Egypt. J. Chem. **Vol. 65,** No. 2 pp. 113 - 121 (2022)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Efficient comparative evaluation of spectrophotometrically determined phosphate in synthetic and in actual human embryos' culture medium

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Abstract

The determination of phosphate in human embryos' culture medium is the main targe of this manuscript using novel mixed reagents. This research also aimed to perform comparative evaluation of spectrophotometrically determined phosphate in synthetic and in actual human embryos' culture medium.The determination of phosphate in human embryos' culture medium under inestigation that used in our hospital in literature is scanty. It depents on the reaction of phosphate with mixed colorimetric reagents. Firstly, the reaction of sodium dihydrogen phosphate with ammonium molybdate was carried out in acidic solution to determine λ_{max} of the blue product formed upon reduction of phosphomolybdic acid with ascorbic acid. The molybdenum blue formed attained a λ_{max} of 825 nm and molar absorptivity of 2.190 x 10^3 L mol^{-1} cm^{-1}. Color development took more than one hour and so, potassium hydrogen tartrate was added to reduce the time of reaction. The product of the reaction in case of adding potassium hydrogen tartrate to the reagent showed a λ_{max} at 830 nm and the molar absorptivity of molybdenum blue was 2.833 x 10^4 L mol^{-1} cm^{-1}. Adding potassium hydrogen tartrate to the reagent increased the rate of reaction, but color development has been taken a long time. So, antimony potassium tartrate was added to the reagent to increase the reaction rate in order to be able to make determination at the maximum absorbance of the product and subsequently increase the sensitivity of the method. This reduced the time of reaction to 5−10 min and wavelength at the maximum absorbance was shifted to be 896 nm with molar absorptivity 1.797 x 10^4 L mol^{-1} cm^{-1}. The intensity of the blue color was proportional to the amount of phosphate initially added into the standard solutions as indicated by the absorbance readings. Tracing the reaction between sodium dihydrogen phosphate and ammonium molybdate under the optimum conditions led to the construction of a calibration curve. The concentration range of the calibration graph was found to be 3.183−17.5 µg mL^{-1} and correlation coefficient of the data obtained was 0.999. The method was applied to a synthetic mixture containing some components of the human embryos' culture medium without interference. This leads to the successful application of the suggested procedures to the spectrophotometric micro-determination of phosphate content in actual human embryos' culture medium; which was in a good matching with the data of determining phosphate in the same media in literature.

Keywords: Spectrophotometric Analysis**,** Phosphate, Molybdenum Blue, Culture Embryo Medium, Synthetic and Actual media.

1. Introduction

The role of embryo culture media in an assisted reproduction is to improve the quality of embryos developing in the laboratory [1]. The composition of embryo culture systems can be divided into the following components: Water, ions, carbohydrates, amino acids, vitamins, nucleic acid precursors, chelators, antioxidants, antibiotics, protein, hormones, growth factors, and a buffer system [2]. Inorganic salts are important component of all types of tissue culture media because they are the major components contributing towards maintenance of osmolality of the media [3]. It was reported that embryos developed in medium with phosphate concentration ranging from 0 mM to 7.2 mM [4]. It was also revealed that, the absence of phosphate in culture media containing amino acids and glucose injures the development of embryos in vitro [5]. Culture media are different in composition, and also the storage and culture affect the concentrations of their components [6]. There are several methods reported for the determination of phosphate such as the volumetric method, quinolone molybdate method which involves the formation of phosphomolybdic acid followed by its precipitation as the salt of quinolone which is titrated with sodium hydroxide [7]. Phosphate was determined by thermometric titration, the principle is based on titration of phosphate ions with magnesium nitrate

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Receive Date: 15 June 2021, Revise Date: 05 August 2021, Accept Date: 08 August 2021

