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Modified Bradford Assay Method of Protein Quantification Utilising Dye Reagents from Four Nigerian Plants

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Abstract: *Aqueous and organic solvents extraction process using ethanol, methanol and chloroform were carried out with four different Nigerian plants namely: Pterocarpus osun (uhe), Lawsonia inermis (lalle), Bixa Orellana (annatto) and Hibiscus sabderriffa (zobo) to extract dye reagents from the plants. The ability of the dye reagents to replace Coomassie Brilliant Blue in the Bradford assay method of protein quantification were determined and compared. The solvents extracts gave good colourful results in the extraction of the dye reagents while only aqueous extract of Hisbiscus sabderiffa (zobo) gave similar results to that of solvent extracts. The solvent extracts obtained from Pterocarpus osun (uhe), Lawsonia inermis (lalle) and Bixa Orellana (annatto) plants could not be used to estimate amino acids from protein samples. However, solvent extracts of Hibiscus sabderriffa (zobo) was able to estimate amino acids from protein samples. The change in maximum wavelength (λmax) and the increased absorption with zobo dye reagent; on addition of protein samples showed that solvent extract of Hibiscus sabderiffa (zobo) dye has the potential to quantify and estimate amino acids in protein samples as much as the Coomassie Blue utilised in the Bradford assay method. The coefficient of correlation (r) values obtained for the proteins are 0.81, 0.92, 0.80 and 0.81 respectively for BSA, haemoglobin, fibrinogen and gamma globulin)*

1. INTRODUCTION

Proteins are biochemical compounds consisting of one or more polypeptides typically folded into a globular or fibrous form, facilitating a biological function.

Like other biological macromolecules such as polysaccharides and nucleic acids, proteins are essential parts of organisms and participate in virtually every process within cells. Many proteins are enzymes that catalyze biochemical reactions and are vital to metabolism. (VanHolde and Matthews, 1996) Protein quantification is often necessary before processing protein samples for isolation, separation and analysis by chromatographic, electrophoretic and immunochemical techniques. Depending on the accuracy required and the amount and purity of the protein available, different methods are appropriate for determining protein concentration. (Krohn, 2002).

Coomassie dye binding assays are the fastest and easiest to perform of all protein assays. The assay is performed at room temperature and no special equipment is required. Standard and unknown samples are added to tubes containing preformulated Coomassie assay reagent and the resultant blue colour is measured at 595nm following a short room temperature incubation. The Coomassie dye-containing protein assays are compatible with most salts, solvents, buffers, thiols, reducing substances and metal chelating agents encountered in protein samples. (Bradford, 1976)

Dyes are chemical substances of chemical or synthetic origin, soluble in a medium used to impart a desired colour to a non-food material like paper, leather, wood, textile and even cosmetics in a process known as dying. (Green, 1995).

Dyes are also referred to as stains and can be used to add colour to tissues and microbes to make them optically distinct. (Ragaswami and Bagyaraj, 1993).

2. EXPERIMENTAL

2.1. Solvents and Equipment Used

All solvents used are of analytical grade. Bradford reagent was made by Sigma-Aldrich, product of USA. The proteins used in this research include: Bovine serum albumin, haemoglobin (from bovine

blood), gamma-globulins (from Human blood), fibrinogen (from human plasma) are all products of Sigma-Aldrich, USA. The UV analysis was carried out using UV-Spectrophotometer Jenway 6405 while the IR analysis was done using FTIR spectrophotometer NICOLET iS10.

2.2. Sampling of Raw Materials

Plants and plant parts used for this research are as follows: (i) seed of *Bixa orellena (annatto) (ii)* heartwood *of Pterocarpus osun (uhe) (iii)* flowers (calyces) of *Hibiscus sabderiffa (zobo) and (iv)* young leaves of *Lawsonia inermis (henna).*

These plants and plant parts were sampled as follows: Zobo calyces was sourced from Bakin dogo market in Kaduna, henna leaves were purchased from Rimi market in Kano metropolis, annatto seeds was collected from the Ahmadu Bello University Herbarium in Zaria, Kaduna State while uhe wood was obtained from an ornamental garden in Owerri, Imo State, Nigeria**.**

2.3. Preparation of Raw Materials

The heartwood of Pt. osun was sun dried and chopped into pieces and further dried in a digital laboratory oven for 4 days at 60°C. The chips were broken into smaller pieces using laboratory mortar and pestle and further reduced to powder by milling (Avwioro *et al*., 2005). The powder was sterilized in a hot air oven at 120°C for 1 h.

