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A New Approach for Micro-Determination of the Free Amino Acids in Presence of Albumin Present in Human Embryos' Culture Medium Using Different Analytical Techniques

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Abstract

The micro-determination of the free amino acids in presence of albumin in human embryos' culture medium using different analytical techniques is the main target of this manuscript. This research also aimed to perform comparative evaluation of spectrophotometric micro-determination of total amino acids present in medium used for human embryos' culture with that obtained by using ion chromatographic method and comparing these results with that had been previously published in literature. Before going to analyze amino acids in actual human embryos' culture medium, that is used in Adam International Hospital (AIH), by using ninhydrin as an indicator, the accuracy, precision and validation of using this indicator had been tested. This test involved using ninhydrin reagent in micro-determination of both glycine and tryptophan as main amino acids components of the tested medium in pure form. The absorption spectra of the colored ninhydrin-glycine or tryptophan products were tested and attain maximum absorbance at λ_{max} = 560 nm and 570 nm respectively with ε = 4.686 x 10^3 L mol^{-1} cm^{-1} in case of glycine-ninhydrin product and 4.883 x 10^3 L mol^{-1} cm^{-1} in case of tryptophan-ninhydrin product. The effect of time of heating on the products was studied and spectra measured showed that molar absorptivity values increase gradually with the period of heating at 60−70°C until 8−10 minutes. The absorbance attained maximum values at 8.0 min. The effect of pH was also studied and showed that pH= 5 is the preferred value for reaction between glycine and ninhydrin while pH = 8 is preferred for tryptophan determination. By studying the stoichiometric ratio, it was shown that ninhydrin reacts with glycine or tryptophan with the ratio 1:1 under optimum conditions (λ_{max} = 560 nm and pH = 5). Calibration graphs were constructed under the optimum conditions for micro-determination of both glycine and tryptophan in pure forms with recovery values ranging from 98.93−101.9 % and 98.06−100.7 % respectively. Studying the interfering possibility of some main components of the tested culture medium like glucose, sodium pyruvate, potassium chloride and sodium bicarbonate showed that there is no interfering possibility of these components in the determination of glycine and tryptophan in a synthetic binary mixture. The calibration curve for determination of the total glycine and tryptophan content in a binary mixture was constructed and it is linear in the concentration range 8.356−66.64 μg mL^{-1}. The obtained values of SD and RSD % are found to be in the range of 0.0011−0.0099 and 0.1098−0.8603 %, respectively. Therefore; this proposed method can be successfully used to determine the total amino acids in the mentioned concentration range (8.356−66.64 μg mL^{-1}) with a high accuracy, precision and sensitivity. This successful application of the proposed method endorsed us to apply this method for micro-determination of total amino acids present in actual embryos' culture medium. The determined amino acids in actual medium, that is used in AIH laboratory, by the proposed spectral method gives concentration of the total amino acids = 510.98 μg mL^{-1}. This value is compared with that obtained by analysis of the same medium using ninhydrin method at Agriculture Research Center (ARC); which is found to be 481 μg mL^{-1}. The identification and individual determination of amino acids present in actual culture medium were performed by using ion chromatographic method and the summation of concentrations of these free amino acids is found to be 301.41 μg mL^{-1}. This result is correlated in a confidence level of 95% with that found in literature on the same medium which is 327.54 μg mL^{-1}.

Keywords: Embryo Culture Medium, Adam International Hospital, Amino Acids, Ninhydrin, Amino Acid Analyzer

1- Introduction

All amino acids ought to be provided to the medium used to culture early embryos. Amino acids profiles of human embryos were related to the chance of an progressing pregnancy and live birth in an assisted conception program [1]. It was also found that the sum of the production and consumption of amino acids was essentially distinctive between hereditarily normal and anomalous embryos on days 2−3 of culture [2]. It was shown that embryos that developed to blastocyst have shown amino acid flux patterns distinctive from those of embryos with comparable morphological appearance which arrested [3]. Analysis of amino acids in culture medium was reported using chromatographic method by derivatization with o-phthaldehyde and 2-

