measurements. However, most studies examining the effects of single/multiple freeze-thaw cycles have been conducted under laboratory conditions, with limited evidence from field survey settings.[1-4]

The 2016–2018 India Comprehensive National Nutrition Survey (CNNS) was conducted to assess the national prevalence of key micronutrient deficiencies, subclinical inflammation, and noncommunicable disease risk factors through the measurement of respective biomarkers among approximately 50,000 children and adolescents ranging from 0 to 19 years of age.[5] Pre- and postanalytical variations arising due to differences in sample handling conditions may alter the properties of the analytes being measured. Although it is ideal to maintain the analytical conditions uniformly, this is not always feasible in large-scale surveys. Therefore, stability of biomarkers needs to be ensured across a variety of anticipated conditions encountered in field settings,

In this study, the stability of analytes such as ferritin, soluble transferrin receptor (sTfR), vitamin B12, vitamin D, C-reactive protein (CRP), total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides (TAG), albumin, creatinine, and total protein in serum and plasma glucose was assessed, following a 1-month single freeze-thaw cycle, a condition commonly practiced in large-scale surveys.

Methods

Sample Collection

A total of 70 subjects who volunteered and consented to provide blood sample were recruited in the study, using a camp approach from the National Capital Territory, India, for studying all the analytes. The study was ethically approved by the ethics committee of the All India Institute of Medical Sciences (AIIMS), New Delhi, India.

Study Protocol

Venous blood samples were collected by trained phlebotomists from subjects after an overnight fast. Blood from subjects were collected in SST vacutainers (Becton Dickinson) for assessing stability of ferritin, vitamin B12, vitamin D, sTfR, total cholesterol, HDL, LDL, TAG, albumin, total protein, and creatinine; for glucose estimation, blood was collected in fluoride vacutainers (Becton Dickinson). Blood was immediately transferred to 2 to 8° C and centrifuged after 30 minutes at 1700×g for 10 minutes to separate serum/plasma, which was analyzed without delay to obtain the baseline value (T_0). Following the analysis, the sample was stored at − 70°C for 1 month after which the samples were taken out from the freezer and allowed to thaw at room temperature and then reanalyzed (T_1).

The methods and platforms/analyzer used to measure baseline and freeze-thaw concentrations for the 13 biochemical analytes examined in the study are presented in ►**Table 1**.

Statistical Analysis

Serum/plasma samples were analyzed at baseline (T_0) (day of blood collection) and following a 1-month freeze-thaw cycle (T_1). Mean T_0 and T_1 concentrations were compared using paired sample *t*-tests, and differences were evaluated according to total allowable error (TEa) limits (desirable bias). The stability of analytes following the freeze-thaw cycle was assessed based on the mean percentage change from T_0 to T_1values (bias).The least significant change (LSC) was calculated based on the coefficient of variation and was considered statistically significant if it exceeded the TEa for the respective analyte.[6] The level of bias and corresponding limits of agreement were presented using Bland–Altman plots. Reported *p* values are 2-sided with $\alpha = 0.05$. Statistical analyses were performed using SPSS Version 22.0 (IBM Inc.)

Results

The mean percentage difference between T_0 and T_1 concentrations (bias) was largest for vitamin D (−12.51%; 95% CI: − 17.00, − 7.83), cholesterol (9.76%; 95% CI: 8.41, 11.10), and HDL (7.98%; 95% CI: 5.33, 10.84) and smallest for ferritin (− 0.58%; 95% CI: − 3.44, 2.29) and LDL (− 0.67%; 95% CI: − 2.16, 1.48) (►**Table 2**). Mean differences for cholesterol, glucose,

Table 1 Details of the biochemical analytes studied for stability in blood sample following exposures to different temperature and time conditions, their methodology for analysis, and the platforms/instruments used