The young fresh leaves of L. inermis were grinded to paste with a laboratory mortar and pestle (Drapper, 1976) and sterilized in a hot air oven at 120°C for 1 h.

The dried calyces of H. sabdariffa were grinded with a laboratory mortar and pestle to a rough powder (Drapper, 1976). The powder was heated and sterilized in a laboratory oven at 120°C for 1 h.

The thorny pods containing the seeds of Bixa orellena were broken with the hand. The seeds were separated from the other particles and sterilized in hot air oven at 120°C for 1h.

2.4. Extraction of Pigment

Two methods employed in extraction of the pigments include:

(a) Aqueous Extraction

(b) Organic solvent Extraction

2.5. Aqueous Extraction

2g of powdered uhe and 10g each of powdered annatto, zobo and lalle were separately boiled in 20 and 100 ml distilled water respectively for 5 min. After boiling these were allowed to cool before purification process. (Braide*et al*.,2010)

2.6. Organic Solvent Extraction

2g of powdered uhe and 10g each of powdered annatto, zobo and lalle were separately soaked in 20ml and 100 ml each of the following solvents (absolute ethanol, 80% ethanol, methanol and chloroform) respectively in 250 ml conical flasks. The mouths of the flasks were stoppered with cotton wool, wrapped with aluminium foil and allowed to stand in the dark for 48 hours at ambient temperature. (Braide *et al*.,2010)

2.7. Purification of Extracts (Dyestuff)

Filtration

The content of each of the flask containing the extracts (aqueous and organic solvent) were filtered using whatman filter paper (Avwioro *et al*., 2005).

Evaporation of Solvents

After filtration, the filtered extracts were subjected to evaporation using the vacuum rotary evaporator type 349/2. The extracts were further dried in the hot air oven at 60^{o}C for 1h in order to eliminate moisture or solvent in the extract.

Preparation of Dye Reagent

100mg of the extracted, purified and evaporated dyestuffs prepared from the four plants were separately weighed and dissolved in 50 ml of 95% ethanol. To this solution 100 ml 85% (w/v)

phosphoric acid was added. The resulting solution was diluted to a final volume of I litre. They were all labelled respectively and stored prior to use.

Protein Preparation

0.1g each of Bovine serum albumin, bovine gamma globulin, fibrinogen and haemoglobin were separately weighed and dissolved in 0.15M NaCl solution and made up to the mark each in individual 100ml volumetric flask. Resulting concentration for each protein was 1mg/ml.

Preparation of Protein Standard

A micropipette was used to measure out 0.1ml, 0.2ml, 0.4ml, 0.6ml, 0.8ml of bovine serum albumin and transferred to test-tubes and the corresponding volume of distilled water added to make up 1ml. The same procedure was repeated for fibrinogen, haemoglobin and gamma-globulin samples.

Protein Assay (Standard Method)

1ml of Protein solution containing 0.1mg/ml-1.0mg/ml was pipetted into different test tubes. 5ml of protein reagent was added to the test tube and the contents mixed by shaking and inversion. The uv absorbance was taken before 10 minutes in 3 ml cuvettes against a reagent blank prepared from 1 ml of the appropriate buffer and 5 ml of dye reagent. The concentration of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in unknown samples. (Zor and Selinger, 1996)

Quality Control Test

The procedure was repeated using the Bradford reagent as the dye reagent on all the proteins so as to ascertain the level of efficacy of the dye reagents obtained from the four Nigerian plants.

3. RESULTS

Table1. *Colours of the dye reagents of the plants in different solvents*

SOLVENTS	COLOUR OF EXTRACT			
	Pterocarpus Osun (uhe)	Lawsonia inermis. (lalle)	Hisbiscus sabdarriffa (zobo)	Bixa orellena (annatto)
Abs. Ethanol	Red	Brown	Mauve	Red
80% Ethanol	Red	Brown	Mauve	Red
Chloroform	Pale amber	Pale brown	Amber	Red
Methanol	Orange	Brown	Mauve	Orange red
Boiled Water	Pale amber	Brown	Red mauve	Amber

Table2. *Wave lengths of maximum absorption of the dyes in different solvents*

SOLVENT	Wavelength (λmax) nm			
	P. osun(uhe)	L. inermis (lalle)	H. sabdarrifa (zobo)	B. orellena (annatto)
Abs ethanol	490	490	520	475
80% ethanol	490	385	515	470
Chloroform	485	675	500	480
Methanol	440	380	515	450
Boiled water	440	380	520	420

