The Haryana Veterinarian (June, 2024) **63(1),** 31-36 ISSN 0033-4359 (P) **Research Article**

SPECTROPHOTOMETRIC METHOD FOR THE RAPID DETERMINATION OF SPERM CONCENTRATION IN MAGRA RAM

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ABSTRACT

The aim of present study was to optimize the methodology for rapid determination of sperm concentration in ram semen by the use of spectrophotometer. In order to fulfil the objective, sperm concentration of Magra rams using 2.94% sodium citrate and TRIS diluents was compared with sperm concentration of Magra rams using haemocytometer. Sperm cell count (Mean±S.E.) as determined manually in a haemocytometer for adult Magra rams (n=5) was 3120 ± 691.12×10^6 per mL (ranging from 1150 to 5100×10^6 per mL). All the five samples were diluted at 1:400, 1:800 and 1:1600 ratio using 2.94% sodium citrate and TRIS diluents and then their regression analyses were carried out to estimate sperm concentration from absorbance. The mean sperm concentrations of these five rams in 1:400, 1:800 and 1:1600 dilutions were 7.80 ± 1.73, 3.89 ± 0.86 and 1.94 ± 0.43 × 10^6 per mL, respectively. The absorbance value of these diluted semen samples measured at three different visible wave lengths i.e., 500, 550 and 600 nm were plotted against their concentrations. The regression equation obtained from the regression of absorbance on the sperm concentration at each of the different wave lengths (i.e., 500, 550 and 600 nm) resulted in the best fit at 600 nm with very high coefficient of determination for 2.94% sodium citrate diluent (R^2 = 0.945, OD range 0.079 - 0.636 and equation Y = 0.046 × -0.043) as well as for TRIS diluent (R^2 = 0.950, OD range 0.073 - 0.589 and equation Y = 0.046 × -0.045). For accuracy of above equations, these equation were employed to estimate the sperm cell concentration in samples during validation of the spectrophotometric method. Semen samples collected randomly from the rams (n=7) were utililized for validation studies and no significant differences (p>0.05) were found between the concentration obtained manually by haemocytometer method and with the use of spectrophotometric method.

Keywords: Equation, Haemocytometer, Ram, Semen concentration, Spectrophotometer, Wave length

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The growing interest in the research of semen biology is largely due to the expanding practice of artificial insemination (AI) and recognition of problems of infertility (Khanal and Munankarmy, 2009). AI ensures better utilization of semen where a single seminal ejaculate can be used to impregnate a large number of females when compared to natural mating where a single male is used on limited number of female animals (Mustafa *et al.*, 2018). The routine evaluation of sperm concentration provides the status of semen quality and helpful in supplying accurate sperm doses required in AI programs (Gaviraghi *et al.*, 2013). Normally, sperm concentration can be estimated using hemocytometeric method which is time consuming and may cause erroneous results due to divergence among counting chambers, manufacturers variations in hemocytometers and technical variations between person to person especially when processing large numbers of samples (Prathalingam *et al.*, 2006). In addition, single hemocytometer counts are not highly precise and variation in results may go up to 20% even in the case of the same technician counting duplicate samples from the same source (Freund and Carol, 1964). Therefore it is a demand of time to investigate a fast and accurate method for estimation of sperm concentration using spectrophotometer by measuring absorbance of diluted sample and emanating sperm concentration from an absorbance vs. concentration standard curve.

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MATERIAL AND METHODS

The present study was undertaken in the Department of Veterinary Biochemistry, College of Veterinary and Animal Science, RAJUVAS and ICAR-CSWRI, Arid region Campus, Beechhwal, Bikaner during summer of the year 2018. Adult Magra rams (n=12) aged between 1.5-3.0 years, with a body condition score of 3+ (scale of 1-5) and having mean body weight of 38 ± 5 kg maintained at ICAR -CSWRI, ARC, Beechhwal, Bikaner were selected for the study. Rams were fed with the standard diet, formulated according to the requirement for a mature ram as per ICAR Nutrient Requirements of Sheep (ICAR, 2013).