DOI: 10.21608/EJCHEM.2021.80863.4006

solution to produce an insoluble precipitate at alkaline pH [8]. The ammonium phosphomolybdate method for determination of phosphate is based on the treatment of the sample at 45ºC with an excess of ammonium molybdate solution in the presence of nitric acid then, the precipitated ammonium phosphomolybdate is washed with dilute potassium nitrate solution, dissolved in an excess of standard sodium hydroxide and titrated with standard hydrochloric acid [9]. Phosphate was determined by complexometric back titration where an excess of bismuth nitrate was added to the sample which results in precipitation of bismuth phosphate and the unreacted bismuth ions are titrated against EDTA [10]. A volumetric method for analysis of phosphates was described; which depends on the conversion of phosphate to the di-acid-ortho-salt and its precipitation as silver phosphate [11]. Determination methods of phosphate have been investigated by gravimetric quimociac technique by precipitation of phosphate as quinoline molybdophosphoric acid [12]. Determination of phosphate was performed by precipitation as insoluble uranyl salt [13]. Nephelometric had been suggested by using the nephelometric precipitant; which was a nitric acid solution of strychnine and molybdic acid [14]. Fluorescence reaction between thiamine and molybdovanadophosphate was applied to a determination of phosphate by measuring the intensity of fluorophore [15]. Amperometric detection of orthophosphate was described in the literature [16]. Phosphate ion selective electrode based on a cobalt matrix has been reported [17]. Electrochemical method for phosphate determination was described by fabrication of an electrochemical cell including electrochemical three electrode system, reaction zone, inlet and outlet to detect phosphate by sequential injection analysis [13]. Electrochemical sensors were adapted for phosphate determination in water samples; the recognition elements were classified as metal based electrodes, modified electrodes with polymers, metal complexes and a biological element [18]. Indirect electrochemical sensing of orthophosphates was performed using screen-printed graphite macro-electrode [18]. A gold micro band array electrode for determination of phosphate was described; the working principle is based on the reduction of molybdophosphate complex using the linear sweep voltammetric method [19]. Detection of phosphate using amperometric was described and depends on the reduction of the phosphomolybdate complex at a carbon paste electrode versus Ag/AgCl [20]. Ion exchange methods using both carbonate / hydrogen carbonate and hydroxide selective columns in combination with self-regenerating membrane and solid-phase based suppressor enable determination of phosphate [21]. Phosphate was determined by gas chromatography via generation of phosphine from phosphate ion using sodium borohydride as a reducing agent [22]. Detergent phosphate was analyzed by ion chromatography using post-column reaction with ferric nitrate and UV detection [23]. Determination of phosphate in plant material by gas chromatography-mass spectrometry and ion chromatography was reported [24].Three techniques for the colorimetric analysis of phosphorus are described in Standard Methods: the vanado-molybdophosphoric acid method [25], the stannous chloride method [26], and the ascorbic acid method [27]. Phosphate level was evaluated in water samples by molybdenum blue phosphorus method; hydrazine sulphate was used to reduce the phosphomolybdate complex [28]. Spectrophotometric determination of phosphate in water was reported [29]. Photometric method for determination of inorganic phosphate in liquid samples which make use of acid reagent and ammonium molybdate reagent was reported [30]. Phosphate in serum was determined by photometric procedure, the method was based on deproteinization of the fresh sample by trichloro acetic acid and the reaction of the protein free filtrate with ammonium molybdate reagent to produce phosphor molybdic acid which is reduced by fresh cuprous oxide to produce a blue colored complex which was measured at 670 nm [31]. Phosphate was determined calorimetrically by using a paired-emitted detector diode using the malachite green spectrophotometric method that is based on the formation of green molybdophosphoric acid complex, the intensity of which is directly related to phosphate concentration [32]. Phosphate was determined in water by flow Coulometry based on the formation of molybdophosphate and its subsequent one-electron electrolytic reduction [33]. Nevertheless, there are few reports about determination of phosphate in human embryos' culture medium. In an attempt to analyze some of the commercially available culture media; phosphorus in the media was quantified using Roche Cobas chemistry analyzer and Roche Cobas reagents and spiked recovery of the analyte was performed [34]. Therefore it can be said that, the methods used in determination of phosphorous or phosphate in human embryos' culture medium; especially spectrometric is scanty. This encourage us to develop and suggest spectrophotometric method using novel suggested mixed reagent in phosphate content determination in human embryos' culture medium

2. Experimental

All chemicals used were of the highest purity available. They included potassium hydrogen tartrate, ascorbic acid, glycine, ammonium molybdate, glucose, and HCl which were provided by EL Nasr pharmaceutical chemicals. Potassium antimonyl tartrate was purchased from Oxford lab chemicals,

India. Tryptophan was supplied from WINLAB. Sodium pyruvate and KCl were purchased from MERCK, and sodium dihydrogen phosphate was obtained from Fluka. Human embryos' culture medium (GLOBAL, TOTAL, W/HSA) supplied from LifeGlobal.

2.1. Solutions

Ammonium molybdate solution (ammonium hepta molybdate tetra hydrate, MW=1235.86 g mol^{-1}) was prepared by dissolving 7.5045 g in 250 mL distilled water; while heating over direct flame to get 2.024 x 10^{-2} M solution and was kept in a dark bottle. To obtain 7.502 x 10^{-3} M solution of potassium hydrogen tartrate $KC_4H_5O_6$ (MW=188.177 g mol^{-1}); 0.3434 g were dissolved in 250 mL distilled water. Stock solution of 1.020 x 10^{-2} M sodium dihydrogen phosphate (MW=156.01 g mol^{-1}) was prepared by dissolving 0.3470 g of solid in the 250 mL of distilled water. The 4.04 x 10^{-4} M solution of $NaH_2PO_4.2H_2O$ was prepared by accurate dilution. Sulfuric acid solution (4.896 N) was prepared by diluting 34 mL of the concentrated acid (36 N) into 250 mL flask. A freshly prepared 25 mL of 3.009 x 10^{-1} M ascorbic acid solution (MW=176.12 g mol^{-1}) was prepared by dissolving 1.3247 g in distilled water. A solution of 7.300 x 10^{-3} M potassium antimonyl tartrate was prepared by dissolving 0.5930 g (MW=324.92 g mol^{-1}) in 250 mL of distilled water. The freshly prepared mixed reagent of components shown in Table 1 is prepared just prior to measurement.