Table3. *Wave lengths of maximum absorption of the prepared dyes*

Dye Reagent	**λmax (nm)**
P.Osun (uhe)	610
L. inermis (lalle)	680
H. sabdariffa (zobo)	650
B. orellena(annatto)	550

Table4. *Spectrophotometric assay of BSA using various extracts of Pterocarpus osun (Uhe)*

Protein conc. (mg/ml)	Absorbance				
	Abs. Ethanol Extract	80% Ethanol Extract	Chloroform Extract	Methanol Extract	Boiled Water Extract
0.1	0.573	0.580	0.588	0.485	0.470
0.2	0.568	0.560	0.561	0.467	0.459
0.4	0.578	0.573	0.578	0.493	0.499
0.6	0.599	0.592	0.590	0.472	0.477
0.8	0.573	0.569	0.567	0.460	0.463
1.0	0.587	0.581	0.580	0.489	0.473

Table5. *Spectrophotometric assay of BSA using various extracts of Lawsonia inermis (lalle)*

Protein conc. (mg/ml)	Absorbance				
	Abs Ethanol Extract	80% Ethanol Extract	Chloroform Extract	Methanol Extract	Boiled Water Extract
0.1	2.281	2.280	1.199	2.479	2.503
0.2	2.272	2.209	1.181	2.350	2.511
0.4	2.254	2.196	1.179	2.250	2.488
0.6	2.262	2.250	1.180	2.500	2.542
0.8	2.268	2.270	1.192	2.601	2.552
1.0	2.276	2.279	1.189	2.708	2.502

Table6. *Spectrophotometric assay of BSA using various extracts of Hibiscus sabdarrifa (zobo)*

Protein conc. (mg/ml)	Absorbance				
	Abs Ethanol Extract	80% Ethanol Extract	Chloroform Extract	Methanol Extract	Boiled Water Extract
0.1	0.073	0.065	0.156	1.185	0.070
0.2	0.075	0.068	0.158	1.186	0.075
0.4	0.076	0.069	0.160	1.188	0.077
0.6	0.077	0.072	0.163	1.189	0.079
0.8	0.079	0.075	0.165	1.190	0.082
1.0	0.081	0.078	0.170	1.198	0.085

Table7. *Spectrophotometric assay of BSA using various extracts of Bixa Orellena (Annatto)*

Protein conc. (mg/ml)	Absorbance				
	Abs Ethanol Extract	80% Ethanol Extract	Chloroform Extract	Methanol Extract	Boiled Water Extract
0.1	2.213	2.587	1.827	1.890	2.256
0.2	2.611	2.411	1.789	1.927	2.281
0.4	2.512	2.660	1.889	1.789	2.240
0.6	2.341	2.670	1.995	1.880	2.234
0.8	2.413	2.500	1.890	1.889	2.251
1.0	2.315	2.511	1.990	1.887	2.249

Table8. *Spectrophotometric assay of various protein samples using extracts of Hibiscus sabdarrifa (zobo)*

Protein conc. (mg/ml)	Absorbance			
	BSA	Haemoglobin	Fibrinogen	Gamma Globulin
0.1	0.177	0.941	1.062	1.081
0.2	0.178	0.957	1.066	1.082
0.4	0.181	1.007	1.068	1.085
0.6	0.183	1.011	1.073	1.115
0.8	0.201	1.042	1.078	1.119
1.0	0.203	1.088	1.099	1.141

Table9. *Spectrophotometric assay of various protein samples using Bradford Reagent. Bradford Protein Assay (Control Experiment)*

Protein conc. (mg/ml)	Absorbance			
	BSA	Haemoglobin	Fibrinogen	Gamma Globulin
0.1	0.693	0.840	0.720	0.906
0.2	1.144	1.027	1.002	1.186
0.4	1.231	1.292	1.257	1.385
0.6	1.447	1.431	1.407	1.495
0.8	1.480	1.498	1.487	1.519
1.0	1.763	1.519	1.577	1.588

4. RESULTS AND DISCUSSION

4.1. Extraction of Dyestuffs

Table 1 gives the colours and extraction efficiency of the dyes from the plants in different solvent media. Boiled water also gave very good extraction efficiency for zobo and lalle while uhe and annatto produced very poor extraction efficiency (Braide *et al*)

However, all dyestuffs were soluble in 80% ethanol and produced colours as strong as absolute ethanol. The effectiveness and or level of extraction were determined by the concentration of the colour as seen in table 1 (Braide *et al*).