Collection of semen samples

Ejaculates were collected from adult Magra rams by using an artificial vagina (AV) made up of hard rubber and measuring 20 cm in length and 4-5 cm in diameter. The semen collections were obtained by exposing the ram to the estrus ewe. The rams were allowed for two false mount

before collection of semen. Immediately after collection, the semen collection cup was maintained in a water bath at 37° C for further evaluation. Each ejaculate was observed for its mass motility and sperm concentration. The samples having mass motility score ≥ 4 (in a scale of 0-5) and sperm concentration > 3×10^9 sperm per mL were only considered for the present study (Evans and Maxwell, 1987).

Sperm cell counting with the hemocytometer

Sperm concentration was estimated with the help of Haemocytometer (Neubaur's counting chamber) by adopting standard procedure (Evan and Maxwell, 1987).

Development of Linear Regressions of absorbance on sperm concentration in two different diluents.

To develop linear regressions of absorbance on sperm concentration for determination of sperm concentration with the spectrophotometer, the first diluents undertaken for study, 2.94% Sodium citrate was placed into a cuvette and inserted into the spectrophotometer to blank the instrument, then diluted semen was added using a positive displacement cuvette. The sample was covered with cuvette coverslip, inverted 10 times and replaced into spectrophotometer. Similarly, the second diluent to be tested for the study, i.e., TRIS buffer was placed into a cuvette and inserted into the spectrophotometer to blank the instrument, then diluted semen was added using a positive displacement cuvette. The sample was covered with cuvette coverslip, inverted 10 times and replaced into spectrophotometer. To demonstrate the repeatability of the spectrophotometer, replicate measures of semen concentrations were determined. To determine the optimum wavelength at a particular dilution of semen sample, the absorption of semen samples from rams (n=5) [whose sperm concentrations were determined through manual counting procedure in a Neubaur's counting chamber under light microscope] were recorded at different dilutions using both 2.94% sodium citrate and TRIS diluents. The rams selected had different sperm concentrations making a range of varied sperm concentration available for plotting against the absorbance at each of the wavelength selected for study (λ = 500, 550 and 600 nm). Serial dilution of the semen from these 5 rams were obtained at dilutions 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600 so that a range of different sperm concentrations could be prepared for absorbance recording in the spectrophotometer. The absorbance of all the samples at each dilution was recorded at each of the above mentioned wavelengths. The standard linear regressions were obtained for absorbance on sperm cell concentrations. The regression equations for best fit were derived to determine the optimum wavelength with maximum coefficient of determination R^2 for each of the diluents (2.94% sodium citrate and TRIS diluent).

Validation of the deduced equations for predicting sperm concentration

Random semen samples were collected from rams (n=7) selected randomly from the ICAR-CSWRI arid region campus (ARC) flock [Other than those selected in the development of standard linear regressions as stated in the above section]. Their sperm counts were determined as described earlier using hemocytometer. The samples were then diluted @ 1:400, 1:800 and 1:1600 in 2.94% Sodium citrate as well as TRIS diluents and absorbance at 600 nm was determined using a spectrophotometer. To estimate the sperm concentration from their absorbance, regression equation derived from the linear regression with best fit of absorbances on sperm concentrations (as described in the previous section) were utilized. The sperm concentration obtained by Spectrophotometric method was compared with the actual sperm concentration determined using hemocytometer.

Statistical analysis

All data including biochemical parameters were presented with their means and standard errors. The data was subjected to analysis of Variance, and Pearson correlation using Graph Pad Software. Significance (Brown -Forsythe and Bartlett's test for pair-wise comparisons of means, and for Pearson's correlation coefficients) were considered at $p<0.05$ unless mentioned otherwise in GraphPad software. All graphs were prepared in MS-EXCEL version 2010.

RESULTS AND DISCUSSION

Determination of sperm concentration by haemocytometer and absorbance spectrum

Sperm cell count (Mean ± S.E.) as determined manually using a hemocytometer for adult Magra rams (n=5) was $3120 \pm 691.12 \times 10^6$/ml (range 1150 to 5100×10^6/ml). All the five samples were initially diluted individually at 1:50, 1:100 and 1:200 dilutions using both 2.94% Sodium citrate and TRIS as diluents. However these dilutions did not elicit a true linear response especially in samples with sperm concentrations above 3000×10^6/ml. Therefore, higher dilutions for the semen samples i.e., 1:400, 1:800 and 1:1600 in both the diluents were prepared and their regression analyses were carried out to estimate sperm concentration from the absorbance values. The mean sperm concentrations of these five rams in both 2.94% sodium citrate and TRIS diluents after 1:400, 1: 800 and 1:1600 dilutions were 7.80 ± 1.73 (2.87, 5.12, 8.75, 9.5, 12.75), 3.89 ± 0.86 (1.44, 2.56, 4.38, 4.75, 6.34), 1.94 ± 0.43 (0.72, 1.28, 2.19, 2.34, 3.19) × 10^6/ml, respectively. The absorbances of these diluted

