Journal of Pharmaceutical and Applied Chemistry
An International Journal

http://dx.doi.org/10.18576/jpac/040101

Spectrophotometric Determination of Glucose in Pure Form and in Human Embryos' Culture Medium Using Selective Reagent via Studying Their Reaction product

M. A. Zayed[*] *and M. M. Taha*

Chemistry Department, Faculty of Science, Cairo University, 12613 Giza A.R. Egypt.

Received: 2 Nov. 2017, Revised: 20 Dec. 2017, Accepted: 22 Dec. 2017.
Published online: 1 Jan. 2018.

Abstract: Simple, rapid and sensitive spectrophotometric method is developed for the determination of glucose in pure form and in human embryos' culture medium. This method is based on the reduction of Triphenyltetrazolium chloride (TTC) by glucose in 4.0 mM NaOH medium to produce a colored stable precipitate. The product was dissolved in 35−45 % ethanol and absorbance was measured at 480 nm. All experimental conditions affecting the development and stability of color were carefully studied and optimized. The calibration graphs are rectilinear in the concentration range of 14.59−29.19 µg $mL^{-1}$for glucose in pure form and of 18.16−39.95 µg mL^{-1} for glucose in a synthetic culture medium mixture. Sandell sensitivity (S), molar absorptivity (ε), correlation coefficient (R2), regression equation, limit of detection (LOD), and limit of quantification (LOQ) are calculated in both cases. The low values of standard deviation (0.0173−0.3958) for glucose in pure form and (0.1440−0.5430) for glucose in the synthetic culture medium mixture, and the relative standard deviation (0.0619−2.009 %) for glucose in pure form and (0.4920−2.750 %) for glucose in the synthetic culture medium mixture reflect the accuracy and precision of the proposed method. The method was applied for the determination of glucose in human embryos' culture medium; which contains glucose as one of its main components. The recovery values, standard deviation and relative standard deviation of glucose determination in the actual human embryos' culture medium are found to be 100.0 %, 0.1891 and 0.3885 %, respectively. The formed reduction product was extracted in chloroform. The proposed reaction pathway of glucose with TTC reagent was postulated and confirmed by separation of the reaction product which was proved to be 1,3,5-triphenylformazan (TPF). The structure of the separated reaction product was confirmed by elemental analysis, FT-IR, and ^{1}H-NMR.

Keywords: Spectrophotometry; Glucose; Human embryos' culture medium; Triphenyltetrazolium chloride; Oxidation-reduction reaction.

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1 Introduction

The high levels of glycol sis exhibited by the mammalian blast cyst have been interpreted as the embryo's adaptation to its imminent invasion of the endometrial; which through histology has been shown to remain a vascular for a period of up to 12 h, and will therefore be relatively anoxic[1,2]. Subsequently, glycol sis will be the sole means of generating energy at this time. However, this may not be the sole explanation for the high levels of glycolysis in the blastocyst. An alternative explanation for the metabolism of glucose by the blastocyst is that as well as being used to generate energy for blastocoel expansion and mitosis, glucose will be required for the synthesis of triacylglycerol and phospholipids, and as a precursor for complex sugars of mucopolysaccharides and glycoproteins. All of these are required by rapidly dividing cells [3–5]. Glucose metabolized by the pentose phosphate pathway (PPP) generates ribose moieties required for nucleic acid synthesis, and the NADPH (reduced nicotinamide adenine dinucleotide phosphate) required for the biosynthesis of lipids and other complex molecules [4, 6]. NADPH is also required for the reduction of intracellular glutathione, an important antioxidant for the embryo [7]. The synthesis of nucleic acids is therefore an important biosynthetic role for glucose in the blastocyst. It has been proposed that high levels of aerobic glycolysis, such as that observed in the mammalian blastocyst, will ensure that there is sufficient substrate available for biosynthetic pathways, such as DNA replication, RNA transcription and synthesis of new membranes, at the required times during cellular

[*] Corresponding author E-mail: mazayed429@yahoo.com

proliferation[8,9]. This in turn suggests that there are times within the cell cycle during which the PPP is more active than others.

The capacity to metabolize glucose increases significantly at the transition from the morula to blastocyst stage and also seems to reflect the embryo's developmental potential and viability. Glucose uptake by microfluorescence in day 4 mouse blastocysts was measured prior to embryo transfer [10]. Embryos that resulted in term pregnancies had a significantly higher glucose uptake in culture than embryos that failed to progress. It was also reported that the glucose consumption by day 4human embryos was higher in embryos that formed blastocysts and correlated with morphologic grade [10]. Meanwhile, several other studies failed to demonstrate a relationship between glucose uptake and blastocyst development in human embryos. It is noteworthy that the media used to assess embryo metabolism in these studies lacked pyruvate, lactate, amino acids and vitamins. Thus, it is possible that there was significant stress on the embryos under these culture conditions [11] and the conclusions drawn may consequently have been limited by this factor.