4.2. Colour of Extract

Absolute ethanol and 80% ethanol gave the best results for the extraction of pterocarpus osun (uhe) and the other plants. The other three solvents that is, chloroform, methanol and boiled water did not give intense colours as did ethanol which can be seen in table 1.

Methanol and boiled water were very good solvents in the extraction of Lawsonia inermis (lalle). Boiled water (aqueous extraction) was good in the extraction of Hibiscus sabdariffa (zobo).

In addition, chloroform is an excellent solvent in the extraction of Bixa orellena(annatto).

4.3. Protein Assay

Extracts of Pterocarpus osun (Uhe), Lawsonia inermis (lalle), Bixa Orellana (annatto) did not estimate protein as can be seen in tables 4, 5 and 7. However, extracts of Hibiscus sabderiffa (zobo) did estimate protein samples. This can be seen in table 6.

Furthermore, the wavelength of maximum absorption of the prepared zobo dye was recorded at 650nm, upon addition of protein sample it changes to 660nm. This also demonstrates that zobo dye can be used for amino acid estimation in protein samples. The other three dye reagents did not change λmax upon addition of protein samples.

4.4. Stability of the Protein-Dye Complex

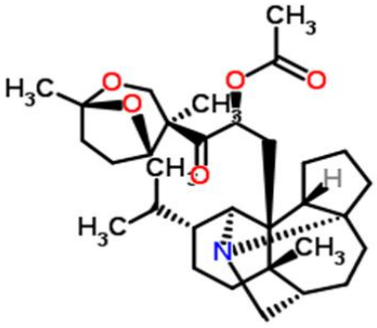
From the experiment, it was observed that once the zobo dye is added to the protein, measurement must be taken within ten minutes in order to achieve accurate results. This may be due to the instability of the protein-dye complex after about fifteen minutes of reaction. Therefore, in order to obtain accurate results, addition of zobo dye to the protein is done only when the uv-visible spectrophotometer instrument is ready for measurement.

4.5. Non-Distinct Colour Formation

In the Bradford assay, there is sudden change in the colour of the dye upon addition of protein samples from brown to blue. However, in this experiment there is non-distinct colour change upon addition of zobo dye to protein sample. The colour of the prepared zobo dye is light brown. Upon addition of zobo dye to protein sample, we have not recorded any clear visible colour change, however, there is a shift in the wavelength of maximum absorption of the zobo dye. The zobo dye estimated amino-acid in various protein samples.

4.6. Principal Constituent of Hibiscus Sabdariffa (Zobo)

The plants are rich in anthocyanins, as well as protocatechuic acid. The dried calyces contain the flavonoids gossypetin, hibiscetine and sabdaretine. The major pigment, formerly reported as hibiscin, has been identified as daphniphylline. Small amounts of myrtillin (delphinidin 3-monoglucoside), Chrysanthenin (cyanidin 3-monoglucoside), and delphinidin are also present. Roselle seeds are a good source of lipid-soluble antioxidants, particularly gamma-tocopherol. (Mohamed *et al*., 2007)



Daphniphylline

- *Molecular Formular* $C_{32}H_{49}NO_5$

As reported the major pigment in zobo is daphniphylline. Thus most probably the reaction between zobo dye and amino acid in protein samples could be between daphniphylline and amino acids.

The ether group was observed at 1008 cm^{-1}.

The IR spectrum frequencies for zobo dye and bovine serum albumin protein shows differences in value to that of the zobo dye alone. The new frequencies obtained shows that there has been bonding between the zobo dye and the protein molecule as shown in figures 2 and 3. The shift in frequencies could be as a result of bonding between the O-H group of the dye and the N-H site on the protein molecule.

5. IR ANALYSIS

The width signal of 3586cm^{-1} confirms the presence of O-H stretch. The double C=C bonds of the aromatic ring were observed for the vibration in 1185 cm^{-1} and the C-H bonds of the aromatic ring were observed for the stretching band at 2383 cm^{-1}. In 1636 cm^{-1} was observed the signal for the C=O bond, assigned to a carbonyl group.