Analyte studied/ Sample type used	Methodology used for analysis	Platforms used in analysis
C-reactive protein (Serum)	Immunoturbidimetric	Beckman Coulter, AU 480, USA
Total protein (serum)	Spectrophotometric, Biuret	Beckman Coulter, AU 680, USA
Albumin (serum)	Spectrophotometric, BCP dye binding	Beckman Coulter, AU 680, USA
Ferritin (serum)	Chemiluminescence/ two-site sandwich immunoassay	Advia Centaur XP, Siemens, USA
Soluble transferrin receptor (serum)	Immunoturbidimetric	Beckman Coulter, AU 480, USA
Vitamin B12 (serum)	Chemiluminescence/ two-site sandwich immunoassay	Advia Centaur XP, Siemens, USA
Vitamin 25 (OH) D (serum)	Chemiluminescence/ two-site sandwich immunoassay	Advia Centaur XP, Siemens, USA
Glucose (plasma)	Spectrophotometric, hexokinase (UV)	Beckman Coulter, AU 680, USA
Total cholesterol (serum)	Spectrophotometric, cholesterol oxidase esterase peroxidase	Beckman Coulter, AU 680, USA
HDL (serum)	Spectrophotometric, direct measure-PEG/ CHOD	Beckman Coulter, AU 680, USA
LDL (serum)	Spectrophotometric, Direct Measure/ CHOD	Beckman Coulter, AU 680, USA
TAG (serum)	Spectrophotometric, enzymatic endpoint	Beckman Coulter, AU 680, USA
Creatinine (serum)	Spectrophotometric, alkaline picrate kinetic (Jaffe's method)	Beckman Coulter, AU 680, USA

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; TAG, triglycerides.

HDL, CRP, vitamin B12 (p = 0.010), sTfR (p = 0.040), and vitamin D were statistically significant (p = 0.000). There were no significant differences between T_0 and T_1 concentrations for LDL, creatinine, albumin, total protein, TAG, and ferritin (►**Table 2**).

►**Table 3** includes the coefficient of variation (CV), LSC and TEa for each bioanalytes. The calculated LSC was less than the respective TEa for all bioanalytes except for serum albumin.

The level of agreement between T_0 and T_1concentrations was relatively consistent for glucose, ferritin, TAG, CRP, sTfR, and vitamin B12 in contrast to cholesterol (1a), LDL (1d), HDL (1c), creatinine (1 g), albumin (1f), total protein (1h), and vitamin D (1l), as can be seen in ►**Fig. 1**.

Discussion

In this study, we examined the stability of 13 bioanalytes following a single one-month freeze-thaw cycle. A significant change in mean concentration from baseline was observed for cholesterol, glucose, HDL, CRP, sTfR, vitamin B12 and vitamin D, although these differences were within TEa limits and therefore considered to be the reliable estimate of these analytes.

We observed more than 12% reduction in vitamin D concentrations following the single freeze-thaw cycle. The susceptibility of vitamin D to freeze-thaw cycles has been shown in other studies. A 2.6% increase in vitamin D concentration after a single freeze-thaw cycle was reported by Wielders et al,[7] using a chemiluminescence platform. Wielders et al have also reported a 4.0% decrease in the mean concentration following storage at – 20°C for up to 2 months. They also observed a mean decrease of 2.3, 3.4, and 8.5% after 72 hours, 24 hours, and 7 days storage of whole blood at room temperature, respectively; however, they were less than the analytical interassay precision. Enko et al studied a new generation of vitamin D assays and have reported a within-run and between-run imprecision of ≤ 20% at each of the evaluated concentration levels.[8]

Riley et al[9] reported a maximum 11% bias for vitamin D levels across three freeze-thaw cycles, when compared with one freeze-thaw process. In contrast, Antonuicci et al[10] reported the stability of vitamin D in serum for up to four freeze-thaw cycles using a DiaSorin RIA assay. However, the study did not assess vitamin D levels prior to freezing, as samples were subjected to at least one freeze-thaw before analysis. Lewis et al[11] also showed the stability of vitamin D in serum during multiple freeze-thaw cycles and exposure to UV and sunlight for up to 8 days using high-performance liquid chromatography (HPLC) tandem mass spectrometry.

In our study, plasma glucose concentrations were approximately 2% higher after the single freeze-thaw, as compared with baseline concentrations. This is in contrast to a study by Clark et al who reported a significant decrease in glucose concentration following a single freeze-thaw cycle using the hexokinase method.[12] Studies have examined the long-term stability of plasma glucose under multiple freeze-thaw exposures and have shown a temperature and time-dependent decrease in concentration.[1,2,13] However, in a study by Flood et al, glucose concentrations was reported to show an increase from 11.8 to 14.0% in human serum refrozen and thawed even once.[3] The values were within the TEa.