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mercaptoethanol [4]. Amino acids content of culture medium was determined by using reverse phase HPLC after the reaction of amino acids with o-phthaldialdehyde to form fluorescent products and using a fluorescence detector [5]. Several methods are reported in literature for the qualitative determination of amino acids [6] such as ninhydrin test, xanthoproteic test, Millon's test, Hopkins-Cole test and nitroprusside test. A modification of Sakaguchi's reaction for arginine quantitative determination which used sodium hypobromite instead of sodium hypochlorite to increase the rate of color development and added urea to stabilize the color and permit colorimetric comparisons was also described [7]. The method depends on employing the color reaction given by certain guanidine derivatives with α-naphthol and sodium hypochlorite as a qualitative test for arginine. Several methods have been modified for the determination of amino acids containing guanidine group [8]. Metol-chloramine-T reagent was used for spectrophotometric determination of arginine and histidine in neutral pH [9]. This method depends on the formation of a colored product with metol and chloramine-T. A spectrophotometric approach for determination amino acids was primarily based on the color produced when the mixed copper is transformed to the copper salt of amino acids. The equimolar concentrations of copper complexes of amino acids do not manifest the same color intensity which make it not possible to determine amino acids in terms of one of them as a reference standard [10]. Leucine dehydrogenase was used to determine Branched chain L-amino acids by spectrophotometric measurement of the formed NADH at 340 nm [11]. Colorimetric estimation of cysteine in pure solution [12] and in presence of different amino acids was also reported via reaction with acid ninhydrin reagent to form a pink product with λmax = 560 nm [13]. Spectrophotometric approach for determination of tryptophan was described which used reaction of diphenylamine sulfonate with sodium nitrite in the sulfuric acid medium to produce a diazotized intermediate which is coupled with tryptophan to form a pink color product [14]. Spectrophotometric method for the determination of L-tyrosine was suggested which was based on the reaction of L–tyrosine with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole in an basic solution (pH 10.0) to form an orange-colored product which exhibits maximum absorbance at 388 nm [15]. Also, determination of tyrosine was performed by the formation of a red colored product with 4-aminophenazone-sodium meta-periodate in an ammoniacal solution which absorbed at 470 nm [16]. A method for specific determination of glycine was developed which is based on the reaction of glycine with benzoyl chloride to produce hippuric acid which is extracted with ethyl acetate and then reacted with acetic anhydride, p-dimethylamino benzaldehyde, and pyridine for color formation [17]. Another method for estimation of glycine was also presented that is based on the reaction of glycine and glutamate with 150 mM o-phthaldialdehyde; which quantitatively converted glycine into a colored o-phthaldialdehyde-glycine-glutamate complex and measuring the absorbance of the complex at 540 nm [18]. A spectrophotometric method for estimation of tryptophan in human urine was reported [19].

A capillary electrophoresis method used for determination of amino acids based on derivatization using 1, 2-naphthoquinone-4-sulfonated was explained. Derivatives of amino acids were spectrophotometrically detected at 480 nm [20]. Underivatized amino acids were analyzed by capillary electrophoresis under alkaline conditions using constant amperometric detection. Amperometric detection was carried out with a copper-disk microelectrode [21]. Another method was described for amino acid analysis which utilized capillary electrophoresis coupled with mass spectrometry which used a low acidic pH electrolyte to produce positive charge on whole amino acids to analyze free amino acids simultaneously. All positively charged amino acids migrated toward the cathode and then were detected by mass spectrometry [22].

Analysis of amino acids to evaluate embryo viability was described using capillary electrophoresis-mass spectrometry [23], infrared spectroscopy [24], NMR [25], FTIR spectroscopy [26] and HPLC [27] were also reported. Spectrophotometric method for determination of tryptophan in human embryos culture medium using ferroin indicator was also described in literature [28]. Determination on amino acids by hydrophilic interaction liquid chromatography-mass spectrometry was reported in literature [29]. Recent developments in amino acids analysis by using HPLC were found in literature [30].

2. Experimental

All of the chemicals used had been of the best purity available. They included, Human embryos' culture medium (GLOBAL, TOTAL, W/HAS; Life Global, US). Glucose purchased from El Nasr Pharmaceutical Chemicals Co., Egypt; and tryptophan from WINLAB, India. Glycine was purchased from ADWIC, Egypt. Potassium chloride was provided from MERCK, Germany. Sodium bicarbonate was provided by El Gomhouria Co. Egypt. Ninhydrin was provided from Aldrich Chemical Co., England.