To investigate complex metabolic profiles of a biological system, non-selective, but specific analytical technologies are required. Many spectroscopic, spectrometric and chromatographic techniques are excellent candidates and provide automated, high-throughput methodologies with information-rich profiles of target biological fluids. Investigators have coined the term ‘bio-spectroscopy’ to designate the analysis of biological fluids by spectroscopic technologies. The scientific platform that incorporates both metabolic profiling and bio-spectroscopy can be referred to as ‘bio-spectroscopy based metabolomics’ or ‘BSM’. Common techniques applied in BSM studies include NMR spectroscopy, mass spectrometry (MS), which can be coupled with separation methods like gas chromatography (GC-MS), liquid chromatography (LC-MS) or HPLC-MS, and capillary electrophoresis (CE-MS).Optical spectroscopies, like Fourier transform infrared (FT-IR), near infrared (NIR) and Raman spectroscopies all provide complementary profiles of the various components within biological fluids due to the similar physical mechanisms involved in each technique. Therefore in the present paper simple, rapid and sensitive spectrophotometric method is developed for the determination of glucose in pure form and in human embryos' culture medium as an alternative to the above mentioned sophisticated tools.

2 Experimental

2.1 Materials and Reagents

All chemicals used were of the highest purity available. They included glucose provided by El Nasr Pharmaceutical Chemicals Co., Egypt; 95 % ethanol was purchased from ADWIC, and Chloroform was supplied from United Company for Chem., Egypt. Potassium chloride; sodium pyruvate, and sodium hydroxide pellets were supplied from MERCK, Germany. Sodium bicarbonate was provided by El Gomhouria Co., Egypt. 2, 3, 5-triphenylTetrazlouim chloride (TTC) was supplied from Sisco research laboratories, India.

2.2 Solutions

A solution of 1.013x 10-3 M glucose was prepared by dissolving 0.0456 g of solid in 250 mL distilled water. Stock solution of 2.156 x 10-3 M 2,3,5-triphenyl Tetrazlouim chloride (TTC) was prepared by dissolving 0.1805 g in 250 mL distilled water. 1.0 x 10-4 M stock solution of synthetic culture medium mixture was prepared by accurately weighing out the calculated weights of glucose, sodium pyruvate, Gly, sodium bicarbonate, and KCl and dissolving them in 250 mL of distilled water. Human embryos' Culture medium used was Quinn's advantage protein plus blastocyst medium from sage In-Vitro Fertilization, Inc, Trumbull, CT06611 U.S.A.Water used in the research was obtained by distillation from all glass equipment. All glassware used were washed carefully with distilled water and dried in the oven before use. Diluted solutions were prepared by accurate dilution from the stock solutions to get the desired concentrations. Solutions were protected from light by keeping them in dark coloured quick fit bottles during the whole work.

2.3 Instruments

Spectrophotometric measurements were carried out using the automatic Optizen pop spectrometer (Mecasys Co., Ltd/ made in Korea) and 1.0 cm optical length quartz cell. FT-IR was performed at the Microanalytical Center, Cairo University. H1-NMR was performed in the NMR laboratory at the department of chemistry, Cairo University. Elemental analysis (C, H and N) was performed at the Microanalytical Center, Cairo University using a CHNS-932 (LECO) Vario elemental analyzer. Small volumes (0.01−1.0 mL) were transferred using automatic pipette (Accupipette; USA.)

2.4 Selection of the Suitable Wavelength (λ_{max})

0.5 mL of TTC (0.9916x 10-2 M) was mixed with 0.5 mL of 0.1 M NaOH; then 0.5 mL of glucose (0.9885x 10-2 M) was added. This mixture, in a test tube, was boiled in water bath for 20 min let to cool to the room temperature. Ethanol was added to dissolve the precipitated product which was transferred to a 10-mL capacity measuring flask. The final mixture was 4-folds diluted with ethanol and the absorption spectrum was scanned in the wavelength range of 400−580

nm against ethanol as a blank.

2.5The Molar Ratio Method (mrm)

In molar ratio method [12], to 2.0 mL of (0.9885x 10-2 M) glucose solution, 1.0 mL of 0.1 M NaOH was added; followed by 0.5−6.0 mL of TTC reagent (1.008x 10-2 M) in different numbered test tubes. These mixtures were boiled in water bath for 20 min; cooled for 40 min to reach room temperature. The least amount of ethanol was added to each mixture to dissolve the product. The mixtures were transferred to measuring flasks and volumes were completed to 10 mL. The absorbance was measured against ethanol as a blank. The glucose: TTC ratio was plotted against absorbance values at the previously selected λmax = 480 nm to check the molar ratio of reactants.