We observed approximately 10% and approximately 8% increase in freeze-thaw concentrations of cholesterol and HDL, respectively, compared with concentrations in fresh (baseline) samples. There is variable evidence on the effects of freeze-thaw cycles on cholesterol and HDL. Ugwuezumba et al reported a nonsignificant reduction in cholesterol and HDL in serum samples, following a freeze-thaw using the enzymatic colorimetric method.[14]

Table 2 Mean baseline and 30-day freeze-thaw concentrations and percentage change from baseline for the biochemical analyte

Analyte	N	Mean baseline concentration (T_0)	Mean freeze-thaw concentration (T_1)	Mean % difference (95% CI)	*p*-Value
Cholesterol (mg/dL)	70	167.09 ± 38.34	183.76 ±44.52	9.76 (8.41,11.10)	0.000
LDL (mg/dL)	67	111.28 ±33.54	110.8 ± 33.54	-0.67(− 2.16, 1.48)	0.619
Glucose (mg/dL)	68	118.79 ±61.47	121.02 ± 62.09	1.93 (1.49, 2.38)	0.000
HDL (mg/dL)	69	40.51 ± 7.5	43.47 ± 7.86	7.98 (5.33,10.84)	0.000
Serum creatinine (mg/dL)	69	0.62 ± 0.15	0.61 ± 0.16	0.94 (− 2.09, 3.97)	0.250
Serum albumin (mg/dL)	70	4.42 ± 0.31	4.46 ± 0.42	0.87 (− 0.55, 2.29)	0.210
Total protein (g/dL)	70	7.85 ±0.48	7.92 ± 0.60	1.00 (− 0.34, 2.34)	0.152
Ferritin (ng/mL)	61	77.52 ± 71.10	78.66± 75.74	− 0.58 (− 3.44, 2.29)	0.413
TAG (mg/dL)	70	171.76 ± 138.42	173.97 ± 120.46	2.82 (1.16,4.49)	0.458
CRP (mg/dL)	67	3.97 ± 5.77	4.12 ± 5.99	3.45 (1.90,5.00)	0.000
Vitamin B12 (pg/mL)	56	254.50 ± 103.61	242.75 ±103.61	− 3.74 (− 6.81, − 3.37)	0.010
sTfR	70	29.13 ± 39.38	27.66 ± 35.05	− 5.49 (− 7.61, 23.48)	0.040
Vitamin D (ng/mL)	60	12.98 ± 8.27	10.53 ± 4.96	− 12.51(− 17.0, − 7.83)	0.000

Abbreviations: CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TAG, triglycerides.
Note: All concentrations are mean ± SD. Percentage differences were calculated using paired sample *t*-tests. $p < 0.05$.

Table 3 Coefficient of variation, least significant change, and total allowable error values for study analytes

Analyte	CV (%)	LSC (%)	TEa (%)
Glucose (mg/dL)	1.84	3.75	5.50
Cholesterol (mg/dL)	4.22	5.69	9.01
LDL (mg/dL)	3.49	5.17	11.90
HDL (mg/dL)	3.70	5.32	11.63
Serum creatinine (mg/dL)	1.82	3.73	8.87
Serum albumin (g/dL)	2.30	4.20	4.07
Total protein (g/dL)	0.98	2.74	3.63
Ferritin (ng/mL)	8.92	8.92	16.90
TAG (mg/dL)	3.47	5.15	25.99
CRP (mg/dL)	2.60	4.46	56.60
Vitamin B12 (pg/mL)	8.79	8.25	30.00
sTfR	3.00	4.79	NA
Vitamin D (ng/mL)	4.76	6.04	17.01

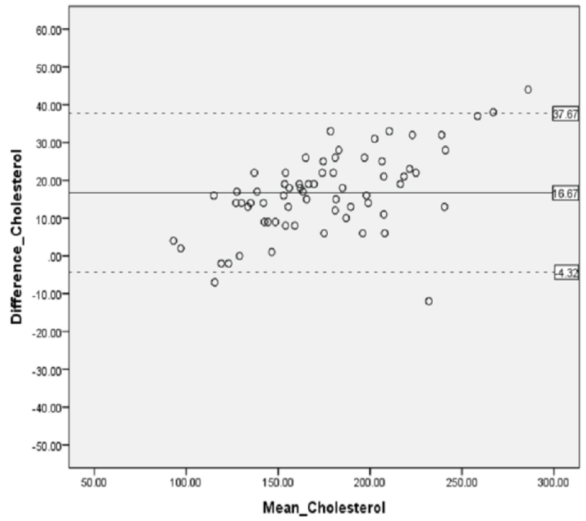
Abbreviations: CRP, C-reactive protein; CV, coefficient of variation; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LSC, least significant change; NA, not available; TEa, total allowable error; TAG, triglycerides.