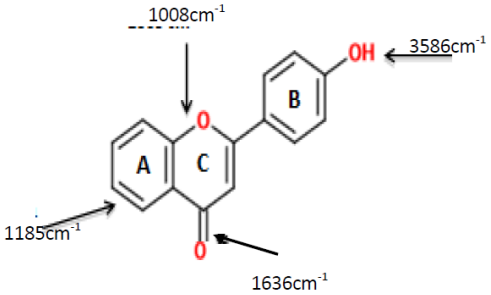
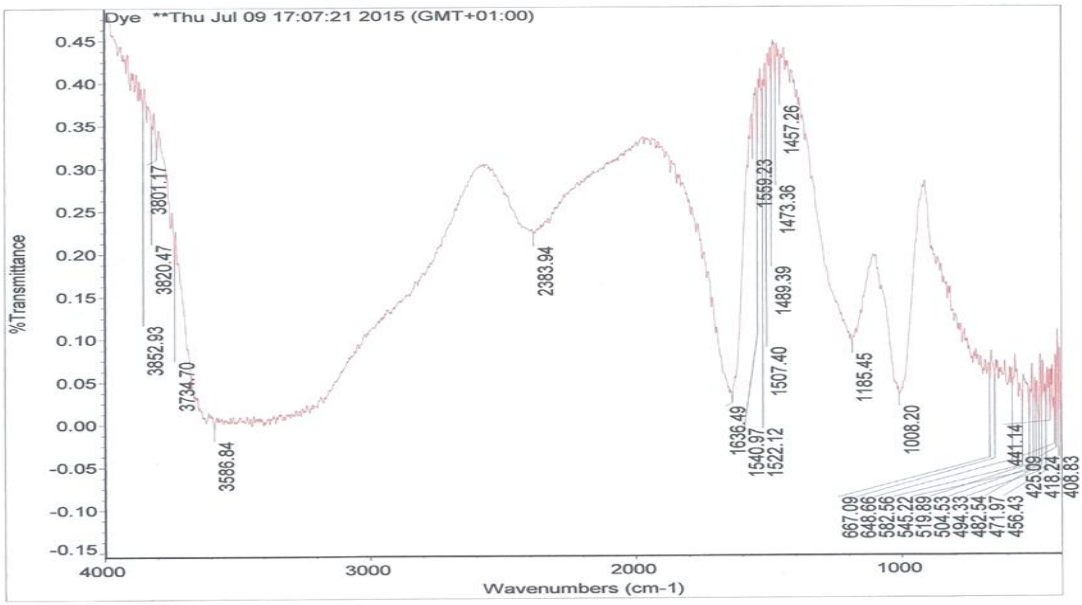


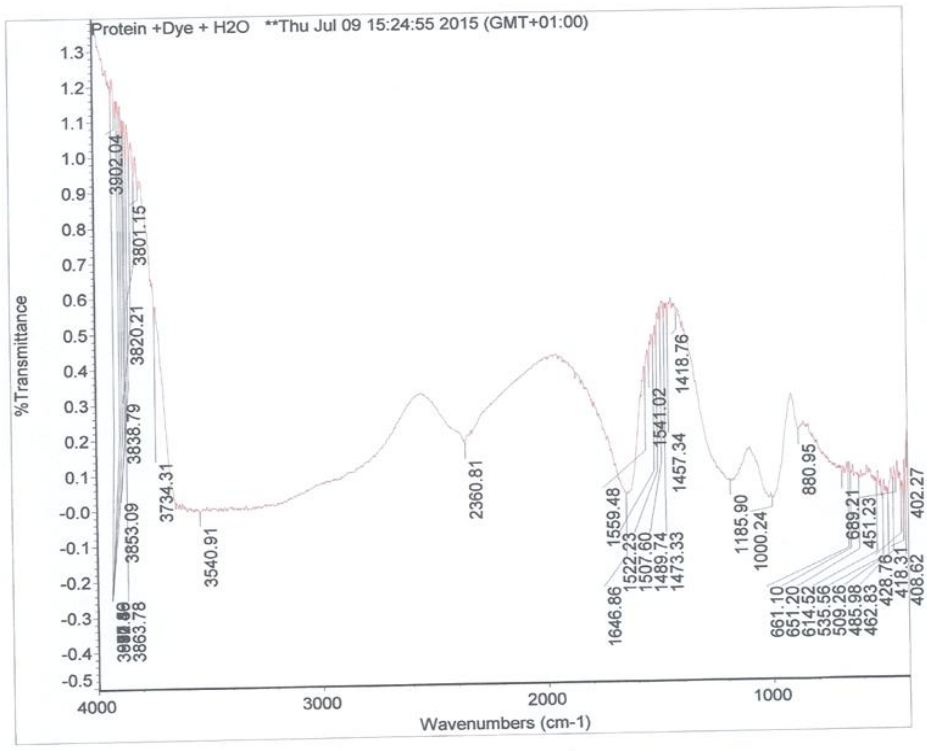
Fig1. *The important functional groups in general structure of flavonoids and their IR frequencies.*