2.6 The Continuous Variation Method

In continuous variation method [13] a series of solutions were prepared by adding different volumes of glucose (0.9885x 10-3 M) to TTC (0.9916x 10-3 M); so that the total number of moles were kept constant. To each mixture was added, 0.5 mL of 0.1 M NaOH and the mixtures, in different test tubes, were boiled in water bath for 20 min. Then, the mixtures were left for 40 min to cool to room temperature. Ethanol was added to each mixture in a measuring flask to complete the volume to 10 mL. The absorbance data obtained at λmax= 480 nm, against ethanol as a blank, were plotted against mole fraction of reactants to check the ratio.

2.7 Validity of Beer's Law

2.0 mL of TTC (2.156x 10-3 M) were mixed with different volumes (0.8−2 mL) of glucose (1.013x 10-3 M) in different numbered test tubes. To each tube was added 0.5 mL of NaOH (0.1 N). The mixtures were boiled in water bath for 20 min and were left to cool to room temperature for 40 min. The content of each tube was transferred quantitatively to 10-mL capacity measuring flask and the volume was completed to 10 mL with ethanol. Absorbance was measured at λ_{max} = 480 nm against ethanol as a blank. Absorbance values were plotted against glucose concentration to construct a calibration curve; to check the validity of Beer's law.

2.8 Determination of Glucose in the Synthetic Mixture

A synthetic mixture (Table 1) was prepared by accurately weighing out the amounts of solids and dissolving them in the least amount of distilled water, then completing the volume to 250 mL using a measuring flask. 1.0 mL of TTC (0.9953x 10^{-3} M) was mixed with different volumes of this synthetic mixture in different numbered test tubes. To each tube was added 0.5 mL of 0.1 N NaOH. The mixtures were boiled in water bath for 20 min, and then were left to cool to room temperature for 40 min. Each tube was transferred quantitatively to 10-mL capacity measuring flask and the volume was completed to 10 mL with ethanol. Absorbance values were measured at λmax= 480 nm against ethanol as a blank. From these values, concentrations of glucose in the synthetic mixture can be calculated. Constituents of the synthetic medium are given in Table 1.

Table1. Synthetic mixture used in microdetermination of glucose in matrix.

Component	Concentration (M)
Glucose	$0.9885x10^{-3}$
KCl	$1.032x10^{-2}$
$NaHCO_3$	$1.004x10^{-2}$
Sodium pyruvate	$1.00x10^{-2}$
Gly	$1.003x10^{-2}$

2.9 Absorption Spectrum of the Product of Reaction between TTC and Actual Human Embryos' Culture Medium

1.0 mL of TTC (0.9953 x 10^{-3} M) was added to 1.0 mL of the culture medium in a test tube; followed by the addition of 1.0 mL of 0.1 M NaOH. The mixture was boiled in water bath for 20 min then was left for 40 min to cool to reach room temperature. After the careful transfer of the mixture to measuring flak; the volume was completed to 10 mL with ethanol and the absorption spectrum was scanned against ethanol as a blank. This was done to check the agreement of λ_{max} at which analysis of actual human embryos' culture medium can be done with that previously selected for pure glucose.

2.10 Microdetermination of Glucose in the Human Embryos' Culture Medium by Using TTC at λ_{max}= 485 nm

To 1.0 mL of the culture medium; 1.0 mL of TTC (0.9953x 10^{-3} M) and 1.0 mL of 0.1 M NaOH were added in a test tube. This mixture was boiled in water bath for 20 min, and was left to cool at room temperature for 30 min. After that, the mixture was carefully transferred to a measuring flask; the volume was completed to 10 mL with ethanol and the absorbance was measured at λ_{max} = 485 nm against ethanol

as a blank. The concentration of glucose in the human embryos' culture medium was calculated from the previously constructed calibration curve. This was repeated in 10 replicates for statistical calculations.

2.11 Isolation and Structure Investigation of Glucose-TTC Reaction Product

40 mL of TTC (1.0x 10^{-2} M) were mixed with 40 mL of 0.1 M NaOH; then 40 mL of glucose (1.0 x 10^{-2} M) was added. This mixture, in a beaker, was boiled on direct flame for 20 min then cooled to room temperature. Excess chloroform was added to dissolve the precipitated product which was transferred to a separation funnel to extract the product. The reaction product melting point was measured and elemental analyses, FT-IR, ^{1}H-NMR of this product were performed in the Microanalytical center, Cairo University.

3 Results and Discussion

3.1 Spectrophotometric Study in Solution

3.1.1 Selection of the Suitable Wavelength (λmax)

The absorption spectrum of the product of reaction between glucose and Triphenyl Tetrazlouim chloride (TTC) in ethanol was scanned against ethanol (35−40 %) as a blank from 400 to 580 nm (Fig. 1).