Another freeze-thaw study using rat serum demonstrated no change in cholesterol and a 2% increase in HDL concentrations following a 24-hour freeze-thaw.[4] Similar effects (< 3% increase) have been observed for HDL concentrations using the heparin manganese procedure and enzymatic methods.[15] Ignatius et al demonstrated a nonsignificant change in concentration of cholesterol (+ 1.5%) and HDL (− 2.9%) after a 2-day freeze-thaw using the enzymatic-spectrophotometric method and precipitation/enzymatic-spectrophotometric method, respectively.[16]

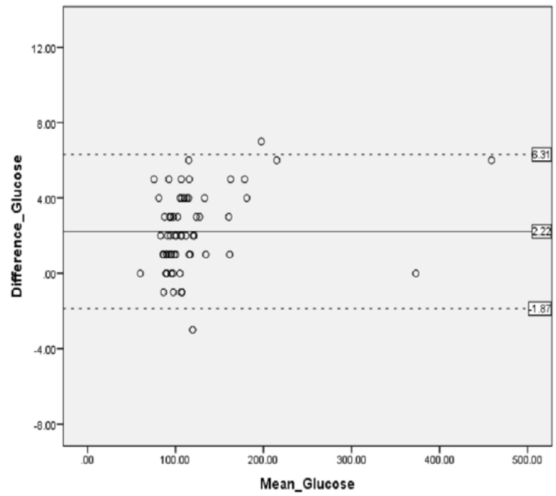
In our study, freeze-thaw concentrations of CRP were approximately 5.5% higher compared with prefreeze baseline concentrations. This is in contrast to studies that have shown CRP to be stable across multiple freeze-thaw cycles. A study by Doumatey et al[17] that used the latex particle-enhanced immunoturbidimetric assay revealed CRP may be stable in serum for an average period of 8.7 years. Ajij et al[18] showed CRP to be stable in serum for up to seven freeze-thaw cycles using the sandwich enzyme immunoassay. In addition, Macy et al[19] observed stable CRP concentrations in serum for up to four freeze-thaw cycles. The increase in CRP observed in our study was well within the TEa limits.

We observed a significant 3.7% decrease in vitamin B12 concentration after one freeze-thaw cycle. Although there is some evidence to support this,[20] most studies suggest vitamin B12 is stable when exposed to freeze-thaw cycles. Jee et al[21] showed a notable decrease in vitamin B12 concentration only after three freeze-thaw cycles for serum stored at – 20°C for 2 to 10 months and analyzed using the Siemens Immulite 2000u. A study by Gislefoss et al[22] that investigated the effects of multiple freeze-thaw (1, 2, 3, 5, and 10) cycles on serum stored at - 25°C using the modular E170

