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R E S E A R C H A R T I C L E

Preliminary Studies on the Stability of Serum Parameters for Antioxidant GAP

C. L. Iwuamadi[1], S. O. Iyam[2*], C. E. Ibeneme[3]

[1]*National Assembly Clinic, Area 11, 1 Ikot-Ekpene Close, Garki 1, Abuja.*
[2]*Department of Microbiology, Faculty of Biological Sciences, University of Calabar, Calabar, P.M.B. 1115.*
[3]*Department of Biochemistry, Faculty of Basic Medical Sciences, University of Calabar, Calabar, P.M.B. 1115.*

A B S T R A C T

The antioxidant Gap of serum can be derived using various widely commercially available assays. In this study, three different assays which include total antioxidant status (TAS), uric acid (UA) and albumin were used on the serum samples from five human volunteers to determine the stability of the antioxidants for a short period of 10 days. The samples were stored in various storage conditions which include the room temperature ranging from (28.3^{o}C, 24.6^{o}C, 23.1^{o}C, 24.6^{o}C), fridge temperature (5.3^{o}C, 4.9^{o}C, 4.7^{o}C, 5.7^{o}C) and frozen temperature at -20^{o}C. The various antioxidant assays, TAS, albumin and GAP showed a statistically significant difference with p-values of 0.036, 0.123 and 0.014 respectively, while uric acid with a p-value of 0.610 was not significantly different. In conclusion, the storage of sample for a short period in the above-listed temperature did affect the performance of the total serum antioxidant and the total serum oxidant assay except in uric acid which was not significan.

Key words: Antioxidant, Gap, stabilities, blood, serum, temperature

A R T I C L E I N F O

Corresponding Author
S. O. Iyam
National Assembly Clinic, Area 11,
1 Ikot-Ekpene Close, Garki 1, Abuja.
Email:soloiyam@gmail.com
MS-ID: IJMPR3964

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CONTENTS

1. Introduction

In clinical, epidemiological, general medical research and very busy laboratories the stabilities of the various biomarkers in blood serum or plasma samples during the time or sample preparation, storage and after storage is generally of vital importance to achieve reliable diagnostic results(Jansen et al., 2013a). This process starts with the

appropriate collection of blood samples, the sample handling, processing the sample to plasma or serum and the appropriate storage of the sample (Lippi et al., 2011). The different storage conditions where samples are stored after preparation should be able to maintain the exact outcome of sample after an assay (Jansen et al., 2013a).

The parameters of antioxidant gap (AOG) are useful assays in nutrition and diet, used to determine the relationship between collective antioxidant food capacity and various chronic medical disorders (Hermsdorff et al., 2011) and ascertain the relationship of plasma or serum with various sicknesses and diseases, like Parkinson's disease (PD) and Alzheimer's disease (AD) (Sofic et al.,2006) and also, relationships between total antioxidant gap and lipid peroxidation regarding the severity Rheumatoid arthritis (RA) disease (Patil et al., 2012). It is necessary to measure the total antioxidant gap of an assay because the antioxidants are made up of various elements that exercise their mode of actions in various ways (Jacob, 1995). The elements of the antioxidant system are both of endogenous and exogenous origin. The endogenous origin includes uric acid, creatinine, bilirubin and albumin and the exogenous origin are mainly vitamins A, C and E (Jansen et al., 2013b). When studying the stability in the changes of the antioxidant gap of different antioxidants, the use of two or more assays for the estimation of the total serum antioxidant and total serum oxidant is necessary (Jansen and Ruskovska, 2013). An easy, fast, efficient, and practical means to determine antioxidant gap is by performing an assay in which all the elements contributes to an integrating value (Koracevic et al., 2001).

The total antioxidant status (TAS) of a sample is measured in an assay because the need of measuring severalantioxidant molecules individually is not a practical approach to use when analyzing a sample(Erel, 2004). Therefore, the TAS of a sample is a quantifiable measurement of the stability state of the various elements under a specified reaction condition (Jacob, 1995).The uric acid (UA) is one of the major serum antioxidants in terms of its activities and mass (Miller et al., 1997). The serum 'antioxidant gap' replicates the antioxidant activity of the following antioxidants, ascorbate, IX-tocopherol, carotene, bilirubin and radical-scavenging antioxidants except for albumin and uric acid (Miller et al., 1997).