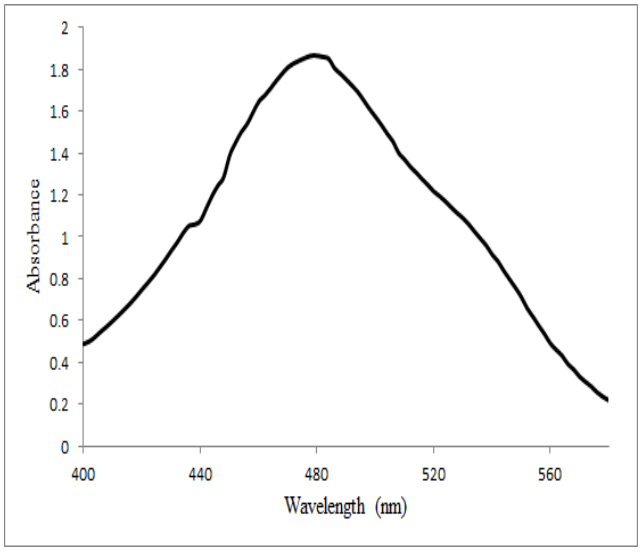


Fig.1 Absorption spectrum of the product of reaction between glucose and TTC in 35−45 % ethanol and [NaOH] = 4.0 x 10^{-3} M.

It is clear from this figure that the formed product attains a maximum absorbance at λ_{max}= 480 nm.

3.1.2. Stoichiometry of the Reaction between Glucose and TTC

The nature of the reduction of TTC by glucose in presence of sodium hydroxide is determined by the molar ratio and the continuous variation methods [12, 13] to check the ratio between TTC and glucose in order to select the optimum conditions for glucose microdetermination.

These results indicate that 1:1 [TTC]: [glucose] is the stoichiometric ratio of reaction through reduction of TTC by glucose in alkaline medium. This is also clarified by the proposed scheme 1.

The proposed scheme of glucose reaction with TTC reagent was confirmed by separation of the reaction product; which is supposed to be 1, 3, 5-triphenylformazan.

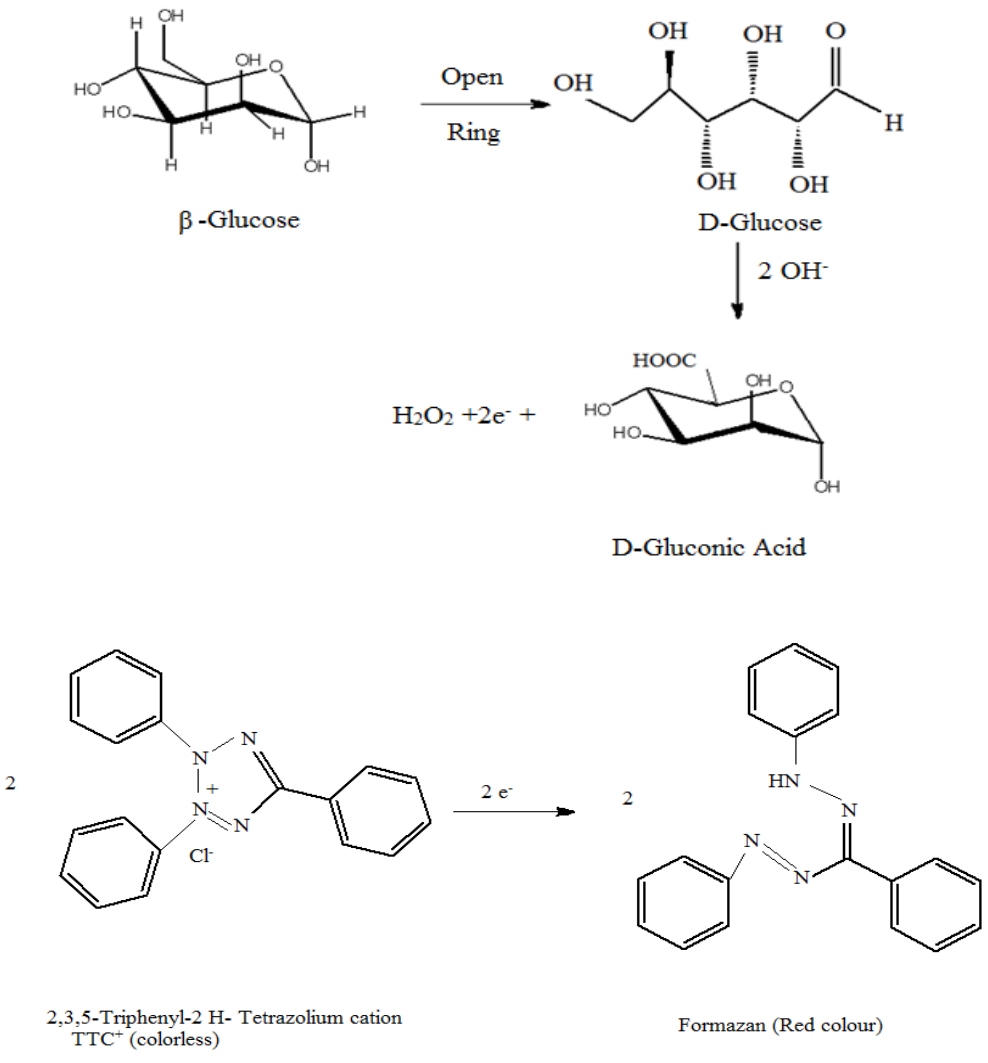
3.1.3 Micro Determination of Glucose in Pure form Using TTC

Spectrophotometric determination of glucose was carried out under the favorable conditions of basicity, reagent concentration, time, temperature, stoichiometric ratio, wavelength and ethanol as a solvent. The data obtained are shown in Table 2.