2.1. Solutions

Stock solution of tryptophan (2.017 x 10^{-4} M) was prepared by dissolving 0.0103 g in 250 mL of

distilled water containing [$NaHCO_3$] = 0.0042 g / 250 mL. Universal Britton [30] buffer solutions were prepared by adjusting 100 mL solutions of the acid mixture to the desired pH value using 0.1 N NaOH.

2.2. Tools, Equipment and Instruments

High precision micropipette (SCILOGEX) 100−1000 µL was used to get the accurate volumes. A single beam visible spectrophotometer, model Unicom 1200, equipped with a 1.0 cm glass cell was used for all spectrophotometric measurements. Spectrophotometric measurements of ninhydrin-pyridine method were carried out using UENWAY 6705 UV/Vis spectrophotometer. Measurements of pH were performed using JENWAY 3505 pH meter. Ion chromatographic analysis was performed by using SYKAM S 4300 amino acid reagent organizer.

2.3. Studying the Reaction between Glycine and Ninhydrin Spectrophotometrically

2.3a. Selection of the Suitable Wavelength (λ_{max}) for Micro-determination of Glycine

To 1.0 mL of 1.003 x 10^{-2} M glycine; 3.0 mL of 2.003 x 10^{-2} M ninhydrin reagent had been added and the solution was boiled in water bath for 8 min. The final volume was adjusted to 10 mL with distilled water and the absorption spectrum of the resulted solution was scanned within the wavelength from 400 to 695 nm.

2.3b. Impact of Time on the Reaction of Glycine with Ninhydrin

Using 10 different test tubes, 1.0 mL of ninhydrin (2.003 x 10^{-2} M) was added to 1.0 mL of glycine (1.003 x 10^{-2} M). Then, each tube was heated in a boiling water bath for different time intervals after which the volume of each one was completed to 10 mL or 25 mL using distilled water, according to the intensity of color of the reaction product. The absorbance was measured at λ_{max} = 560 nm against water. Molar absorptivity against heating period was plotted. From this curve the optimum time of heating for micro-determination of glycine was determined.

2.3c. Effect of pH on λ_{max} and on the Absorptivity of the Product of Reaction between Glycine and Ninhydrin

In 11 different test tubes; 0.4 mL of 2.007x 10^{-2} M of ninhydrin were added to 0.4 mL of 1.019 x 10^{-2} M of glycine and all tubes were boiled in water bath for 8 min. The tubes were left to cool for 30 minutes to reach room temperature. After that, 2.0 mL of the universal buffer solution were added in each tube to adjust the pH of the mixture to a certain value (3−12). The solution mixture of each tube was completed to 10 mL with distilled water. Absorption spectrum of each sample was scanned against water at λ_{max}= 560 nm. The plot of the molar absorptivity against pH values was used to select suitable pH for the reaction of glycine with ninhydrin.

2.3d. Stoichiometric ratio of the Reaction between Glycine and Ninhydrin

The stoichiometry of the reaction between ninhydrin and glycine was examined by applying the continuous variation method [31] in which a series of solutions had been organized via adding different volumes of ninhydrin reagent (2.003 x 10^{-2} M) to glycine solution (1.003 x 10^{-2} M), so that the molarity was kept constant. The procedure was performed as mentioned before and the absorption spectrum data obtained at λ_{max}= 560 nm were plotted against mole fraction of glycine.

2.4. Study of the Reaction between Tryptophan and Ninhydrin Using Spectrophotometric Measurements

2.4a. Selection of the suitable wavelength (λ_{max}) for Micro-determination of Tryptophan

To 1.0 mL of 1.022 x 10^{-3} M of tryptophan, 3.0 mL of 2.003 x 10^{-2} M ninhydrin reagent were added and boiled in water bath for 8 min. The mixture was diluted by distilled water to 10 mL. The absorption spectrum of the resulted mixture was scanned from 400 to 675 nm to select the best wavelength for tryptophan determination.