TABLE 1. Composition of the mixed reagent used to quantify phosphate.

Reagent	Volume	Final concentration
Ammonium molybdate (2.428 x 10^{-2} M)	25 mL	4.837 x 10^{-3} M
Sulfuric acid (5.0 N)	63 mL	2.510 N
Ascorbic acid (3 x 10^{-1} M)	25 mL	5.976 x 10^{-2} M
Potassium antimonyl tartrate (7.300 x 10^{-3} M)	12.5 mL	7.271 10^{-4} M

Some components of the human embryos' culture media were selected to study the interfering possibility with the determination of phosphate using the prepared mixed reagent. Solutions of these components were prepared 10 folds more concentrated than the working phosphate solution. Table 2 shows weights needed to prepare 100 mL of 2 x 10^{-3} M solution of each component.

TABLE 2. Weights needed to prepare 100 mL of 2x 10^{-3} M mixture of each component used to study interference with phosphate determination.

Component	Weight needed (g)
Glucose	0.0360
KCl	0.0149
$NaHCO_3$	0.0168
Sodium pyruvate	0.0220
Glycine	0.0150
Tryptophan	0.0408

2.2. Tools, Equipment and Instruments

All glassware and sample containers were cleaned and dried followed by rinsing with 10% HCl and three rinses with deionized water before use. High precision micropipette (SCILOGEX) 100-1000 µL was used to get the accurate volumes.

A single beam visible spectrophotometer, model Unico 1200, equipped with a 1.0 cm glass cell was used for all spectrophotometric measurements.

2.3. Spectrophotometric Procedures

2.3a. Selection of the Suitable Wavelength (λ_{max})

The spectrum of the product of reaction between the mixed reagent (Table 1) and phosphate was checked for λ_{max}. In 10 mL volumetric flask; 2 mL of phosphate solution (2.04 x 10^{-4} M); 2 mL of the mixed reagent were added and mixed thoroughly by stirring and then, the volume was adjusted to 10 mL with distilled water. After 10 min from addition of reactants; the development of color appeared to be completed and absorption spectrum of the final solution was scanned in the wavelength region (400−1004 nm) using water as blank.

2.3b. Effect of Time on the Development of Color

The mixed reagent (2 mL) was added to 3 mL of 2.04 x 10^{-4} M phosphate solution and the volume of the mixture was diluted to 10 mL with distilled water. Absorbance at λ_{max}= 896 nm was recorded at 5 min intervals until it has almost stopped increasing for about 3 readings.

2.3c. Validity of Beer's Law

A series of 10 mL volumetric flasks were arranged. To each flask aliquots of 2.04 x 10^{-4} M phosphate solution, corresponding to 3.183−17.5 µg mL^{-1} (1.0−5.5 mL), and 2 mL of the mixed reagent were added to each flask. Then each solution was let at room temperature for 10 min. Absorbance of solutions were measured at λ_{max}= 896 nm against water as a blank. The absorbance values obtained were plotted against phosphate concentration to check the validity of Beer's law and to construct a calibration curve.

2.3d. Interference Effect

A volume of 2 mL of the mixed reagent were added to 2 mL of 2.04 x 10^{-4} M of phosphate solution in a 10 mL measuring flask. In other separate flasks; were added the same previous mixture and mixed with 2 mL of 10-folds molar excess of interfering materials (2 x 10^{-3} M of glucose, KCl, $NaHCO_3$, sodium pyruvate, glycine and tryptophan). After 10 min, the mixtures were diluted to the mark with distilled water.

The absorbance was measured at λ_{max}= 896 nm against water. The absorbance value of each mixture, containing a different interfering material, was compared with that of the mixture which does not contain any interfering component to examine the interference effect.

2.3e. Validity of Beer's Law for Determination of Phosphate in Synthetic Embryos' Culture Medium Using the Mixed Reagent

A volume of 0.5−6 mL of the synthetic mixture containing 3.183−19.1 µg mL^{-1} of phosphate in a 10-mL calibrated flask was mixed with 2 mL of the mixed reagent. After 10 min, the mixture was diluted to the mark with distilled water. The absorbance was measured at 896 nm against water as a blank. Absorbance was plotted against phosphate concentration in the synthetic mixture aliquots to check the validity of Beer's law and construct a calibration curve.