It is obvious from Fig. 2 that the calibration curve is rectilinear in the concentration range of 14.59−29.19 μg mL^{-1}. Above this limit, a negative deviation is observed. The mean recovery values obtained are in the range of 98.77−101.0 %.The analytical parameters for the determination of glucose are listed in Table (3).The limits of detection (LOD) and quantification (LOQ) are found to be 0.4641 and 1.407 μg mL^{-1}, respectively. The standard deviation values (SD) are found in the range of 0.0173−0.3958 and the relative standard deviation values (RSD) are in the range of 0.0619−2.009.The low values of the calculated standard deviation and relative standard deviation indicate the high accuracy and precision of the proposed method. This is also supported by the calculated values of Sand ell sensitivity that is 1.015x 10^{-7} μg cm^{-2} which indicates the high sensitivity of the method.

Analytical parameters deduced from the calibration curve (Fig. 2) such as molar absorptive and regression equation for glucose determination are tabulated in Table 3.

The correlation coefficient of the data obtained is found to be 0.9993. Finally it is concluded that, this spectrophotometric method can be applied successfully for the determination of glucose in the concentration range mentioned above with high accuracy, precision and sensitivity as indicated by the values of SD and RSD. The successful application of TTC reagent in micro determination of glucose in pure form supported using this procedure in analysis of glucose in synthetic and actual embryos' culture medium.



Scheme 1. Proposed pathways of oxidation-reduction reaction between glucose and TTC reagent

Table2. Microdetermination of glucose in pure form using TTC

Glucose weight taken (µg mL^{-1})	Glucose weight found (µg mL^{-1}) (*)	Recovery (%)	SD	RSD (%)
14.59	14.74	101.0	0.1386	0.9504
18.24	18.18	99.67	0.3958	2.009
21.89	21.62	98.77	0.0751	0.3700
23.71	23.78	100.3	0.0231	0.0972
25.54	25.57	100.1	0.0635	0.2454
27.36	27.37	100.0	0.0675	0.2463
29.19	29.25	100.2	0.0173	0.0619

*Average value of five replicates
The found glucose weight values are calculated from the standard calibration curve (Fig.6).

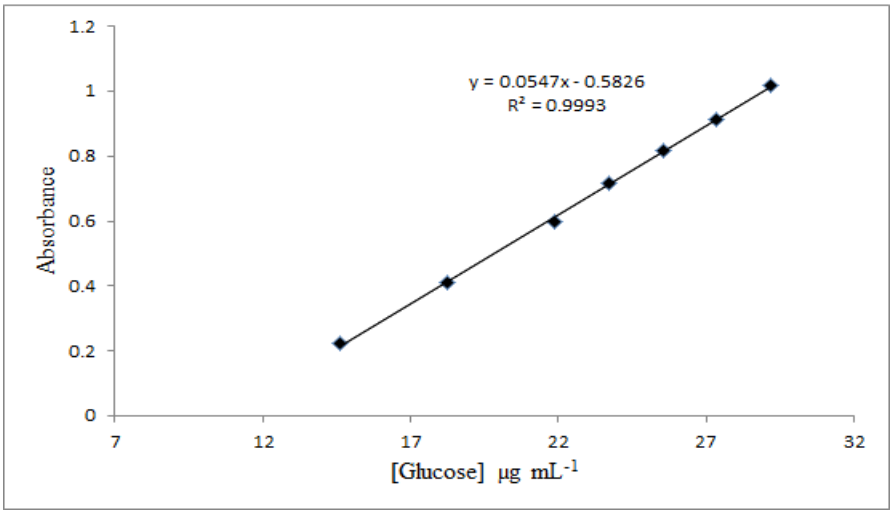


Fig2. Microdetermination of glucose in pure form using TTC.

Table3. Analytical parameters for micro-determination of glucose in pure form using TTC.