2.4b. Effect of pH on λ_{max} and Absorption Spectrum of the Product of Reaction between Tryptophan and Ninhydrin

In 10 different test tubes, 0.4 mL of 2.007 x 10^{-2} M ninhydrin was added to 0.4 mL of 1.009 x 10^{-2} M tryptophan; each mixture was boiled for 8 min. The tubes were left to cool for 30 minutes to reach room temperature and then 2.0 mL of various pH values of the universal buffer solutions were added to the tubes to adjust the pH of each mixture to a certain value (3−12). The solution of each tube was adjusted to 10 mL and the absorption spectrum of each sample was recorded against water. The values of molar absorptivity were plotted against pH at λ_{max}= 570 nm to select the suitable pH range for tryptophan reaction with ninhydrin.

2.4c. Stoichiometry of the Reaction between Tryptophan and Ninhydrin Using the Method of Continuous Variation

Different volumes of ninhydrin reagent (1.013 x 10^{-2} M) were added to tryptophan solution (1.005 x 10^{-2} M), so that the molarity was kept constant. The mixtures were boiled in water bath for 8 min then, were left at room temperature for 30 min to cool. After that, the volume of each tube was completed to 10 mL by distilled water. The absorbance data obtained at λ_{max}= 570 nm were plotted against mole fraction of tryptophan.

2.5. Micro-determination of Glycine in Pure form by using Ninhydrin

Two mL of ninhydrin (2.007×10^{-2} M) were added to (0.2−2.0 mL) of glycine (1.04915×10^{-2} M). The mixtures were boiled in water bath for 8 minutes, let to cool, transferred to measuring flask and diluted to 50 mL. Absorption spectra of the product formed was scanned at λ_{max} = 560 nm. Calibration curve was constructed by plotting the absorbance values for different mixtures against [glycine] µg mL^{-1}.

2.6. Micro-determination of Tryptophan in Pure Form Using Ninhydrin

Two mL of ninhydrin (2.007×10^{-2} M) had been added to different volumes (0.2− 2.0 mL) of tryptophan (1.012×10^{-2} M) in test tubes and boiled in water bath for 8 min, then were cooled to reach room temperature. The mixtures were diluted to 50 mL and the absorbance of the formed product was measured at λ_{max}= 570 nm against water. The absorbance values were plotted against [tryptophan] µg mL^{-1} to construct a calibration curve used for micro-determination of tryptophan using ninhydrin.

2.7. Selection of the Suitable Wavelength (λ_{max}) for Micro-Determination of the Total Amino Acids in Binary Mixture of Glycine and Tryptophan.

To 3.0 mL of ninhydrin solution (2.003×10^{-2} M), 1.0 mL of glycine (1.003×10^{-2} M) and 1.0 mL of tryptophan (1.022×10^{-3} M) were mixed and the mixture was boiled in water bath for 8 min, cooled and diluted to 10 mL. The absorption data were recorded in the wavelength region from 400 to 675 nm from which the best wavelength for determination of the total glycine and tryptophan in binary mixture using ninhydrin was selected.

2.8. Interference Effect

To fixed volumes of glycine (0.5 mL of 1.019×10^{-2} M), tryptophan (0.5 mL of 1.0×10^{-2} M) and ninhydrin (1.5 mL of 2.003×10^{-2} M); different volumes (0.1−0.3 mL) of 1.0×10^{-1} M of glucose, sodium pyruvate, KCl and $NaHCO_3$ were added in different test tubes to study the interfering possibility of these components of culture medium in the determination of amino acids. The mixtures were boiled for 8 min, cooled, and diluted to 10 mL. The absorbance values of these mixtures in comparison with the blank samples (containing the reaction mixture with no interfering components) were measured to determine the interfering limits of synthetic embryo medium constituents.

2.9. Validity of Beer's Law for Micro-determination of the Total Amino Acids Content (Glycine and Tryptophan) Using Ninhydrin

To check the validity of Beer's law for micro-determination of the total amino acids (glycine and tryptophan) in a binary mixture using ninhydrin indicator; the following procedure was proposed. Two mL of ninhydrin (2.025×10^{-2} M) were mixed in test tube with different volumes (0.1− 1.0 mL) of equimolar amounts of glycine (1.0492×10^{-2} M) and tryptophan (1.012×10^{-2} M) and boiled for 8 minutes. The mixtures were cooled and diluted 50 mL. The absorbance values were measured at λ_{max}= 560−570 nm. From these values; total amino acid concentrations were calculated using the calibration curve under the same conditions.