2.3f. Determination of Phosphate in Actual Human Embryos' Culture Medium by Standard Addition Method

In ten separate 10-mL volumetric flasks were transferred even aliquots of the culture medium (0.5 mL in each flask). The first flask was then diluted to the mark with distilled water. A standard containing 2.014 x 10^{-4} M of phosphate solution was then added in increasing volumes (1−5.5 mL) to the subsequent flasks and each flask was then diluted to the mark with distilled water. The absorbance was measured for all of the diluted solutions at 896 nm and the data was plotted with concentration of added standard. The concentration of analyte was determined by extending the line created down to the horizontal axis; at that point the response is zero.

3. Results and Discussion

3.1. Selection of the Suitable Wavelength (λ_{max})

Reaction product of the mixed reagent with phosphate solution was scanned against water as a blank from 400−1000 nm (Fig. 1). The resulting spectrum shows that the product attains maximum absorbance at λ_{max}= 896 nm with molar absorptivity (ε = 1.797 x 10^4 L mol^{-1} cm^{-1}).

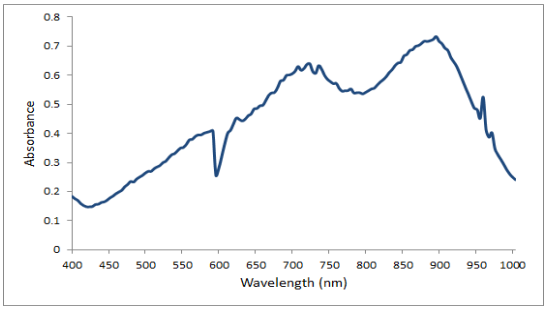

Fig. 1. Absorption spectrum of 4.08 x 10^{-5} M reduced phosphomolybdate complex formed using the mixed reagent reaction with phosphate solution; λ_{max}= 896 nm).

3.2. Effect of Time on the Development of Color

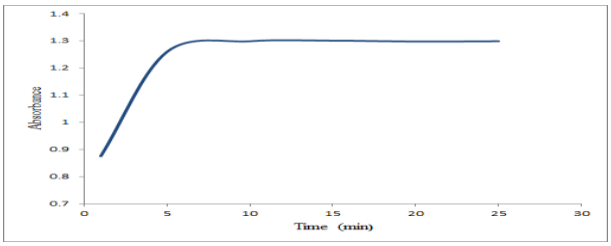

Fig. 2. Effect of time on the development of the color of the product of reaction of phosphate with the mixed reagent.

It is clear from Fig. 2 that the reaction takes 5−10 min to be completed.

3.3. Validity of Beer's Law for Micro determination of Phosphate in Pure Form Using the Mixed Reagent

Spectrophotometric determination of phosphate was carried out under the favorable conditions of reagent components concentrations, acidity and wavelength. The data obtained are shown in Table 3.

It is obvious from data presented in Table 3 that, the calibration curve is rectilinear in the concentration range of 3.183−17.5 µg mL^{-1}. Above this limit; a positive deviation is observed. The mean recovery values obtained are in the range of 97.66−104.7 %. The analytical parameters for the determination of phosphate in pure solution are listed in Table 4. The limit of detection (LOD) and quantification (LOQ) are found to be 0.0661 and 0.2002 µg mL^{-1}; respectively. The standard deviation values (SD) are in the range of 0.0005−0.01983 and the relative standard deviation values (RSD) are in the range of 0.006347−0.186614 %. The low values of the calculated standard deviation and relative standard deviation indicate the high accuracy and precision of the method. This is also supported by the calculated values of Sandell sensitivity that is 5.565 x 10^{-8} µg cm^{-2}; which indicates the high sensitivity of the method.

TABLE 3. Micro determination of phosphate in pure form using the mixed reagent.

Analyte	Weight taken (µg mL^{-1})	Weight found (µg mL^{-1})	Recovery (%)	SD	RSD (%)
Phosphate	3.183	3.334	104.7	0.003578	0.107258
	4.774	4.766	99.83	0.00866	0.1819
	6.365	6.242	98.07	0.001732	0.027744
	7.957	7.878	99.00	0.0005	0.006347
	9.548	9.706	101.65	0.018099	0.186614
	11.14	11.02	98.92	0.008367	0.075936
	12.73	12.85	100.9	0.005	0.038903
	14.32	14.32	100	0.01983	0.132214
	15.91	15.85	99.62	0.009574	0.060396
	17.5	17.09	97.66	0.009574	0.056031

TABLE 4. Analytical parameters for spectrophotometric determination of phosphate in pure form using the mixed reagent.