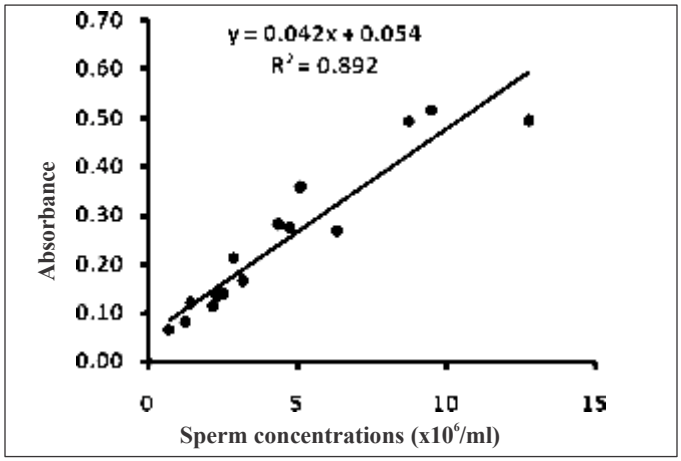

Fig. 1. Regression of absorbance on sperm concentrations with 2.94% Sodium Citrate diluent at 500 nm

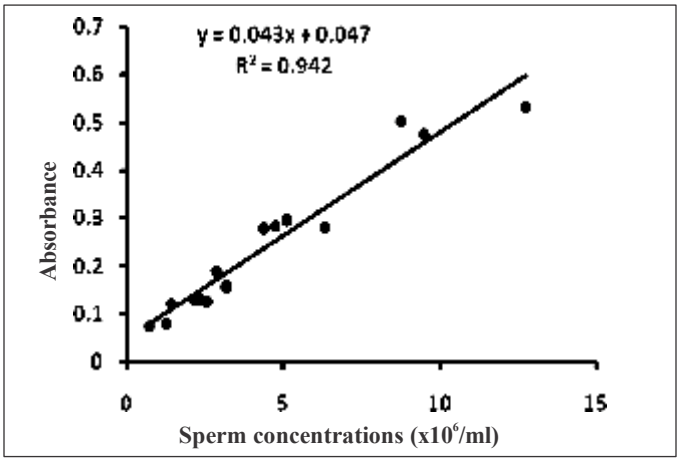

Fig. 2. Regression of absorbance on sperm concentrations with 2.94% Sodium Citrate diluent at 550 nm

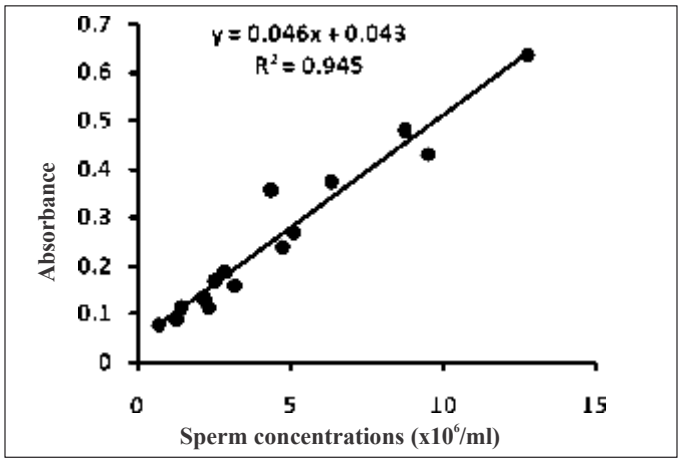

Fig. 3. Regression of absorbance on sperm concentrations with 2.94% Sodium Citrate diluent at 600 nm