Therefore, the aim of this study is to use three commercially available antioxidants assays like the total antioxidant status (TAS), uric acid (UA) and albumin (ALB), to estimate the stability of the antioxidant gap (AOG) and to produce guidelines on the optimal storage conditions at different temperatures and storage periods. Thus, calculating the antioxidant gap as an additional parameter to the routine diagnostic will establish its usefulness in clinical settings.

2. Materials and Methods

Human participants:

For the 10 days antioxidant gap stability study, five (5) healthy human participants took part in this study. Healthy volunteers were used because, using samples from unhealthy individuals can disturb the correlation due to the presence of unexpected and unknown disturbances (Jansen and Ruskovska, 2013). The five participants involved were also non-fasting.The study was conducted after the five participants read the provided participant information sheet and authorised the consent forms by signing in their signatures and dates.

Health and safety measures:

The TAS, uric acid and albumin are carefully considered as not particularly hazardous, however a Control of substance hazardous to health (COSHH) assessment and virtual research environment (VRE) system was undertaken prior to the beginning of this study. VRE with Ethics ID ETH1617-1031 was approved on the 16thof March 2017 by the FST Research Ethics Committee.

Sample collection:

Using a serum tube containing activating gel, tourniquet, sterile swabs, dry cotton wool and plasters. The samples were collected intravenously from the antecubital fossa of the participants into a clot activator vacuum blood tube container by a trained phlebotomist. Thesample bottles were labelled 1 to 5 from the various participants respectively.

Processing the sample:

Within two hours after sample collection, the five sampleswere transferred to a centrifuge where they were centrifuged at a speed of 3000rpm for 15minutes. They were spun for 15minutes to achieve a proper separation of the serum.The supernatant, serum was carefully separated from the cells and divided into 300µl in Eppendorf tubes and stored immediately in their various storage temperatures, room temperature, fridge temperature (4^{o}C) and freezer temperature at (-20^{o}C). The initial concentration of the parameters that is the baseline values and all other assay values were determined in duplicate on the day of sample collectionand all through this study.

Calibration and Measurements of Parameters:

The calibration and measurement of the uric acid, albumin and TAS assay were carried out in an ILab Aries analyser. For TAS assay, the Randox TAS kit (2,2'-Azino-di-[3-ethylbenzthiazoline sulphaonate]) was used and a TAS QC as a control, while for the uric acid and albumin, the uric acid liquid (IL Test ^{TM} 0018257740) and the albumin (IL Test ^{TM} 0018481800) was used respectively. For the calibration and control of uric acid and albumin, ReferrILG and SeraChem 1 and SeraChem 2 were used respectively. SeraChem 1 and SeraChem 2 are two levels of control used to control the accuracy and precision of the method used, while ReferrILG is the standard needed to draw a calibration graph.The measurement of all the assays carried out was performed in duplicate.

Statistical Analysis:

Descriptive statistical analysis was carried out using a statistical software, such asIBM andSPSS 23.The one-way analysis of variance (ANOVA) was used to determine a significant statistical difference between the mean of the measured sample at the different storage temperatures for the different days.The values were considered significant when $p< 0.05$.Microsoft Excel has also been used to generate graphs for the AOG as shown in (Figure 1) and (Table 2).

Total Antioxidant Status (TAS)Assay (Randox):

The assay principle according to the instruction from the manual, ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) was incubated with a peroxidase (metmyoglobin) and H_2O_2 to yield the radical cation ABTS+. It has a blue-green colour that is measured at an optical density of 600 nm. Antioxidants from the samples added will inhibit the production of colour to a certain degree that is proportional to its concentration.

Stability and preparation of TAS:

Reagent bottle R1 contains buffer. The buffer is ready for use.Reagent bottle R2 contains chromogen. One vial of the chromogen R2 was reconstituted with 10 ml of buffer R1. Reagent bottle R3 contains substrate. 1ml of substrate R3 was diluted with 1.5 ml of buffer R1. For the calibration Standard, one vial of the standard was reconstituted with 1ml of double deionised water. The Intra-Assay Coefficient of Variation (CV) showing the calibration precision of TAS, values were recorded at 0.06%, 0.66%, 0.40%, 0.36%,0%, and 0.17% which was obtained from the calibration graph.