Analytical Parameter	Value
λ_{max} (nm)	480
[glucose] (µg mL^{-1})	14.59−29.19
ε (L mol^{-1} cm^{-1})	0.9855x 10^4
SD	0.0231−0.3958
RSD (%)	0.0619−2.009
Sandell sensitivity (µg cm^{-2})	1.015x 10^{-7}
The straight line equation is Y = ax+ b, a	0.0985
b	-0.5832
R^2	0.9993
Recovery (%)	98.77−101.0
LOD (µg mL^{-1})	0.4641
LOQ (µg mL^{-1})	1.407

3.1.4 Test of Interference of Embryos' Medium Components on Determination of Glucose in the Synthetic Medium Matrix

The effect of various components of the synthetic embryo culture medium on the determination of glucose was studied. Microdetermination of glucose using TTC reagent in presence of different concentrations of interfering materials, ranging from one to ten times more than glucose, was done. This was performed by observing the change in the absorbance value due to interferents' abundance. The concentration of glucose during each measurement was maintained at a fixed value of 0.9885×10^{-4} M (17.81 µg mL^{-1}). The absorbance value of the blank sample, glucose without any interfering material, was taken as the average of triplicates. The results obtained are shown in Table 4.

It is clear from the data obtained that, no pronounced variation in absorbance value (absorbance = 0.585 at λ_{max} = 480 nm, in presence of glucose alone as a reactant) due to the presence of the other medium constituents of 1 to 10 times concentration more than glucose.

This non-interfering effect of embryos' culture medium constituents on glucose determination using TTC reagent supported its use in glucose analysis in this synthetic medium.

3.1.5 Spectrophotometric Determination of Glucose in the Synthetic Embryos' Culture Medium Using TTC

Spectrophotometric determination of glucose is carried out under the favorable conditions of basicity, reagent concentration, time, temperature, stoichiometric ratios, wavelength and ethanol as a solvent. The data obtained are shown Table 5.

The limits of detection (LOD) and quantification (LOQ) are found to be 2.700 and 8.200 µg mL-1, respectively. The standard deviation values (SD) are found in the range of 0.1440−0.5430 and the relative standard deviation values (RSD) are in the range of 0.4920− 2.750 %.The low values of the calculated standard deviation and relative standard deviation indicate the high accuracy and precision of the proposed method to determine glucose in synthetic embryo medium using TTC reagent under favorable conditions. This is also supported by the calculated values of Sandell sensitivity that is 1.509x 10-7 µg cm-2 which indicates the high sensitivity of the method to determine glucose in synthetic embryo medium using TTC reagent. This successful application promoted the use of TTC in glucose analysis in actual embryo medium.

3.1.6 Spectrophotometric Determination of Glucose in Human Blastocyst culture Medium Using TTC Reagent

The spectral measurement refers to the possible application of the proposed procedure at λ_{max}= 485 nm for glucose determination in actual embryos' culture medium under the selected proper conditions. The data obtained of glucose determination in 1.0 mL of actual embryos' culture medium repeated 10 times (each one are of five replicates at different days) are shown in Table 7 in which the calculated weight taken of glucose is 48.88 µg mL^{-1}.

Table 7 shows the found glucose concentration from 47.01 to 50.29 µg mL^{-1} determined in actual embryos' culture medium using TTC reagent under the favorable conditions. These found values are obtained from the measured absorbance values and using the calibration curve of synthetic mixtures (Fig. 7). The obtained values of SD= 0.1891 and RSD= 0.3885 % refer to high accuracy and precision of the successful application of TTC reagent in determination of glucose in actual human embryos' culture medium for the first time using very cheap visible spectral technique without interference of other constituents of this medium.

3.2 Spectroscopic Studies of Reaction Product

The structure of the separated reaction product was confirmed by elemental analysis, FT-IR, and ^{1}H-NMR.

3.2.1 Elemental Analysis of Glucose-TTC Redox Reaction Product

The isolated solid product has a red color and is characterized using elemental analysis which indicates the formation of 1:1 [glucose]: [TTC] with calculated C % = 76, H % = 5.333, N % = 18.67 and found C % = 76.51, H % = 5.10 and N % = 18.42. These data obtained are calculated proposing that this product is 1, 3, 5-triphenyl formazan (TPF) of general formula C19H16N4 and M.Wt. = 300 g/mol.

3.2.2 UV-Visible Spectrum of Tetrazlouim Formazan

The spectra of triarylformazans exhibit four distinctive absorption bands (A, B, C, and D), one in the visible and the others in the UV range [14]. The first band (A) observed in the wavelength range of 216−239 nm is assigned to that of the phenyl moiety. The second band (B, 240−285 nm) is attributed to the low energy π-π* transition of the phenyl moiety. The third band (C) within the 300−350 nm range and the sharp peak is due to the π-π* transition within the hydrogen chelate ring formed by the

Table4. Test of interference of embryos medium components on the determination of [glucose] = 0.9885 x 10^{-4} M; Absorbance = 0.585 at λ_{max} = 480 nm.