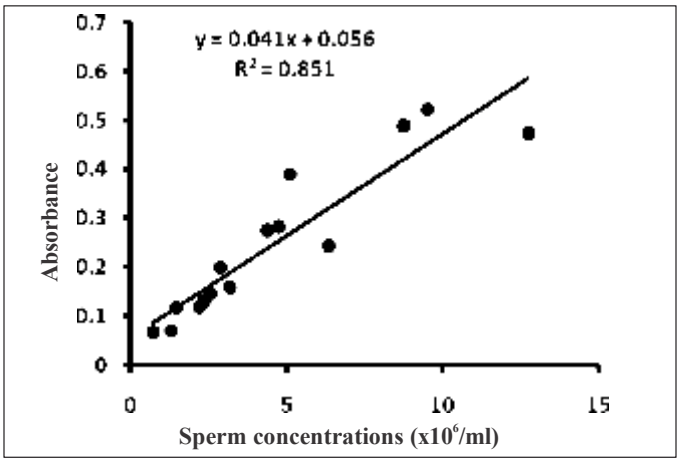

Fig. 4. Regression of absorbance on sperm concentrations with TRIS diluent at 500 nm

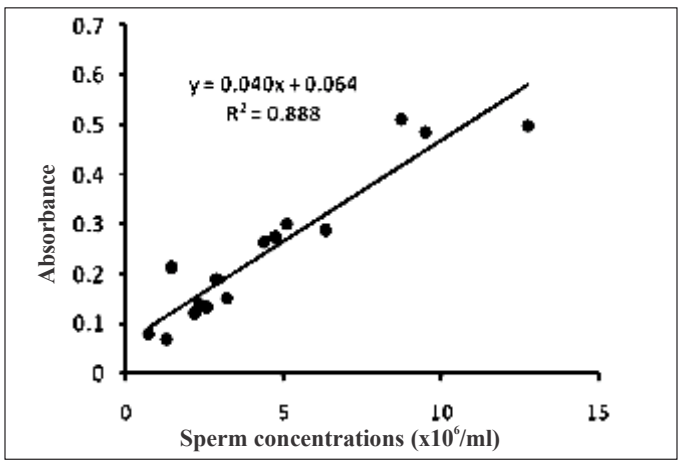

Fig. 5. Regression of absorbance on sperm concentrations with TRIS diluent at 550 nm

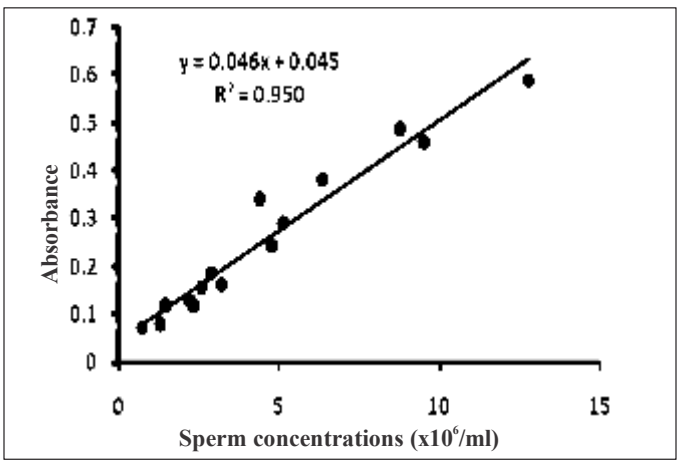

Fig. 6. Regression of absorbance on sperm concentrations with TRIS diluent at 600 nm

samples were plotted against their concentrations at three different wavelengths i.e., 500, 550 and 600 nm. Their regression equations are presented in Table (1) and figure (1-6). The regression equations obtained from the regression of absorbance on the sperm concentration at each of the different wavelengths resulted in the best fit at 600 nm with very high coefficient of determination (R^2 = 0.945, OD range 0.079 - 0.636 for 2.94% sodium citrate diluent (Fig. 3) and R^2 = 0.950, OD range 0.073 - 0.589 for TRIS diluent (Fig. 6). There was no significant difference ($p<0.05$) between the optical densities (absorbances) as measured by the two diluents. However, within a wave length in either of the diluents, the absorbances observed for different sperm concentrations were significantly