Uric Acid Liquid (IL Test ™ 0018257740)

Uric acid + $2H_2O + O_2 \longrightarrow H_2O_2$ + Allantotoin + CO_2

Peroxidase

H_2O_2 + 4-AAP + TBHB ⟶ Quinoneimine dye + $2H_2O$

4-AAP = 4- amino antipyrine

TBHB = 2,4,6-Tribomo-3-hydroxybenzoic acid

The concentration of this dye is proportional to the uric acid concentration in the sample.The Intra-Assay Coefficient of Variation (CV) showing the calibration precision for uric acid, the values recorded were0.08%, 0.80%, 0.80%, 0.03%, 0.08%, 0.03%, respectively, for the various days which was obtained from the calibration graph with the SeraChem1 and SeraChem2 as sample quality control.

Albumin (IL Test ™ 0018481800):

The albumin present in the sample will bind Brom cresol green (BCG) which causes a change in spectral of the dye from yellow to green. Furthermore, the increase in absorbance due to the colour that is developing is proportional to the albumin concentration in the sample.The Intra-Assay Coefficient of Variation (CV) showing the calibration precision for albumin, values were recorded at0.19%, 0.14%, 0.13%, 0.14%, 0.43%, 0.03%, obtained from the calibration graph.

Temperature Range:

Day 1 baseline value recorded. Room temperature (RT) for day 2, 3, 7 and 10 was recorded at 28.3°C, 24.6°C, 23.1°C, 24.6°C, respectively, fridge temperature was recorded for day 2, 3, 7 and 10 at 5.3°C, 4.9°C, 4.7°C and 5.7°C respectively and frozen temperature for the day 10 assay was recorded at -20°C.

3. Results and discussion

In table 1, the mean values and standard deviation of the antioxidant assay (TAS, uric acid, albumin, and antioxidant gap) were recorded and summarised. In table 2, the percentage change of TAS, uric acid,GAP and albumin at each day was compared to the baseline value. The value of the percentage change was derived by dividing the various day's value by the day 1 baseline value of each antioxidant and multiply by a 100. Antioxidant Gap was obtained by subtracting the Total Antioxidant Status (TAS) attributed to albumin and uric acid from the total antioxidant value for each sample recorded (Miller et al., 1997). Therefore, the antioxidant gap values were calculated using the formula;

AOG = TAS (mmol/L) - [(serum albumin mmol/L x 0.69) + uric acid mmol/L].The average values of all the antioxidant assays were calculated using excel at the beginning of this study.

Table 1: Descriptive Statistics of the mean and standard deviation (± SD)

	Mean	Std. Deviation	N
Uric Acid	.2659	.06439	60
Albumin	.6713	.03283	60
TAS	1.7755	.20566	60
GAP	1.0437	.22181	60

Table 1: Descriptive Statistics of the mean and standard deviation (± SD) for the various assays, N = total number of samples stored in the Eppendorf tubes.

Table 2: The percentage change of TAS, UA, GAP and albumin at each day compared to the baseline value. % change= Days / Day 1 (baseline value) *100. All the values for the percentage in TAS, uric acid, albumin and GAP were recorded to their nearest whole number.

Testing of the performance of the TAS assay for this 10 days stability study, the sera from five (5) human participants were kept under different storage conditions (RT,FT and F temperature) and the assay performed using an ILab Aries analyser. A decrease was observed in all three temperatures from the day 2 of the study. At room temperature and fridge temperature there was a decrease from the day 2 to 7 and gradually starts increasing by the day 10 of the assays. While for the frozen temperature the assay continuously decreases from the day 1 down to the day 10 and no form of an increase was noticed. The means of the TAS assay for the different temperatures were statistically significant with p-values of 0.036 which is less than 0.05.Since the p-value is less than 0.05, it therefore means that the null hypothesis is rejected and therefore a significant difference does exist.