Analyte	Analytical Parameter	Value
Phosphate	λ_{max} (nm)	896
	[phosphate] (µg mL^{-1})	3.183− 17.5
	ε (L mol^{-1} cm^{-1})	1.797 x 10^4
	SD	0.0005− 0.01983
	RSD (%)	0.006347− 0.186614
	Sandell sensitivity (µg cm^{-2})	5.565 x 10^{-8}
	The straight line equation is Y = ax+ b	
	a	0.1787
	b	0.031
	R^2	0.999
	% recovery (%)	97.66− 104.7
	LOD (µg mL^{-1})	0.0661
	LOQ (µg mL^{-1})	0.2002

The correlation coefficient of the data obtained is found to be 0.999. Finally it is concluded that, this spectrophotometric method can be applied successfully for the determination of phosphate in the concentration range mentioned above with a high accuracy, precision and sensitivity; as indicated by the values of SD and RSD. The successful application of the mixed reagent in micro determination of phosphate in pure form encourages the use of this procedure in analysis of sodium dihydrogen phosphate in synthetic embryos' culture medium.

3.4. Interference Study

The effect of interfering materials on the efficiency of phosphate determination using the mixed reagent was evaluated and the results obtained are depicted in Table 5. The interference effect is defined as the difference in the absorbance value between the solution containing phosphate alone as a reactant and that in presence of the other medium constituents of 10-folds more concentrated than phosphate. The percent error is the difference in the absorbance value between the solution containing phosphate alone as a reactant and that in presence of the interfering materials divided by the absorbance value in presence of phosphate alone and multiplying the product by 100.

TABLE 5. Test of interference of synthetic embryos' culture medium components with the determination of [phosphate]= 4.08x 10^{-5} M. Absorbance of blank phosphate = 0.903 at λ_{max}= 896 nm.

Component	Absorbance	Interference effect	Error %
Glycine	0.908	± 0.005	0.554
Sodium pyruvate	0.907	± 0.004	0.443
Glucose	0.940	± 0.037	4.097
KCl	0.916	± 0.013	1.440
$NaHCO_3$	0.881	± 0.022	2.436
Tryptophan	0.906	± 0.003	0.332

It is clear from the data obtained (Table 5) that, no pronounced variations in the absorbance values due to the presence of other medium constituents. This non-interfering effect of embryos' culture medium constituents on phosphate determination using the mixed reagent encouraged the application of the method to determine phosphate in synthetic embryos' culture medium.

3.5. Spectrophotometric determination of phosphate in synthetic embryos' culture medium using the mixed reagent

Under the optimized experimental conditions the calibration curve of phosphate solution was constructed. The data obtained are shown in Table 6 and Fig. 3.

TABLE 6. Micro determination of phosphate in synthetic culture medium mixture using the mixed reagent.

Analyte	Weight taken (µg mL^{-1})	Weight found (µg mL^{-1})	Recovery (%)	SD	RSD (%)
Phosphate	3.183	3.197	100.4398	0.005715	0.178776
	4.774	4.857	101.7386	0.0035	0.072087
	6.365	6.402	100.5813	0.026432	0.413005
	7.957	8.09	101.6715	0.007659	0.094654
	9.548	9.625	100.8065	0.012884	0.133833
	11.14	11.19	100.4488	0.017078	0.152587
	12.73	12.75	100.1571	0.005	0.039208
	14.32	14.16	98.88268	0.05252	0.370837
	15.91	15.76	99.0572	0.092916	0.651278
	17.5	17.22	98.4	0.105987	0.611466
	19.1	18.73	98.06283	0.073485	0.392337

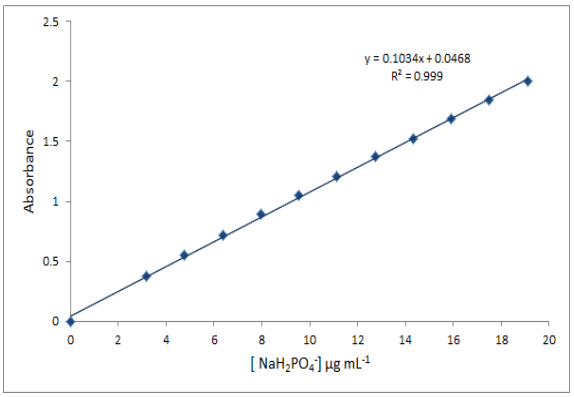

Fig. 3. Micro determination of phosphate in synthetic culture medium mixture using the mixed reagent.

The method exhibited a wide linear range (3.183−19.1 µg mL^{-1}) and the coefficient of correlation (R^2) was 0.999. The limit of detection and the limit of quantification were 0.1169 and 0.3543 µg mL^{-1}; respectively. The standard deviation values (SD) are found to be in the range of 0.0035−0.105987 and the relative standard deviation values (RSD) are 0.039208−0.651278 %. The low values of the calculated standard deviation and relative standard deviation indicate the high accuracy and precision of the method. This is also supported by the calculated value of Sandell sensitivity which is 6.150 x 10^{-8} that indicates the high sensitivity of the method. The molar absorptivity and regression equation for the analyte are listed in Table 7.