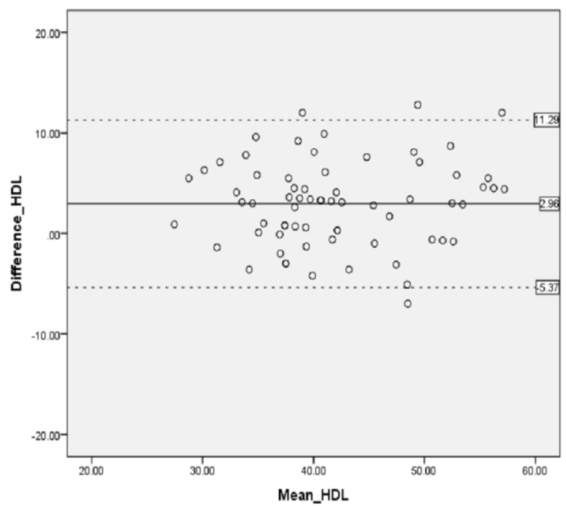
(a) Cholesterol



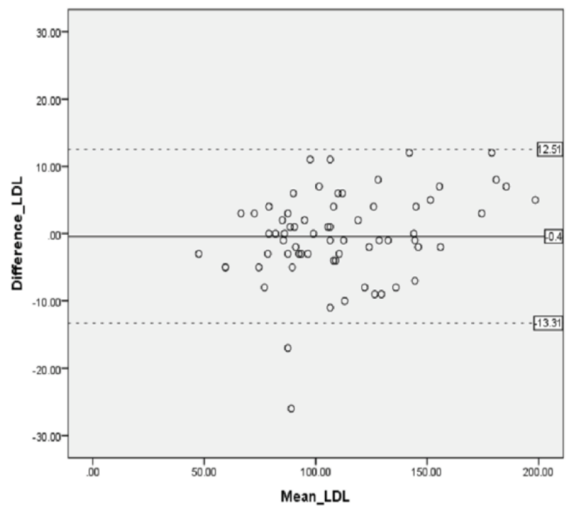
(b) Glucose



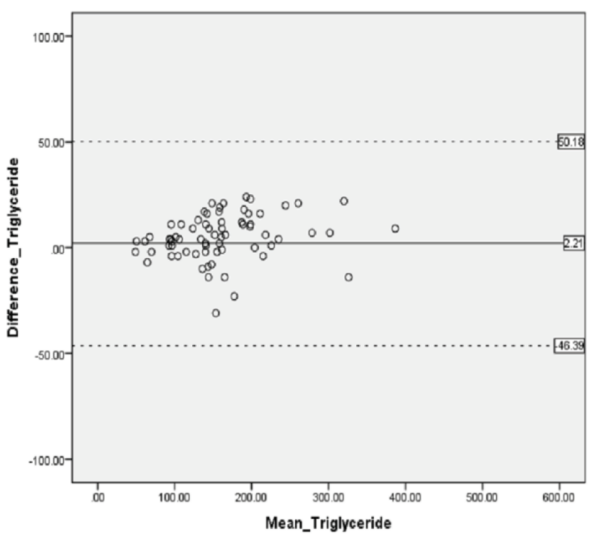
(c) HDL



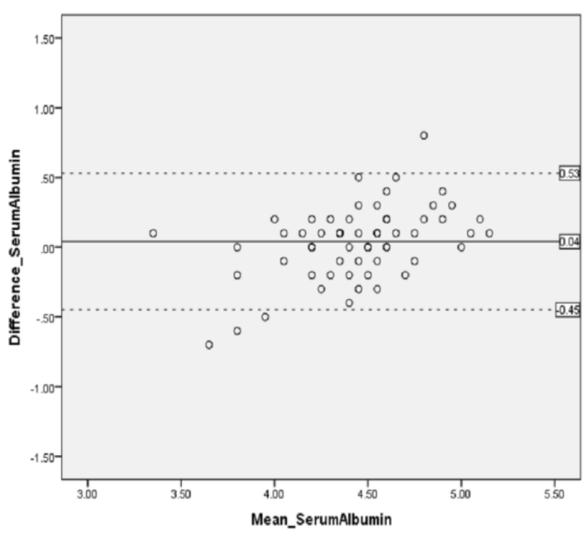
(d) LDL



(e) TAG



(f) Albumin



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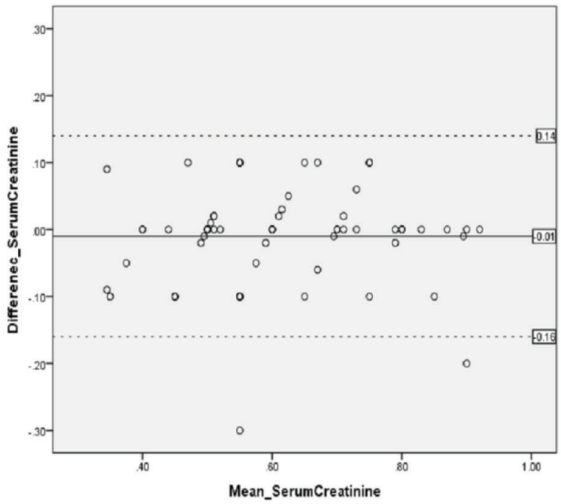