2.10. Determination of the Total Free Amino Acids in Actual Human Embryos' Culture Medium Using Ninhydrin

In a test tube, 1 mL of ninhydrin (2.004×10^{-2} M) was added to 0.5 mL of the culture medium. Then, the pH of the mixture was adjusted to 10 with the use of universal buffer solution to prevent thermal coagulation of albumin in the culture medium [32]. The test tube was heated in a boiling water bath for 8 min. After that, it was removed and let for 30 min at room temperature to cool. Then, the content of the test tube was diluted to a 10 mL and the absorbance of the final solution was measured at 560 nm. The total concentration of amino acids in culture medium was determined from the calibration curve constructed.

2.11. Determination of the Total Free Amino Acids Present in Actual Human Embryos' Culture Medium by Using Ninhydrin-pyridine Method

The total free amino acids were determined depending upon the procedure in which 1 mL of culture medium was reacted with 1.0 mL of 10 % pyridine and 1.0 mL of 2 % ninhydrin solution. [33]. The optical density of the colored solution was read at 570 nm using UENWAY, 6705 UV / Vis spectrophotometer.

2.12. Identification and Determination of Amino Acids Present in Actual Human Embryos' Culture Medium Using Amino Acid Analyzer

Identification of amino acids present in culture medium was carried out at Soils, Water and Environmental Res. Inst. (SWERI), Agricultural Research Center following the method of AOAC (2012) [34]. Analysis was performed using amino acid analyzer SYKAM S 4300. In a centrifuge tube, 800 µL of culture medium were poured and 200 µL of sulfo-salicylic acid (10 %) were added; the tube was shaken well and the solution was stored in a refrigerator at 4°C for 30 min. After that, the tube was centrifuged for 10 min and the upper clear solution was mixed with the sample diluting buffer, then sample was injected for analysis.

3. Results and Discussion

3.1. Study of the Reaction between Glycine and Ninhydrin through Spectrophotometric Measurements

Before going to analyze the actual human embryos' culture medium using ninhydrin as an indicator for amino acids; it is recommended to check the accuracy, precision and validation of using this indicator to determine glycine in pure form, as one of the main culture medium components, and applying the suggested procedure in analysis of the actual human embryos' culture medium. This study involved the effect of different conditions on the reaction between glycine and ninhydrin; such as the effect of heating time, pH and ratio of reactants. These parameters affect the formation of the chromophore, Ruhemann's purple, usually formed between amino acids and ninhydrin [35].

3.1a. Selection of the Suitable Wavelength (λ_{max}) for Glycine Micro-determination

The absorption spectrum of the colored ninhydrin chromophore (1.003 x 10^{-3} M), formed when glycine was reacted with ninhydrin in the wavelength region 400– 695 nm (Fig. 1).

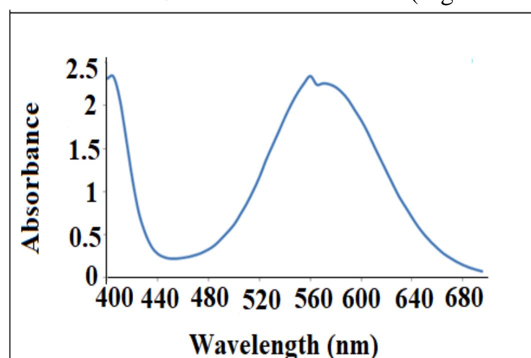

Fig. 1. Absorption spectrum of ninhydrin chromophore during reaction with glycine (λ_{max} = 560 nm).

It is inferred from Fig. 1 that the formed product undergoes a maximum absorbance at λ_{max} = 560 nm ($\varepsilon = 2.337x\ 10^3$ L mol^{-1} cm^{-1}).

3.1b. Impact of Time on the Reaction between Glycine and Ninhydrin

The effect of heating time in boiling water on the formation of the chromophore, Ruhemann's purple was carefully studied and the results are illustrated in Fig. 2.