Interfering components	Molarity x10^{-3} M	Absorbance	Interference effect
Sodium pyruvate	1.0	0.427	± 0.04
Glycine	0.1	0.501	± 0.034
KCl	0.1	0.403	± 0.064
$NaHCO_3$	0.1	0.308	± 0.159

Table5. Microdetermination of glucose in the synthetic mixture using TTC at λ_{max} = 480 nm.

Glucose weight taken (µg mL^{-1})	Glucose weight found (µg mL^{-1}) (*)	Recovery (%)	SD	RSD (%)
18.16	18.20	100.2	0.5434	2.750
21.79	21.57	98.99	0.2081	1.016
27.24	27.39	100.6	0.1446	0.4920
36.32	36.66	100.9	0.3873	1.050
39.95	39.65	99.25	0.2663	0.6746

*Average of five replicates

The mean recovery values obtained are in the range of 98.99−100.9 %. The analytical parameters for the determination of glucose in synthetic embryo medium are listed in Table 6.

Table6. Analytical parameters for the determination of glucose in the synthetic medium using TTC reagent

Analytical Parameter	Value
λ_{max} (nm)	480
[glucose] (µg mL^{-1})	18.16−39.95
ε (L mol^{-1} cm^{-1})	0.6627x 10^4
SD	0.1440−0.5430
RSD (%)	0.4920−2.750
Sandell sensitivity (µg cm^{-2})	1.509 x 10^{-7}
Recovery (%)	98.99−100.9
LOD (µg mL^{-1})	2.700
LOQ (µg mL^{-1})	8.200

Table7. Microdetermination of glucose in actual human embryos' culture medium using TTC at λ_{max}= 485 nm.

No. of replicates	Glucose weight taken (µg mL^{-1})	Glucose weight found (µg mL^{-1}) (*)	Recovery (%)	SD	RSD %
1		50.29			
2		49.52			
3		47.60			
4		50.26			
5	48.88	48.63	100.0	0.1891	0.3885

azo and hydrazone group and the tautomerization occurring within this ring. The fourth broad band is characteristic of the formazan structure due to π-π* transitions within the N=N group influenced by charge transfer within the whole molecule. The band is generally observed at 410−500 nm and shifted to 600 nm depending upon the structure. The visible absorption is very intensive, with the extinction coefficients registering values between 13.000−23.000 for mono-formazan and 35.000−50.000 for di-formazan [15]. The spectra of the reaction product (Fig. 1) refer to the band at λ_{max} = 480 nm which is in good agreement with the above mentioned literature [14, 15]. This indicates that Triphenyl formazan is the possible reaction product.

3.2.3 FT-IR of Glucose-TTC Reaction Product

There are notable absorption bands in the IR spectra of formazan. These are C=N, N-H and N=N absorption bands. Shifting toward lower or higher frequency of these bands determines chelate or non-chelate structure. The C=N stretching band at 1500−1510 cm^{-1} shows chelate structures. On the contrary, the C=N stretching band at 1551−1561 cm-1shows non-chelate structures [17, 18]. The shifts of these bands to higher frequencies are explained in terms of the rupture of hydrogen bond and the loss of the resonance stabilization of the six-membered chelate ring. Similarly, the lower values of the N=N stretching band are due to the intra molecular hydrogen bond and to some chelate ring resonance. The N=N stretching band of the chelate form of TPF is located at 1357 cm^{-1} while non-Chelate form is located at 1418 cm^{-1}[16]. In addition, the lower values of absorption bands N-H at 3011−3090 cm^{-1}, showed chelate structure. The majority of formazans with this form are generally characterized by the lack of N-H absorption band. Chelate structures have a six-membered Conjugate systems that p-electrons are delocalized. Because of this, double bond character decreases. And the stretching bands of C=N, N=N and N-H were observed at lower frequencies. The FT-IR spectra of the red colored product obtained via reaction of glucose and TTC is shown in Fig. 3; which may confirm the possibility of triphenylformozan formation.

From these data the appearance of the C=N stretching band at 1501.31 cm^{-1} (T% = 85.59) shows chelate structures; the N=N stretching band of the chelate form of TPF is located at 1345.11 cm^{-1} (T% = 90.91) [16]. In addition, the lower values of absorption bands N-H at 3058.55 cm^{-1} (T% = 95.14) shows chelate structure. Therefore; the obtained red colored product may have hydrogen bonding ring structure (Fig. 4).

3.2.4 ^{1}H-NMR spectra of glucose-TTC reaction product

The N-H signal of formazan in the 1H-NMR spectrum is indicative in evaluating the structure [19]. The obtained 1H-NMR of the red product is given in Fig. 5.