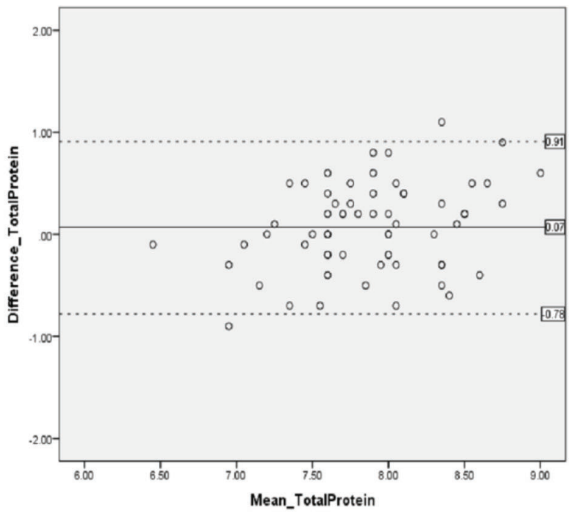
(g) Creatinine



(h) Total protein



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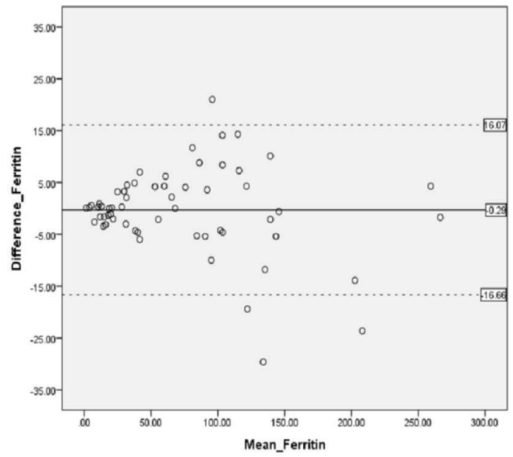
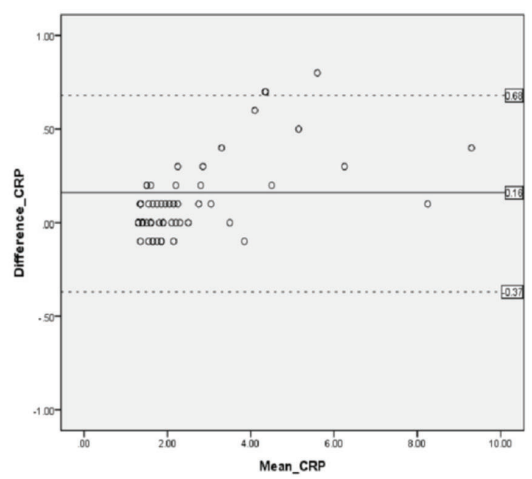
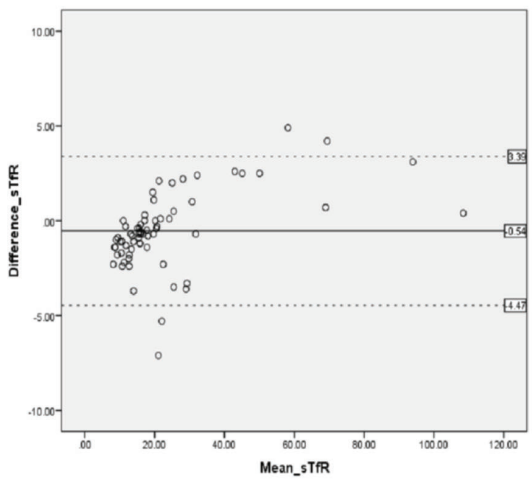