It is obvious from Fig. 2 that the molar absorptivity of Ruhemann's purple increases gradually with the period of heating at 60–70°C until 8–10 min; where a maximum constant value is attained after 8.0 min of heating in a boiling water bath.

3.1c. Effect of pH on λ_{max} and Molar Absorptivity of the Product Formed due to Reaction of Glycine with Ninhydrin

Absorption spectral data of glycine, as a primary aliphatic amino acid present essentially in actual human embryos' culture medium, when reacted with ninhydrin reagent at different pH values of the universal buffer of pH range 3–12 are represented graphically in Fig. 3.

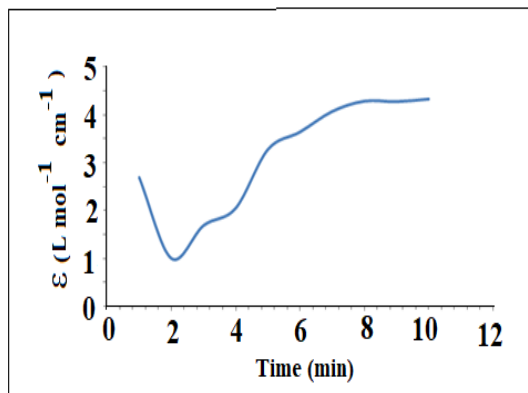

Fig. 2. Effect of time of heating in a boiling water bath on the formation of reaction product of glycine and ninhydrin at λ_{max}= 560 nm.

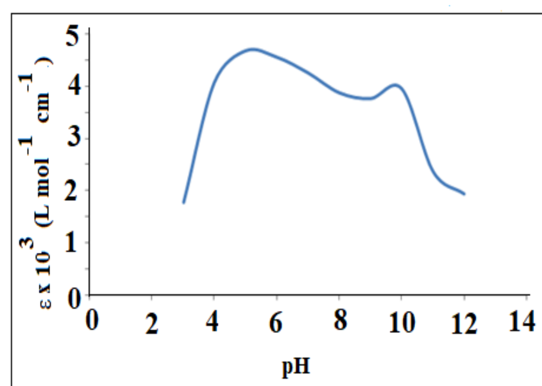

Fig. 3. Effect of pH on molar absorptivity of glycine and ninhydrin product of reaction

These data show that, ε attains its maximum value (4.686 x10^3 L mol^{-1} cm^{-1}) at pH= 5 (λ_{max}= 560-570 nm).

3.1d. Stoichiometric ratio of the Reaction between Ninhydrin and Glycine

The stoichiometry of reaction of ninhydrin with glycine is determined using the continuous variation method (Fig. 4) in order to select the proper ratio of glycine: ninhydrin.

The results of the continuous variation indicate that, ninhydrin reacts with glycine in the ratio 1:1 at pH= 5.0.

3.1.e. *Micro-determination of Glycine in Pure Form Using Ninhydrin*

Therefore; glycine can be determined in pure form using ninhydrin at the selected proper conditions of pH= 5.0, time of heating (8–10 min at 60–70°C) and

in the ratio 1:1. The validity of Beer' law is represented in Fig. 5.

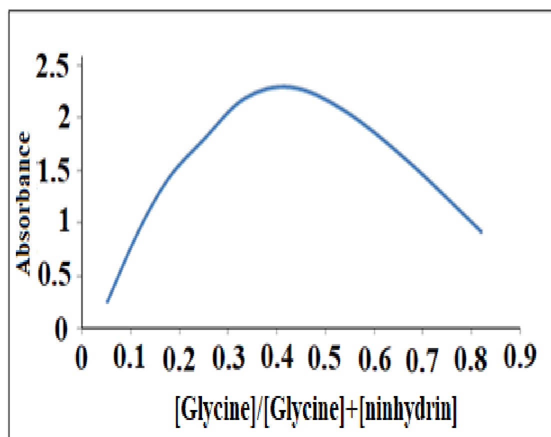

Fig. 4. Stoichiometric ratio of the reaction between glycine and ninhydrin at λ_{max} = 560 nm by using the continuous variation method.