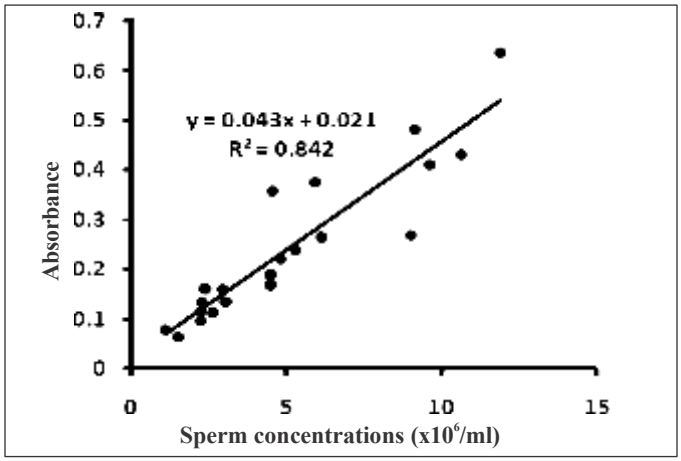

Fig. 7. Validation of regression of absorbance on sperm concentrations with 2.94% Sodium Citrate diluent at 600 nm in test samples (n=21)

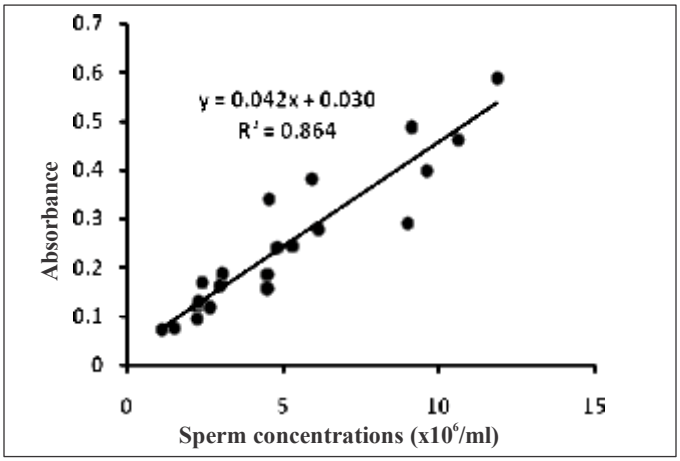

Fig. 8. Validation of regression of absorbance on sperm concentrations with TRIS diluent at 600 nm in test samples (n=21)

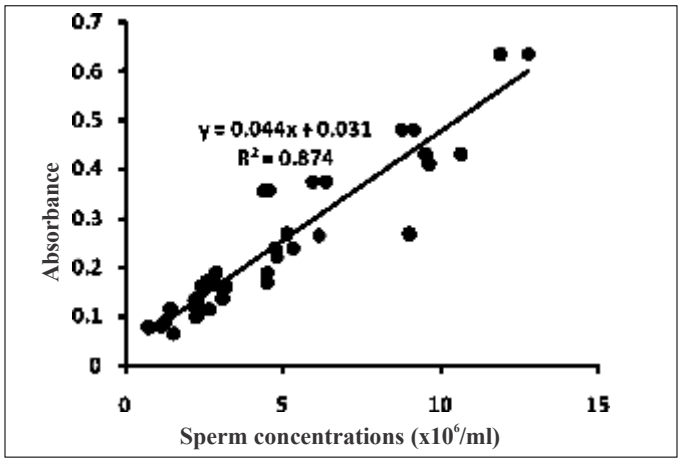

Fig. 9. Validation of regression of absorbance on sperm concentrations with 2.94% Sodium Citrate diluent at 600 nm in all samples in the study (validation + standardization, n=36)

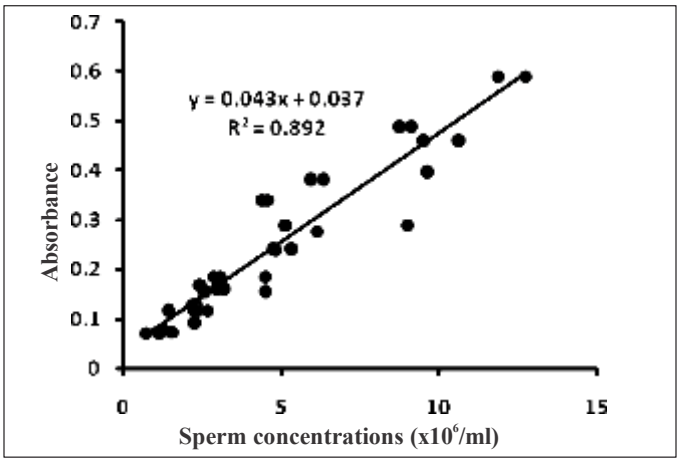

Fig.10. Validation of regression of absorbance on sperm concentrations with TRIS diluent at 600 nm in all samples in the study (validation + standardization, n=36)