Thu Jul 09 17:09:13 2015 (GMT+01:00)
FIND PEAKS:
Spectrum: Dye **Thu Jul 09 17:07:21 2015 (GMT+01:00)
Region: 4000.00 400.00
Absolute threshold: 0.417
Sensitivity: 46
Peak list:

Position:	408.83	Intensity:	0.0084
Position:	418.24	Intensity:	-0.0174
Position:	425.09	Intensity:	0.0024
Position:	441.14	Intensity:	0.0071
Position:	456.43	Intensity:	0.00014
Position:	471.97	Intensity:	0.0206
Position:	482.54	Intensity:	-0.0024
Position:	494.33	Intensity:	0.0146
Position:	504.53	Intensity:	0.0271
Position:	519.89	Intensity:	0.0011
Position:	545.22	Intensity:	0.0185
Position:	582.56	Intensity:	0.0402
Position:	648.66	Intensity:	0.0445

Fig2. *IR Spectrum of Zobo dye*



Thu Jul 09 15:31:00 2015 (GMT+01:00)
FIND PEAKS:
Spectrum: Protein +Dye + H2O **Thu Jul 09 15:24:55 2015 (GMT+01:00)
Region: 4000.00 400.00
Absolute threshold: 1.276
Sensitivity: 66
Peak list:

Position:	402.27	Intensity:	0.128
Position:	408.62	Intensity:	0.0113
Position:	418.31	Intensity:	-0.0988
Position:	428.76	Intensity:	0.0231
Position:	451.23	Intensity:	0.0453
Position:	462.83	Intensity:	0.0491
Position:	485.98	Intensity:	0.0154
Position:	509.26	Intensity:	-0.0054
Position:	535.56	Intensity:	0.0380
Position:	614.52	Intensity:	0.0618
Position:	651.20	Intensity:	0.0707
Position:	661.10	Intensity:	0.0751
Position:	689.21	Intensity:	0.0693

Fig3. *IR Spectrum of Zobo dye with protein sample*

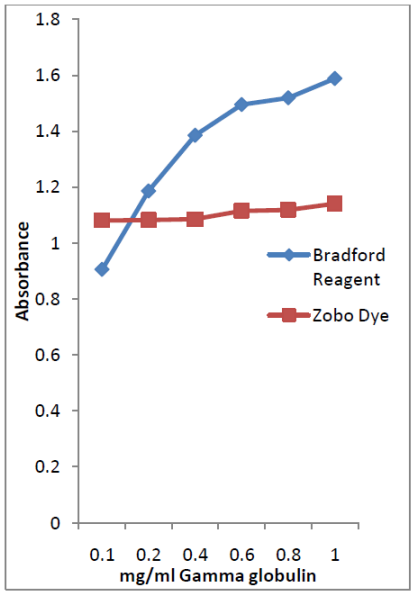


Fig4. *Comparative Protein-Dye Response pattern for Zobo and Coomassie dyes using Gamma Globulin*

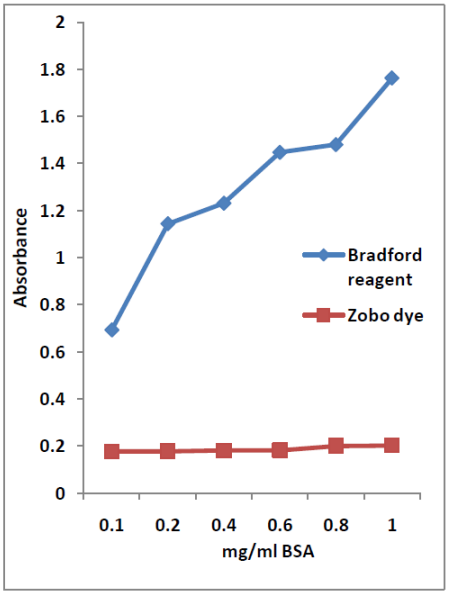


Fig5. *Comparative Protein-Dye Response pattern for Zobo and Coomassie dyes using BSA*