TABLE 7. Analytical parameters for spectrophotometric determination of phosphate in synthetic mixture using the mixed reagent.

Analyte	Analytical Parameter	Value
Phosphate	λ_{max} (nm)	896
	[phosphate] (µg mL^{-1})	3.183−19.1
	ε (L mol^{-1} cm^{-1})	1.626 x 10^4
	SD	0.0035−0.105987
	RSD (%)	0.039208−0.651278
	Sandell sensitivity (µg cm^{-2})	6.150 x 10^{-8}
	The straight line equation is	
	Y = ax+ b	
	a	0.1613
	b	0.0468
	R^2	0.999
	% recovery (%)	98.06283−101.7386
	LOD (µg mL^{-1})	0.1169
	LOQ (µg mL^{-1})	0.3543

All of the above data refer to a final conclusion that the method is feasible for micro determination of phosphate in synthetic embryos' culture medium.

3.6. Determination of Phosphate in Human Embryos' Culture Medium by Standard Addition Method

The values of SD and RSD obtained for micro determination of phosphate in synthetic embryos' culture medium refer to the reliability of the method to determine phosphate in the complex matrix of culture medium. Determination of phosphate in Human Embryos' Culture Medium by Standard Addition Method considered a good way to reduce the error in determination of the unknown concentration of phosphate in culture medium. It involved a modification of the standard calibration curve to deal with the low absorbance value of the analyte and the complicated matrix effect. The standard phosphate concentration of 1.639 µg mL^{-1} were added to 10 variable mL volumes of Human Embryos' Culture Medium and absorbance values of these solutions are plotted. As a result the standard addition plot as standard curve (Fig 4) intersects with the x-axis represents the concentration of phosphate (absolute value) in the 10-mL flask.

Fig. 4 Show the plot of absorbance versus concentration of added standard phosphate.

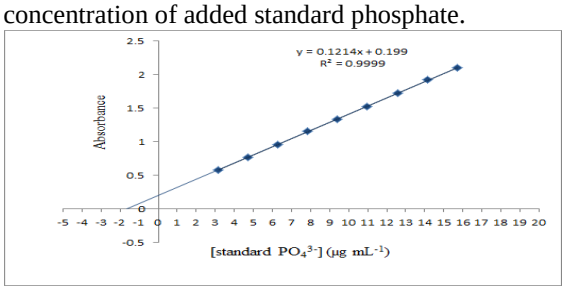

Fig. 4. Standard addition curve for determination of phosphate$^-$ in culture medium.

The phosphate concentration determined by standard addition method in actual Human Embryos' Culture Medium is found to be 32.78 µg mL^{-1}; which compared well with the average value of determining phosphate in the embryos' culture medium (35.99 µg mL^{-1}) which was previously published [35]. This is matching the data of determining phosphate in the same media in literature.

3.7. *Statistical evaluation of the obtained data by F-test*

The statistical evaluation of the obtained data by F-test is presented in Table 8.

Table 8. Statistical evaluation of the obtained data by F-test

	average	N	SD
In pure form	33.36	5	0.0040
In synthetic mixture	31.97	5	0.00572
In actual medium	30.68	5	0.00420
In literature	35.99 (35)	5	0.00420

The standard deviations of the results of the application on the actual media and the determination of phosphate in the synthetic mixture were 0.0304 and 0.0367 respectively (each obtained from five measurements).

$F = 0.0367^2 / 0.0304^2 = 1.457$

Taking the numbers of degrees of freedom of both numerator and denominator as 4, the critical value is $F_{4,4} = 9.605$. The calculated value is less than the tabulated one, so there is no significant difference between the two variances at the 5% level.

The standard deviations of the results of the application on the actual media and the determination of phosphate in the synthetic mixture were 0.00420 and 0.00572 respectively (each obtained from five measurements).

$F = 0.00572^2 / 0.00420^2 = 1.854$

Taking the numbers of degrees of freedom of both numerator and denominator as 4, the critical value is $F_{4,4} = 9.605$ (36). The calculated value of 1.854 is less than the tabulated value of 9.605, so there is no significant difference between the two variances at the 5% level.

The standard deviations of the results of the application on the actual media and the determination of phosphate in pure form were 0.00420 and 0.004 respectively (each obtained from five measurements).

$F = 0.00420^2 / 0.004^2 = 1.103$

The critical value is $F_{4,4} = 9.605$ (36). The calculated value of 1.103 less than the tabulated value of 9.605, so there is no significant difference between the two variances at the 5% level.

The standard deviations of the results of the application on the actual media and the determination of phosphate in previously published paper (35) were 0.00420 and 0.00430 respectively (each obtained from five measurements).