Table 1. Regression parameters of the regressions* of absorbance on sperm concentrations obtained at different wavelength in two different diluents

Wavelength (nm)	Regression Parameters	2.94% Sodium Citrate Buffer	TRIS Buffer
500 nm	Equation	Y=0.042 x -0.054	Y=0.041 x-0.056
	R^2-value	0.89	0.85
	Absorbance range	0.065-0.514	0.068-0.521
550 nm	Equation	Y=0.043 x -0.047	Y=0.040 x -0.064
	R^2-value	0.942	0.888
	Absorbance range	0.075-0.531	0.07-0.498
600 nm	Equation	Y=0.046 x -0.043	Y=0.046 x -0.045
	R^2-value	0.945	0.950
	Absorbance range	0.079-0.636	0.073-0.589

Y= absorbance and x= sperm concentration; number of observations =15. The equations used for final estimation of the sperm concentration from absorbances are indicated in bold.

different ($p<0.01$). The equations at 600 nm were employed to estimate the sperm cell concentration in samples during validation of the spectrophotometric method. Very high Pearson correlation coefficients with high significance ($p<0.01$) were observed between the absorbance recorded for various sperm concentrations at different wavelengths and in two different diluents. These correlations derived in the trial for development of linear regression equations from various dilutions developed from the samples of 5 adult rams (standardization) are presented in Table 2.

Validation of the deduced equations for determination

Table 2. Standard Pearson Correlation Matrix showing statistical significance (p<0.01) between the sperm concentrations of Magra Rams as measured by various methods

	AC	NC500	NC550	NC600	TRIS500	TRIS550	TRIS600
AC	1.000	0.945**	0.971**	0.972**	0.923**	0.943**	0.975**
NC500	0.945**	1.000	0.988**	0.926**	0.996**	0.977**	0.952**
NC550	0.971**	0.988**	1.000	0.960**	0.975**	0.984**	0.975**
NC600	0.972**	0.926**	0.960**	1.000	0.898**	0.931**	0.994**
TRIS500	0.923**	0.996**	0.975**	0.898**	1.000	0.967**	0.929**
TRIS550	0.943**	0.977**	0.984**	0.931**	0.967**	1.000	0.954**
TRIS600	0.975**	0.952**	0.975**	0.994**	0.929**	0.954**	1.000

**Significance of correlation at (p<0.01); AC: Actual sperm concentration in semen samples by haemocytometer method
NC500 : Estimated sperm concentration in spectrophotometer in 2.94% sodium citrate diluent at 500 nm wavelength for samples (n=15) at different concentrations; NC 550 : Estimated sperm concentration in spectrophotometer in 2.94% sodium citrate diluent at 550 nm wavelength for samples (n=15) at different concentrations; NC600 : Estimated sperm concentration in spectrophotometer in 2.94% sodium citrate diluent at 600 nm wavelength for samples (n=15) at different concentrations; TRIS500 : Estimated sperm concentration in spectrophotometer in TRIS diluent at 500 nm wavelength for samples (n=15) at different concentrations; TRIS550 : Estimated sperm concentration in spectrophotometer in TRIS diluent at 550 nm wavelength for samples (n=15) at different concentrations; TRIS600 : Estimated sperm concentration in spectrophotometer in TRIS diluent at 600 nm wavelength for samples (n=15) at different concentrations

Table 3. Sperm concentrations (Mean±S.E) at 600nm estimated from standard regression equations during validation of spectrophotometric method

Sperm Concentration ($\times10^6$/ml) obtained in hemocytometer	Dilutions [conc in ($\times10^6$/ml)]	Final Sperm concentration ($\times10^6$/ml)* at 600 nm estimated from standard regression equations		
		2.94% Sodium Citrate Diluent	TRIS Diluent	Significance
3478.57±386.35	1:400 (8.7±0.97)	2959.01±504.64 (OD:0.38±0.06)	2954.04±461.66 (OD:0.38±0.05)	ns
	1:800 (4.35±0.48)	3254.66 ±675.09 (OD:0.23±0.03)	3371.43±629.33 (OD: 0.24±0.03)	ns
	1:1600 (2.18±0.24)	2514.29 ±500.30 (OD:0.115±0.014)	2514.29±514.64 (OD:0.117±0.015)	ns
	Overall	2909±317.3 (OD: 0.243±0.03)	2947±305.9 (OD: 0.247±0.03)	ns
	Significance	(P<0.001)	(P<0.001)	