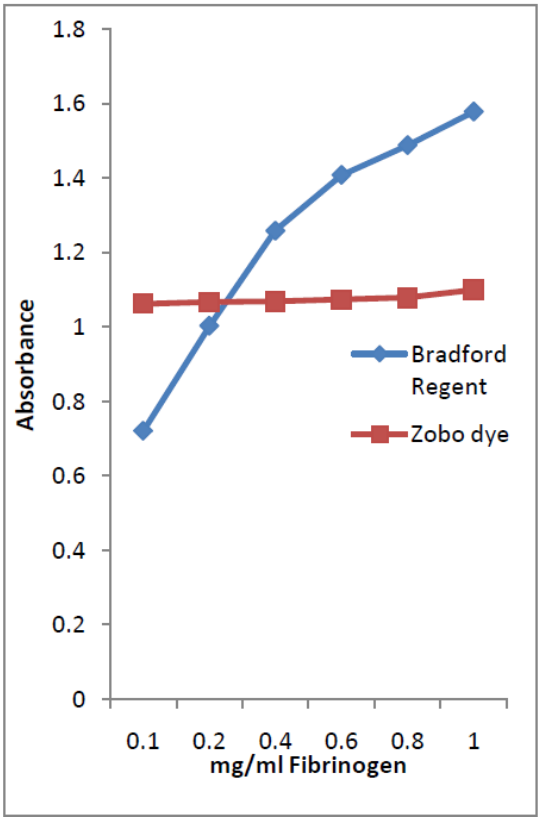


Fig6. *Comparative Protein-Dye Response pattern for Zobo and Coomassie dyes using Fibrinogen*

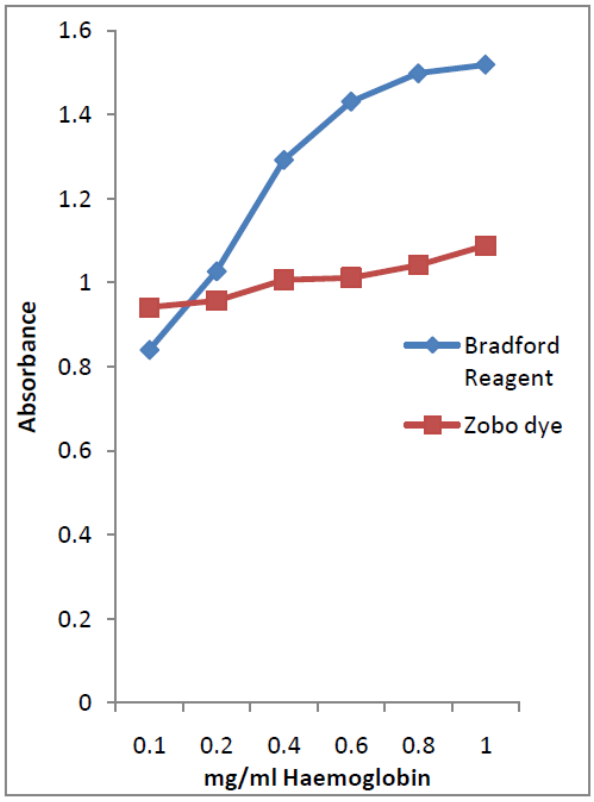


Fig7. *Comparative Protein-Dye Response pattern for Zobo and Coomassie dyes using Haemoglobin*

The comparative plot of absorbance versus protein concentration (figures 4-7) for gamma globulin, BSA, Fibrinogen and haemoglobin shows that zobo dye has the potential of estimating protein as compared to Bradford reagent. The statistical analysis of the results obtained in tables 8 and 9 showed that the values for Hibiscus sabderiffa(zobo) dye and coomassie brilliant blue are significantly correlated with coefficient of correlation values (r) being 0.81, 0.92, 0.80 and 0.81 respectively for BSA, haemoglobin, fibrinogen and gamma globulin.

6. CONCLUSION

The results show that extracts of Hibiscus sabdariffa (Zobo) can be used for protein assay. It estimates amino acid in protein samples as much as the coomassie blue used in the Bradford assay. There are possible bonding sites between the dye and amino acids, hence its usefulness in quantitation of amino-acid in protein samples.

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