$F = 0.00430^2 / 0.00420^2 = 1.048$

The calculated value of 1.048 is less than the tabulated value of 9.605 (36), so there is no significant difference between the two variances at the 5% level.

Finally, checking the accuracy of the mixed reagent method in determination of phosphate by F-test shows the high precision of the method in determination of phosphate in actual culture medium and the good correlation between the results of the mixed reagent method and the published method.

4. Conclusions:

The determination of phosphate in human embryos' culture medium is the main targe of this manuscript using novel mixed reagents. This research also aimed to perform comparative evaluation of spectrophotometrically determined phosphate in synthetic and in actual human embryos' culture medium.The determination of phosphate in human embryos' culture medium under inestigation that used in our hospital in literature is scanty. It depents on the reaction of phosphate with mixed colorimetric reagents. The method was applied to a synthetic mixture containing some components of the human

embryos' culture medium without interference. This leads to the successful application of the suggested procedures to the spectrophotometric micro-determination of phosphate content in actual human embryos' culture medium; which was in a good matching with the data of determining phosphate in the same media in literature.

5. Acknowledgement

Thanks are acknowledged to Chemistry Department, Faculty of Science, and Cairo University who give a support to this research with chemical, Lab instruments and measurements. Thanks are present to Adam International Hospital of Human Fertility, Giza, Egypt, who provided us with Actual human embryos' culture medium and permits successful applications of suggested and tested spectrophotometric method**.**

Conflict of Interest: All authors are declared that there is no conflict of interest.

Role of Authors: Prof. Mohamed A. Zayed supervised the whole work presented in this manuscript revised its whole content and follow its submission to the journal. **The Ph.D. student Mohamed Mustafa Taha did the whole work in lab and Hospital applications. Tabulate the results and wrote the draft of the whole text.**

References

1. Gruber I, Klein M. Embryo culture media for human ivf: Which possibilities exist? J Turkish Ger Gynecol Assoc. 2011;
2. Gardner D. In vitro fertilization: a practical approach. 2007; Available from: http://evilevil.pixub.com/o/in-vitro-fertilization-a-practical-approach-by-david-k-gardner.pdf
3. Mehta RH. Growth of human preimplantation embryos in vitro. Reprod Biomed Online [Internet]. 2001;2(2):113–9. Available from: http://dx.doi.org/10.1016/S1472-6483(10)62235-3
4. Wales RG. Effects of ions on the development of the pre-implantation mouse embryo in vitro. Aust J Biol Sci. 1970;23(2):421–30.
5. Reed L, Gardner DK. Phosphate-Free Culture Media Impairs ICM Development and Alters Blastocyst Metabolism. Fertil Steril. 2005;
6. Tarahomi M, Vaz FM, van Straalen JP, Schrauwen FAP, van Wely M, Hamer G, et al. The composition of human preimplantation embryo culture media and their stability during storage and culture. Hum Reprod. 2019;
7. Hoffman WM, Ferretti RJ, Breen HJ. Quinoline Molybdate Method for the Determination of Water-Soluble and Available Phosphorus in Fertilizers. J AOAC Int [Internet]. 1963 Aug 1 [cited 2021 Feb 14];46(4):570–9. Available from: https://academic.oup.com/jaoac/article/46/4/570-579/5732070
8. Determination of phosphate by magnesium titration [Internet]. [cited 2021 Feb 11]. Available from: https://www.metrohm.com/en-au/applications/AN-H-008
9. of Indian Standards B. IS 5305 (1969): Method for volumetric determination of phosphorus.
10. Phosphate Determination by Complexometric Back Titration [Internet]. [cited 2021 Feb 11]. Available from: https://www.mt.com/hk/en/home/supportive_content/ana_chem_applications/titration/M682.html
11. Cullum DC, Thomas DB. The volumetric analysis of phosphates. Anal Chim Acta. 1961 Jan 1;24(C):205–13.
12. Shaver LA. Determination of phosphates by the gravimetric quimociac technique. J Chem Educ [Internet]. 2008 [cited 2021 Feb 11];85(8):1097–8. Available from: https://pubs.acs.org/doi/abs/10.1021/ed085p1097
13. Lewis DT. The gravimetric determination of phosphate and vanadate. Analyst [Internet]. 1940 Jan 1 [cited 2021 Feb 11];65(775):560–1. Available from: https://pubs.rsc.org/en/content/articlehtml/1940/an/an9406500560
14. Kobbr PA, Eobrbr G. Nephelometric estimation of phosphorus. J Am Chem Soc. 1915;
15. Kishida M, Aoki T. Determination of Phosphate Utilizing Fluorescent Reaction of Thiamine with Molybdovanadophosphate by Flow Injection Analysis. undefined. 1998;
16. Harden SM, Nonidez WK. Determination of Orthophosphate by Flow Injection Analysis with Amperometric Detection. Anal Chem [Internet]. 1984 [cited 2021 Feb 8];56(12):2218–23. Available from: https://pubs.acs.org/doi/abs/10.1021/ac00276a053
17. Xiao D, Yuan HY, Li J, Yu RQ. Surface-Modified Cobalt-Based Sensor as a Phosphate-Sensitive Electrode. Anal Chem. 1995;
18. Forano C, Farhat H, Mousty C. Recent trends in electrochemical detection of phosphate in actual waters. Vol. 11, Current Opinion in Electrochemistry. Elsevier B.V.; 2018. p. 55–61.