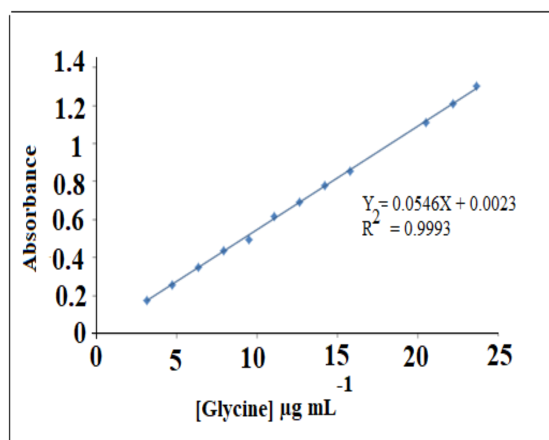

Fig. 5. The validity of Beer's law for micro-determination of glycine by using ninhydrin at λ_{max}= 560 nm.

The analytical parameters deduced from this calibration curve are given in Table 1

Table 1: Analytical parameters for micro-determination of glycine using ninhydrin.

Parameters	The value
λ_{max} (nm)	560
[glycine] µg mL^{-1}	3.197–23.69
ε (L mol^{-1} cm^{-1})	0.04116 x 10^{5}
Recovery %	98.93–101.9
SD	0.0346–0.3848
RSD (%)	0.2756–1.879
LOD (µg mL^{-1})	2.886
LOQ (µg mL^{-1})	8.759
Y= aX+ b, a b	 0.054 0.002
R^2	0.999
S (µg cm^{-2})	2.430x 10^{-7}

Therefore, the proposed method was used to determine glycine in pure form in the range 3.197–23.69 µg mL^{-1} (Table 2).

Table 2: Micro-determination of variable [glycine] using excess [ninhydrin] = 8.028 x 10^{-4} M at λ_{max} = 560 nm.

Weight taken of glycine (µg mL^{-1})	Weight found of glycine (µg mL^{-1})	Recovery (%)	SD	RSD (%)
3.151	3.197	101.5	0.0360	1.767
7.875	7.968	101.2	0.0843	1.062
12.60	12.62	100.2	0.0346	0.2756
14.17	14.19	100.1	0.0569	0.4393
20.48	20.28	99.03	0.3848	1.879
22.16	22.07	99.59	0.1386	0.6235
23.62	23.69	100.3	0.1360	0.5542

The data in Table 2 show that, calibration curve is linear in the range of concentrations of glycine (3.197– 23.69 µg mL^{-1}). The obtained average recovery values are in the range of 99.03–101.5%. The detection (LOD) and quantification limits (LOQ) are found to be 2.886 and 8.759 µg mL^{-1}, respectively. The standard deviation (SD) values are in the range of 0.0346 to 0.3848 and the relative standard deviation (RSD %) values vary from 0.2756 to 1.879. The obtained standard deviation and the relative standard deviation have low values which demonstrate the suggested method's high accuracy and precision. The calculated results of the Sandell sensitivity of 2.430 x 10^{-7} µg cm^{-2} also reflect the high sensitivity of the method.

3.2. Spectrophotometric Study of the Reaction between Tryptophan and Ninhydrin Reagent Through Spectrophotometric Measurements

3.2a. Selection of the Suitable Wavelength (λ_{max}) for Tryptophan Micro-determination

The absorption spectrum of the reaction product of the ninhydrin (1.022 x 10^{-4} M) with tryptophan at pH = 8.0 was scanned in the wavelength region 400– 675 nm (Fig. 6). It is evident from this figure that the formed product exhibits a maximum absorbance at λ_{max} = 570 nm (ε= 4.883x 10^{3} L mol^{-1} cm^{-1})

3.2b. Influence of pH on the Absorption of the Tryptophan-Ninhydrin Reaction Product

Absorption spectra of tryptophan reaction with ninhydrin reagent at different pH values of the universal buffer (3–12) are illustrated in Fig. 7.

These data show that, ε attains its maximum value (6.217x 10^{3} L mol^{-1} cm^{-1}) at pH= 8.0 (λ_{max}= 570 nm).

3.2c. Stoichiometry of Tryptophan-Ninhydrin Reaction

The continuous variation method is used to identify the stoichiometry of the reaction of ninhydrin with

tryptophan in order to choose the right tryptophan ratio: ninhydrin. The data obtained are displayed in Fig. 8. The results in Fig. 8 refer to the formation of tryptophan-ninhydrin product of reaction at 1:1 ratio.