Uric acid Assay:

The sera stored at room temperature for the 10 days stability study, there was a slight increase in the uric acid from the day 1 to the day 2 of the study and was stable from the day 2 to the day 7 then later a slight but not too obvious decrease was noticed on the day 10. The sera stored at the fridge temperature were stable from the baseline value to the day 2, a gradual increment was noticed from the day 2 to 10 of the study. While for the frozen temperature a steep and steady increase was noticed from the day 1 until the day 10 of the study (Suppl. Item III). The means of the uric acid assay for the different temperatures were not significant with a p-value of 0.610 which islarger than 0.05, we, therefore,cannotsay that a significant difference exists.

Albumin Assay:

The albumin on storage for 10 days at room and fridge temperatures showed irregular patterns. From the day 1(base-line value),Increases on the day 2, reduced on day 3,

increased again on day 7 and, reduced again on day 10. While for those stored at a frozen temperature, it continuously increasesfrom day 1 down to the day 10 (Suppl. Item II). There was a significant difference as p-value was 0.123 which is higher than 0.05.

Total antioxidant Gap Assay:

The stability of the GAP was also performed for the different storage temperature conditions. The sera stored at room temperature and fridge temperature decreases from the point of the baseline value day 1 to day 7 and a gradual increase was noticed from the day 7 to day 10. While those stored at the frozen temperature decreased from the day 1 to the day 10. The means of the GAP assay for the various temperatures were statistically significant with p-values of 0.014 which is less than 0.05, therefore a significant difference does exist.

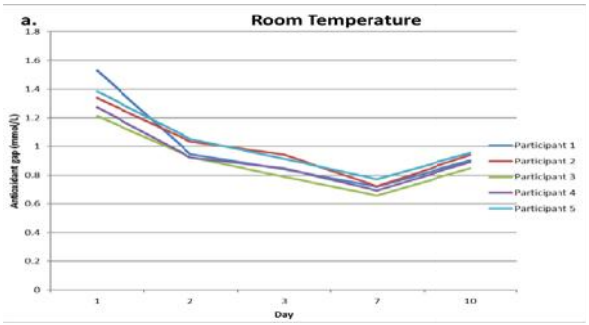

Fig 1 (a): Antioxidant gap graph plot

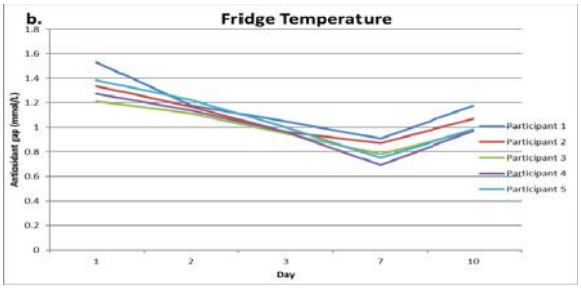

Fig 1(b): Antioxidant gap graph plot

Figure 1a and 1b. Day 1 to day 10 stability study showing the antioxidant gapchange for five participants, human sample serum stored at a Room temperature from28.3^{o}C, 24.6^{o}C, 23.1^{o}C, 24.6^{o}C recorded in each of the days. **Figure 1b.** Day 1 to day 10 stability study showing the antioxidant gap change for five participants, human sample serum stored at fridge temperature recorded at 5.3^{o}C, 4.9^{o}C, 4.7^{o}C, 5.7^{o}C for the different days.

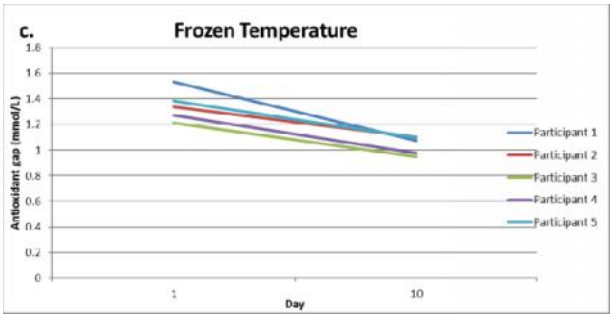

Fig 1©: Antioxidant gap graph plot

Supplementary Items:

- ✓ Mean graph plot
- ✓ Graph plot of albumin
- ✓ Graph plot of uric acid
- ✓ Graph plot of TAS
- ✓ ANOVA

Supplementary Item I:

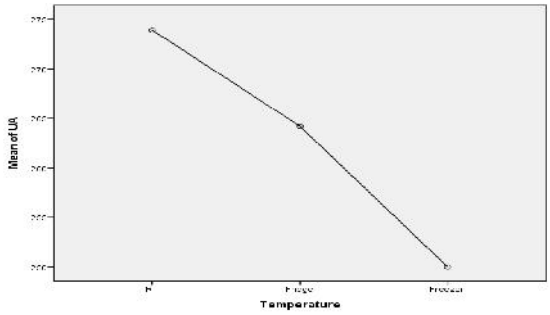

Fig 2(a): Mean Plot of Uric acid

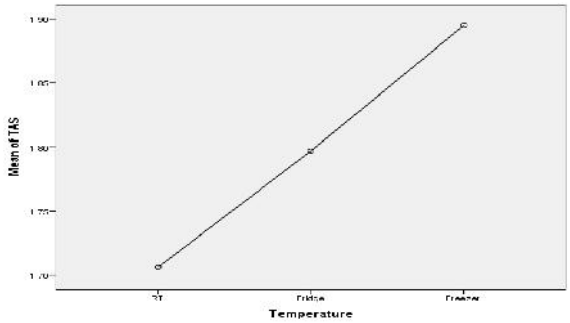

Fig 2(b): Mean Plot of TAS

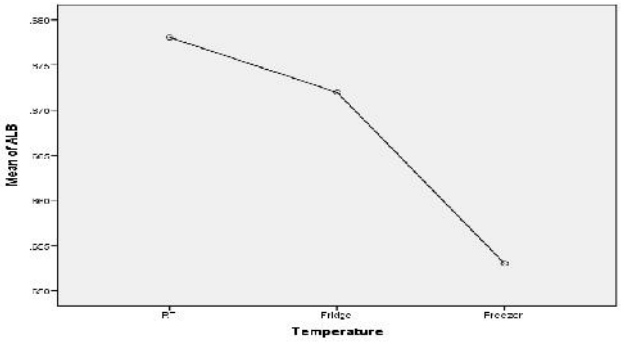

Fig 2(c): Mean Plot of Albumin

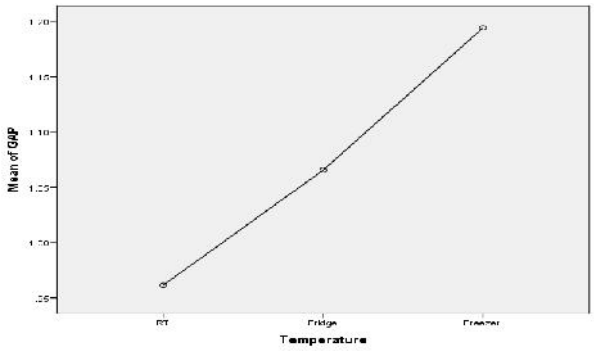

Fig 2(d): Mean Plot of AOG

The mean graph plot using ANOVA to determine the means difference

Supplementary Item II:

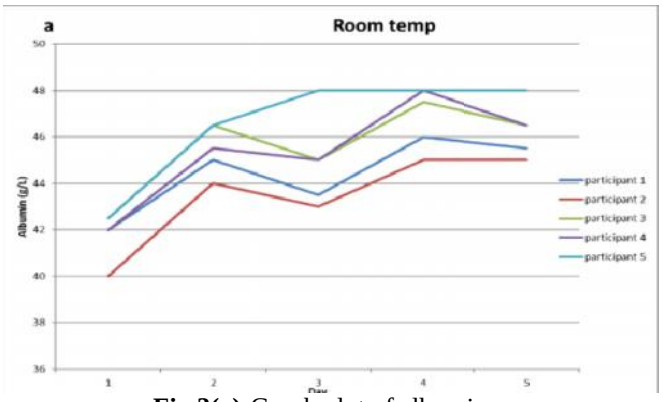

Fig 3(a): Graph plot of albumin

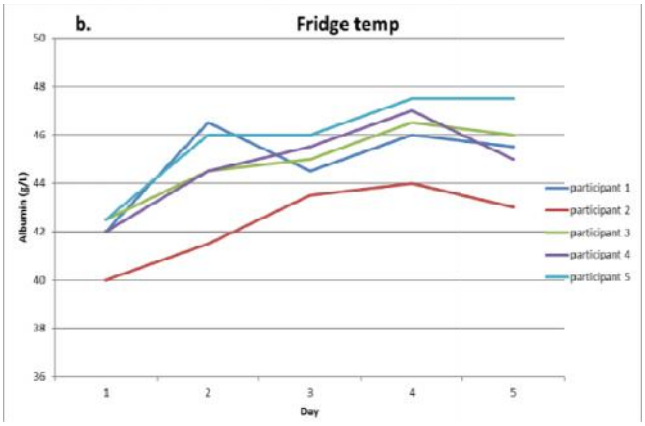

Fig 3(b): Graph plot of albumin

Suppl. Item IIa:
Day 1 to day 10 stability study showing the graphical change for five participants, human sample serum stored at Room temperature from 28.3^{o}C, 24.6^{o}C, 23.1^{o}C, 24.6^{o}C recorded in each of the days.

Suppl. Item IIb:
Day 1 to day 10 stability study showing the albumin change in temperature for five participants, human sample serum stored at fridge temperature recorded at 5.3^{o}C, 4.9^{o}C, 4.7^{o}C, 5.7^{o}C for the different days.

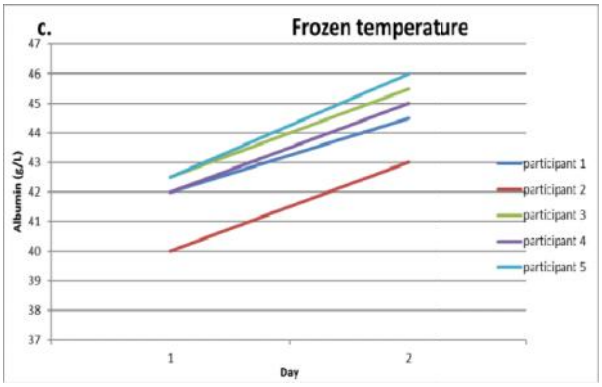

Fig 3©: Graph plot of albumin

Suppl. Item. IIc: Stability study for albumin with human sera stored at a temperature of -20^{o} C. An increase was observed at this temperature.

SupplementaryItem IV:

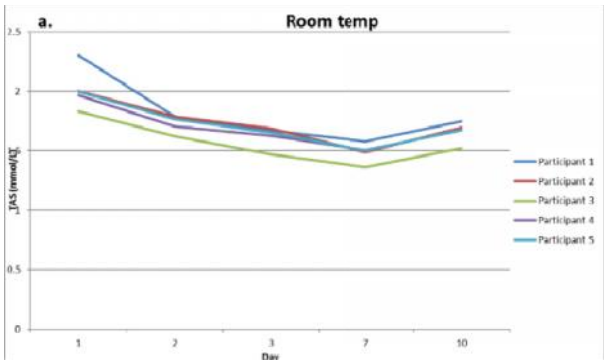

Fig 4(a): Graft plot of TAS

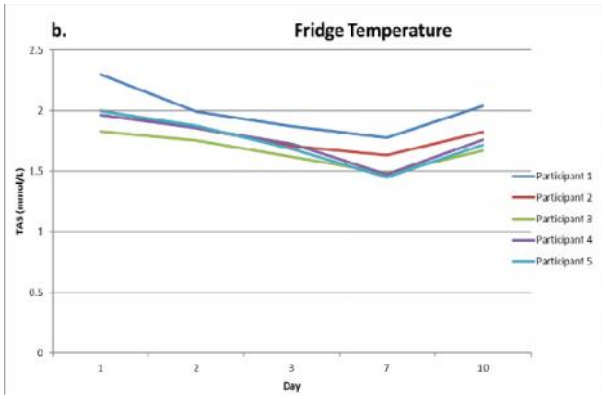

Fig 4(b): Graft plot of TAS

Suppl. Item IVa: The total antioxidant status of five participants, human sample serum stored at a Room temperature from 28.3^{o}C, 24.6^{o}C, 23.1^{o}C, 24.6^{o}C recorded in each of the days. **Suppl. Item IVb.** TAS for five participants, human sample serum stored at fridge temperature recorded at 5.3^{o}C, 4.9^{o}C, 4.7^{o}C, 5.7^{o}C for the different days.