19. Wang F, Tong J, Li Y, Bian C, Sun J, Xia S. An electrochemical microsensor based on a AuNPs-modified microband array electrode for phosphate determination in fresh water samples. Sensors (Switzerland) [Internet]. 2014 Dec 19 [cited 2021 Feb 11];14(12):24472–82. Available from: /pmc/articles/PMC4299121/
20. Calvo Quintana J, Idrissi L, Palleschi G, Albertano P, Amine A, Rhazi M El, et al. Investigation of amperometric detection of phosphate Application in seawater and cyanobacterial biofilm samples. Talanta. 2004;63:567–74.
21. Ruiz-Calero V, Galceran MT. Ion chromatographic separations of phosphorus species: A review. Vol. 66, Talanta. Elsevier; 2005. p. 376–410.
22. Hashimoto S, Fujiwara K, Fuwa K. Determination of Phosphate Ion by Gas Chromatography with the Phosphine Generation Technique. Anal Chem [Internet]. 1985 Jun 1 [cited 2021 Feb 13];57(7):1305–9. Available from: https://pubs.acs.org/doi/abs/10.1021/ac00284a030
23. Chester TL, Smith CA, Culshaw S. Note Determination of inorganic phosphates in detergents by high-perform-ance liquid chromatography on PRP-1 with phosphorus-selective de-tection. Vol. 287, Journal of Chromatography. 1984.
24. Smillie RH, Grant B, Cribbes RL. Determination of phosphate and phosphite in plant material by gas chromatography-mass spectrometry and ion chromatography. J Chromatogr A. 1988 Jan 1;455(C):253–61.
25. Lim S. Determination of phosphorus concentration in hydroponics solution. Methodology. 1991;1–4.
26. Harvey HW. The estimation of phosphate and of total phosphorus in sea waters. J Mar Biol Assoc United Kingdom. 1948;27(2):337–59.
27. Murphy J, Riley JP. A modified single solution method for the determination of phosphate in natural waters. Anal Chim Acta. 1962;
28. Oladeji S, Adelowo F, Odelade K. Evaluation of Phosphate Level in Water Samples (Ogbomoso Rivers) Using UV-Visible Spectrophotometric Method. Int J Sci Res Environ Sci. 2016 Apr 1;4(4):102–8.
29. Yogendra Kumar MMS, Abdul Galil MS, Suresha MS, Sathish MA, Nagendrappa G. A simple spectrophotometric determination of phosphate in sugarcane juices, water and detergent samples. E-Journal Chem. 2007;
30. Hahn BA, Kaufman RA, Wesolowski AF. United States Patent (19) Hahn et al. 54 PHOTOMETRIC METHOD FOR THE DETERMINATION OF INORGANIC PHOSPHATE IN LIQUID SAMPLES. 1983 Dec.
31. Determination of serum inorganic phosphate by a new photometric procedure [Internet]. [cited 2021 Feb 14]. Available from: https://www.researchgate.net/publication/288565099_Determination_of_serum_inorganic_phosphate_by_a_new_photometric_procedure
32. O'Toole M, Lau KT, Shepherd R, Slater C, Diamond D. Determination of phosphate using a highly sensitive paired emitter-detector diode photometric flow detector. Anal Chim Acta. 2007 Aug 10;597(2):290–4.
33. Manová A, Beinrohr E. Determination of phosphate in water by flow coulometry. Acta Chim Slovaca [Internet]. 2020 Nov 6 [cited 2021 Feb 14];13(1):102–7. Available from: www.istran.sk
34. Morbeck DE, Krisher RL, Herrick JR, Baumann NA, Matern D, Moyer T. Composition of commercial media used for human embryo culture. Fertil Steril. 2014;
35. Morbeck DE, Krisher RL, Herrick JR, Baumann NA, Matern D, Moyer T. Composition of commercial media used for human embryo culture. Fertil Steril [Internet]. 2014 [cited 2021 Apr 5];102(3). Available from: https://pubmed.ncbi.nlm.nih.gov/24998366/
36. James N. Miller, Jane Charlotte Miller, Statistics and Chemometrics for Analytical Chemistry,Pearson/prentice Hall 2005, Science-268 pages