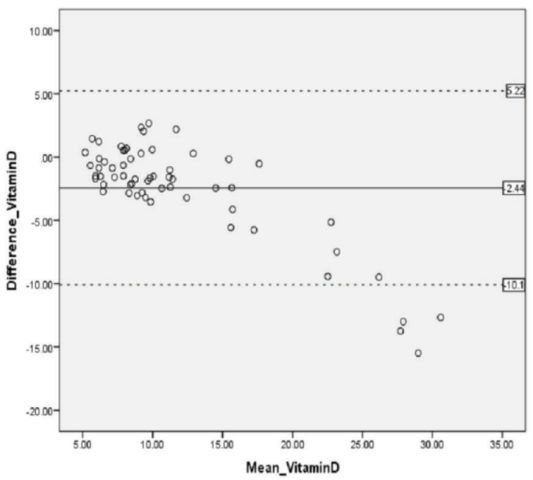
(j) CRP



(k) sTFR



(l) Vitamin D



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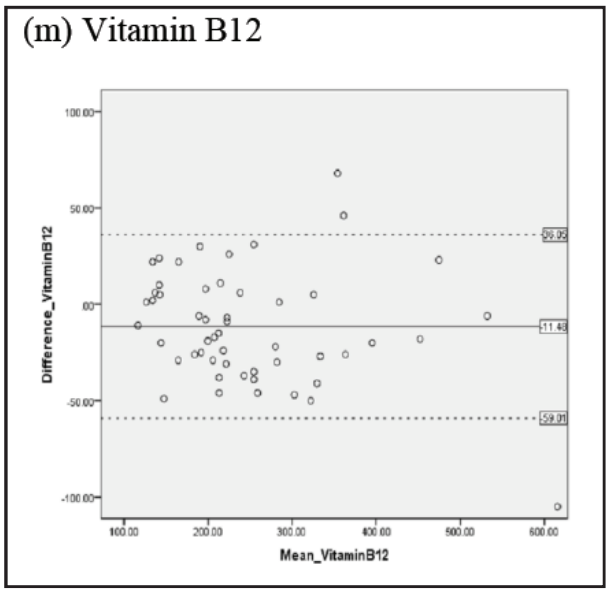

Fig. 1 Bland–Altman plots for study analytes. (**a**–**m**) Bland–Altman plots for biochemical analytes with the mean of two measurements, at baseline and after freeze thaw cycle, for each subject on X-axis and difference in two measurements on Y-axis. In each figure, the horizontal lines above and below the mean indicate the two SD limits.

(Roche) electrochemiluminescence immunoassay showed a nonsignificant change between vitamin B12 concentrations in fresh versus single freeze-thaw samples and between concentrations in samples exposed to one versus multiple freeze-thaw cycles.

For sTfR, the mean T_1 concentration was significantly lower (5.5%) than the baseline concentration. Pfeiffer et al[23] reported a nonsignificant decrease (< 1%) in sTfR concentration after one freeze-thaw cycle, when compared with samples stored at – 70°C, and observed a nonsignificant decrease of 2% in sTfR concentrations following subsequent freeze-thaw cycles.

There was no significant change in mean concentration after the freeze-thaw cycle for LDL, serum creatinine, serum albumin, total protein, ferritin, and TAG. Kachwaa et al in a similar study where serum was stored at – 20°C for 30 days have reported stability for creatinine, total protein, albumin, cholesterol, and TAG levels.[24] Albumin, cholesterol, creatinine, C-reactive protein, glucose, TAG, and vitamin B12 were seen to be robust among the studied analytes for up to 10 repeated thaw cycles compared with baseline level in the study conducted by Gislefoss et al.[22]

With the exception of serum albumin, all LSC concentrations were within acceptable TEa limits. The observed changes in freeze-thaw concentrations may be due to the leakage from blood cells, exchange of serum and erythrocyte content, serum clot formation, increased hematocrit, degradation of proteins, differential storage conditions and methods of assessment.

It is important to distinguish our results from studies comparing the stability of analytes exposed to single versus multiple freeze-thaw cycles, as our values are based on comparisons between single freeze-thaw and fresh samples (prior to freezing). As Kronenberg[25] notes, differences have shown to be greater after the first freeze-thaw cycle and thus may explain the larger differences in analyte concentrations observed in our study. Our findings suggest a single 1-month freeze-thaw cycle has no meaningful effect on serum and plasma concentrations of key micronutrient, noncommunicable disease (NCD), and inflammatory biomarkers. Further evidence is needed on the stability of analytes undergoing freeze-thaw processes compared with fresh samples.

Authors Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ransi Ann Abraham, Garima Rana and the Population Council team. The first draft of the manuscript was written by Ransi Ann Abraham, and all authors commented on the previous versions of the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest. The views expressed are those of the authors and do not necessarily reflect those of their organization.

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