*Final concentration derived after correction for dilution factor as indicated.Values underlined are nearest to the actual values. ns: non-significant

Table 4. Standard Pearson Correlation Matrix with significance (p<0.01) between the sperm concentrations of Magra Rams as measured by various methods

	AC	NAC-ESTI	TRIS-ESTI
AC	1.00	0.92**	0.93**
NAC-ESTI	0.92**	1.00	0.99**
TRIS-ESTI	0.93**	0.99**	1.00

** Significance of correlation at (p<0.01) Actual sperm concentration in semen samples by hemocytometer method
NAC-ESTI : Estimated sperm concentration in spectrophotometer in 2.94% sodium citrate diluent at 600 nm wavelength for samples (n=21) at different concentrations
TRIS-ESTI : Estimated sperm concentration in spectrophotometer in TRIS Diluent at 600 nm wavelength for samples (n=21) at different concentrations

of sperm concentration

Semen samples collected randomly from the rams (n=7) were utilized for validation studies and their data are presented in table 3. No significant (P>0.05) differences were found between the concentrations obtained manually by haemocytometer method and by spectrophotometric method. There were significantly (p<0.01) high correlations between the actual values as determined manually and those calculated by employing the regression equations (Table 4, r > 0.92, and near absolute i.e., r = 0.99 between the actual and those estimated by TRIS diluent at 600 nm). The regressions were also plotted for the absorbances at 600 nm (recorded with each of the diluents) on the actual sperm concentrations in the semen of the animals undertaken for validation study. These are presented in the figures 7-10. The regression equation revealed the best fit with R^2=0.864 for absorbances in TRIS diluent which was non significantly better than that observed with 2.94% Sodium citrate (R^2 = 0.842). Additionally the regressions

were plotted for the absorbances recorded in each of the two different diluents for the 36 different sperm concentrations (0.72 to 12.75 millions/ml) derived from dilution of all the 12 animals undertaken in this research. These regressions equations with best fit linear curves are presented in the figures 9, and 10. Amongst these two, the TRIS diluent yielded better results with R^2 = 0.892 (correlation coefficient r = 0.945 p<0.001) against an R^2 = 0.874 with sodium citrate diluent (correlation coefficient r = 0.935, p<0.001).

The present study was conducted to evaluate the use of a spectrophotometer for determination of sperm concentration in ram semen. Analysis of the results revealed that spectrophometric method was not only efficient, time-saving and sample-sparing but also gives an accurate measurement of sperm concentration (Dong *et al.*, 2005; Tan *et al.*, 2010; Eric *et al.*, 2012). Previous studies used wavelength range of 500-600 nm (Foote, 1978) and 546 nm (Atiq *et al.*, 2011) for cattle, 520 nm for rabbit (Castellini *et al.*, 2005), 350 nm for buffalo bull (Rehman *et al.*, 2019). Therefore sperm concentration in the present study was determined with absorbance values in the afore mentioned wavelengths. Also, in present study, highest R^2-value of 0.945 and 0.950 for 2.94% sodium citrate and TRIS, respectively was obtained at 600 nm, hence it was considered best wave length for determination of sperm concentration and there was no significant difference between the absorbance as measured by the two diluents at this particular wave length. It can be expected that the difference in wave length in present study with that of other studies is obviously due to species difference. On the other hand, haemocyometric method is time consuming, laborious and is unable to efficiently examine large number of semen samples, therefore, it is difficult to be used routinely for semen evaluation in AI laboratory (Cadena-Herrera *et al.*, 2015).

CONCLUSION

The spectrophotometric method for rapid determination of sperm concentration was optimized in TRIS and 2.94% sodium citrate diluents and compared with that of hemocytometer method for Magra ram semen. Measurement of absorbance at 600 nm wavelength, provided best coefficient of determination than at 500 and 550 nm in either of the diluents. The spectrophotometric method for determination of sperm concentration of ram semen was accurate, precise, requires limited technological skills and therefore could be successfully employed in large commercial sheep flocks for use in assisted reproductive technologies, especially artificial insemination (AI) programs.

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