This curve refers to chemical shifts values of protons of glucose-TTC reaction product that is in good agreement with protons assigned in the given structure (Fig. 6)

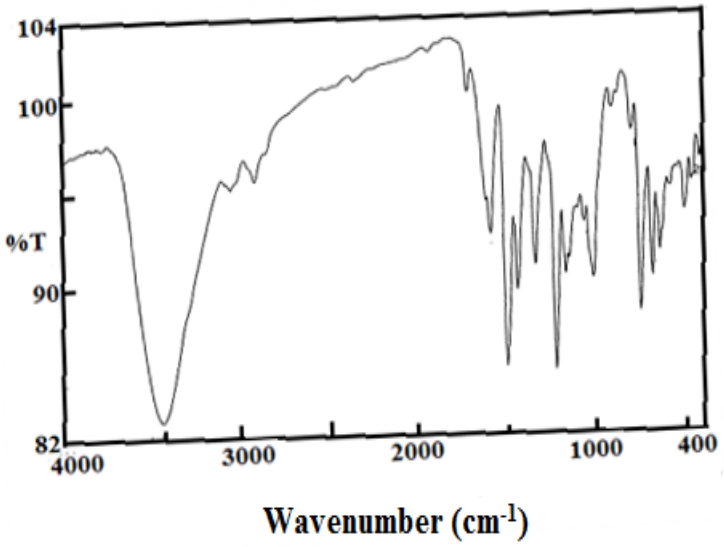
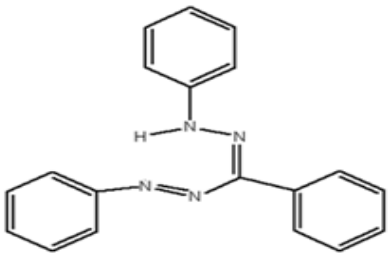


Fig3. FT-IR Spectra of Tetrazlouim-Glucose reaction product.



Chemical Formula: $C_{19}H_{16}N_4$
Exact Mass: 300.14
Molecular Weight: 300.36
m/z: 300.14 (100%), 301.14 (20.7),
302.14 (2.3%), 301.13 (1.5%)
Elemental Analysis: C; 75.98, H; 5.37,
N; 18.65

Fig 4. Structural formula of the reaction product 1,3,5-triphenyl formazan (TPF)

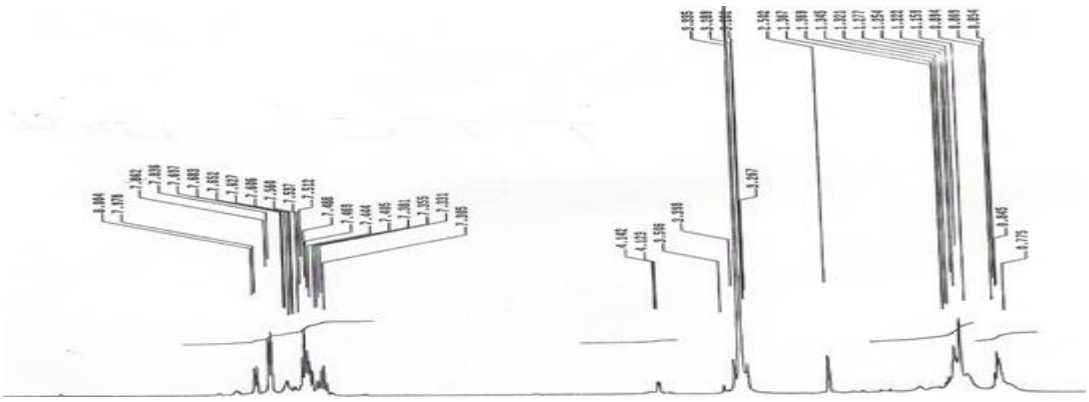


Fig 5.^{1}H-NMR Spectra of Tetrazlouim-Glucose reaction product

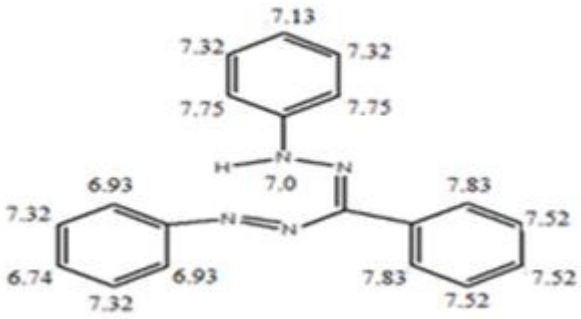


Fig 6. Estimated chemical ^{1}H-NMR shifts of the red colored reaction product

4 Conclusions

A simple and sensitive method to determine glucose was developed and optimized. This method was applied to determine glucose in the complex matrix of human embryos' culture medium without interference. The method gives comparable analytical results to previously techniques and can afford high sensitivity, selectivity, accuracy, precision, and a wide linear range. The method is based on the formation of a coloured compound due to reduction of TTC by glucose. In addition, the reaction product was characterized using elemental analysis, IR and NMR techniques which proved that it was TPF.

Acknowledgement

Our research group presents their deep thanks to chemistry Department, Faculty of Science Cairo University for supporting this research with chemicals and instruments used for different measurements.

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