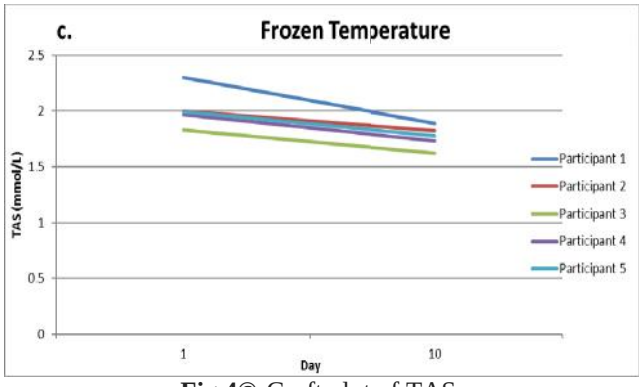

Fig 4©: Graft plot of TAS

Suppl. Item. IVc: The TAS stability storage with human sera stored at a temperature of -20^{o} C. A decrease was observed at this temperature.

4. Conclusions

The total antioxidant gap or status of serum can be obtained using various commercially available assays (Jansen et al.,

2013a).In this study, the antioxidant gap and status were achieved using three of those available assays which include (TAS, uric acid and albumin) were used and experimented in human serum against various temperatures [room temperature, freezer temperature (4OC) and frozen temperature (-20OC)] for a period of 10days. The TAS assay showed that a significant difference does exist between the increase and decrease during the study.There was a decrease in all three storage conditions, but gradually an increase was observed in the day 10 of both the room temperature and fridge temperature. A short stability study carried out by Jansen et al. (2013a) showed a statistically significant decrease in TAS during 2 days of sample storage under room temperature.While day 1 to 10 of the frozen temperatures in this decrease and remained unchanged. On the contrary, in a study by (Jansen et al., 2013b) the storage of TAS at -200C (frozen temperature) showed a statistically significant increase of 11-16% of the baseline value. The reason for the increase in the assay in the study was due to error in the quality control measurement (Jansen et al., 2013b). There were also irregularities from the TAS control which might be a contributing factor to the error described.

The storage stability outcome of uric acid was demonstrated because they substantially contribute to the antioxidant assays(Lamont, Campbell and Fitzgerald,1997). On storage for 10 days, the uric acid showed a slight increment from day 1 to 2 and decrease on day 10 at room temperature, uric acid showed an increment from day 2 to day 7 of the fridge temperature this may be due to irregular temperature of the fridge recorded at day 2 was 5.3OC and day 7, 4.7OC and gradual reduces when the temperature dropped to 5.3OC on day 10. The sera stored frozen continuously decreases at a temperature recorded at -20OC, although the p value of 0.610 showed that there was no significant difference. In addition, there was no significant difference between the average values of uric acid stored at -20°C, -70°C and -196°C for a period of 12 months carried out in a different study as reported by Jansen et al., 2013b.

The albumin showed an irregular pattern of increment and decrease at both room temperature and fridge, while the frozen temperature showed a continuous increase at a temperature of -20°C. The irregularities were also observed in the quality control SeraChem 2 for albumin, this could one of the major cause of the irregular pattern of increment and decrease.

The total antioxidant gap assays showed a total decrease from the day 1 to the day 7 of both the room temperature and fridge temperature, but a gradual increase was noticed on the day 10 of the two storage conditions, while for the frozen temperature at -20Oc there was steady and continuous decrease from the day 1 to the day 10. The overall conclusion is that all the assays showed a statistical difference, excluding uric acid, which indicated no significant difference amongst the various storage conditions. TAS, uric acid, albumin and the AOG values derived from the temperature stored at -20OC should be used with care as in this study the values either increased as in albumin or reduced drastically as seen in TAS, uric acid and AOG. For this study, storage of uric acid at room temperature seems as a preferable means of storage. While for the TAS, albumin and AOG seem rather not appropriate, however, study carried out (Jansen et al., 2013a; Jansen et al., 2013b) showed a contrary result. Merely a few studies have been carried out regarding the stability study of total serum antioxidant gap, about a few can be found (Jansen et al., 2013a; Jansen et al., 2013b). Although there is stability study done on the various antioxidants seen in serum and plasma which include carotenoids and vitamins. In that study, it was concluded that the serum samples for carotenoids were stable and best stored for at -70oc and deteriorates within months (Mathews-Roth and Stampfer,1984).Finally a large study between the various antioxidant status should be carried out comparing the difference between different sex, children and adults.

Table 2: Suppl. Item V: Anova showing the square mean, significant difference of the various assays

		Sum of Squares	df	Mean Square	F	Sig.
UA	Between Groups	.004	2	.002	.498	.610
	Within Groups	.240	57	.004		
	Total	.245	59			
ALB	Between Groups	.005	2	.002	2.172	.123
	Within Groups	.059	57	.001		
	Total	.064	59			
TAS	Between Groups	.274	2	.137	3.521	.036
	Within Groups	2.221	57	.039		
	Total	2.496	59			
GAP	Between Groups	.406	2	.203	4.635	.014
	Within Groups	2.497	57	.044		
	Total	2.903	59			

Table 3: The percentage change of TAS, UA, GAP and Albumin

	Day/Temperature	% change of albumin	% change of Uric Acid	% change of Tas	% change of GAP
Participant 1	Day 1				
RT (Room Temp)	Day 2 (28.3°C)	107	112	78	62
	Day 3 (24.6°C)	104	114	73	55
	Day 7 (23.1°C)	110	115	69	47
	Day 10 (24.6°C)	108	112	76	59
Participant 1	Day 1				
FR (Fridge Temp)	Day 2 (5.3°C)	111	98	87	77
	Day 3 (4.9°C)	106	108	82	69
	Day 7 (4.7°C)	110	116	77	59
	Day 10 (5.3°C)	108	118	89	77
Participant 1	Day 1				
F (Frozen Temp)	Day 10 (-20°C)	106	106	82	70
Participant 2	Day 1	95	74	87	87
RT (Room Temp)	Day 2 (28.3°C)	105	88	78	68
	Day 3 (24.6°C)	102	88	73	62
	Day 7 (23.1°C)	107	89	65	47
	Day 10(24.6°C)	107	86	74	61
Participant 2	Day 1	95	75	87	87
FR (Fridge Temp)	Day 2 (5.3°C)	99	77	81	76
	Day 3 (4.9°C)	104	87	74	63
	Day 7 (4.7°C)	105	92	71	57
	Day 10 (5.3°C)	102	93	80	70
Participant 2	Day 1	95	75	87	87
F (Frozen Temp)	Day 10 (-20°C)	102	81	80	72
Participant 3	Day 1	101	53	80	79
RT (Room Temp)	Day 2 (28.3°C)	111	64	70	60
	Day 3 (24.6°C)	107	65	64	51
	Day 7 (23.1°C)	113	63	59	43
	Day 10(24.6°C)	111	57	66	55
Participant 3	Day 1	101	53	80	79
FR (Fridge Temp)	Day 2 (5.3°C)	106	54	76	73
	Day 3 (4.9°C)	107	61	70	62
	Day 7 (4.7°C)	111	66	65	51
	Day 10 (5.3°C)	110	66	73	63
Participant 3	Day 1	101	53	78	79
F (Frozen Temp)	Day 10(-20°C)	108	60	70	61
Participant 4	Day 1	100	77	86	83
RT (Room Temp)	Day 2 (28.3°C)	108	93	74	60
	Day 3 (24.6°C)	107	94	71	55
	Day 7 (23.1°C)	114	95	65	45
	Day 10 (24.6°C)	111	91	73	58
Participant 4	Day 1	100	77	85	83
FR (Fridge Temp)	Day 2 (5.3°C)	106	77	81	74
	Day 3 (4.9°C)	108	87	75	63
	Day 7 (4.7°C)	112	88	64	45
	Day 10 (5.3°C)	107	97	77	63
Participant 4	Day 1	100	77	85	84
F (Frozen Temp)	Day 10 (-20°C)	107	88	75	64
Participant 5	Day 1	101	51	87	90
RT (Room Temp)	Day 2 (28.3°C)	111	69	77	69
	Day 3 (24.6°C)	114	71	72	60
	Day 7 (23.1°C)	114	69	65	50
	Day 10 (24.6°C)	114	68	73	62
Participant 5	Day 1	101	51	87	90
FR (Fridge Temp)	Day 2 (5.3°C)	110	53	82	80
	Day 3 (4.9°C)	110	62	73	65

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