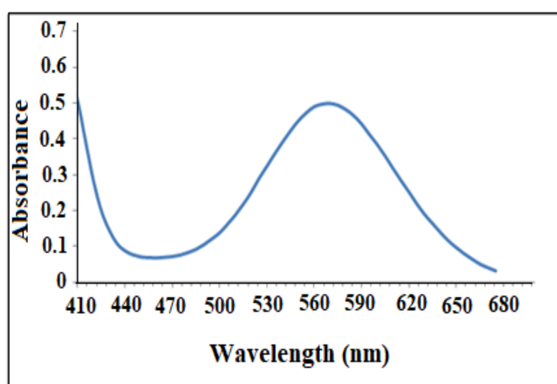

Fig. 6. Absorption spectrum of ninhydrin reaction product with tryptophan in pure form (λ_{max}= 570 nm).

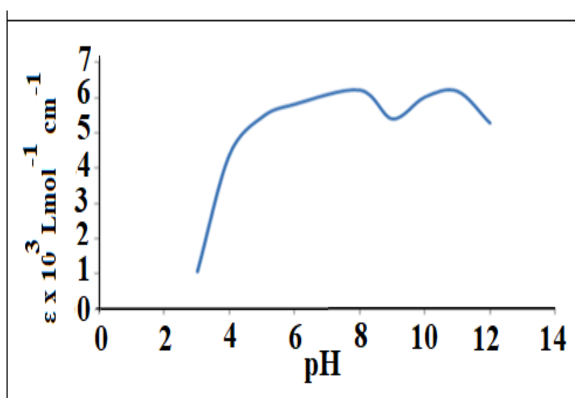

Fig. 7. The impact of pH on ε of tryptophan reaction product with ninhydrin

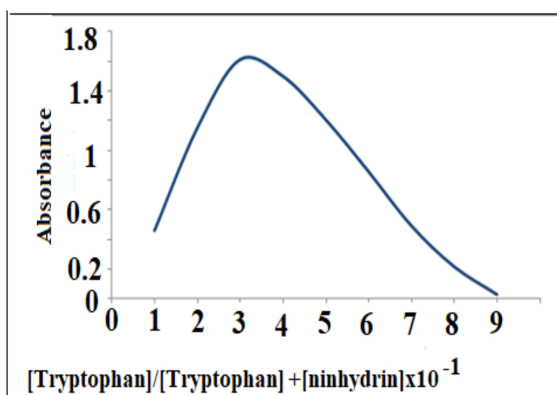

Fig. 8. Absorption spectrum of tryptophan reaction with ninhydrin at λ_{max}= 570 nm using the continuous variation method.

3.3. Micro-determination of Tryptophan in Pure Form using Ninhydrin

Therefore, tryptophan can be determined in pure form using ninhydrin at the selected proper conditions of pH = 8.0, time of heating (8–10 min at 60–70 ºC) and in1:1 ratio. The validity of Beer' law is shown in Fig. 9.

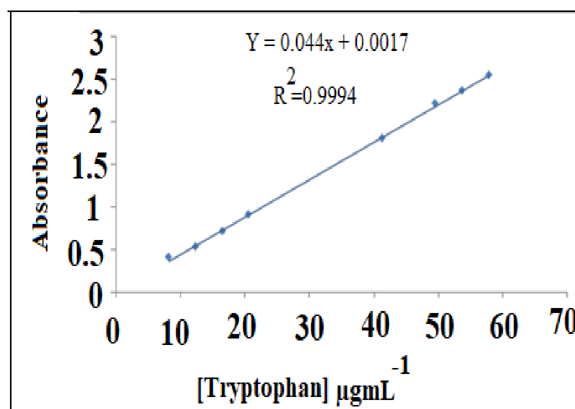

Fig. 9. The calibration graph for the determination of tryptophan in pure form using ninhydrin at λ_{max} = 570 nm, pH= 8, heating for 8–10 min at 60–70ºC.

Therefore the proposed method was used for tryptophan micro-determination in pure state in the range of 8.10– 57.54 µg mL^{-1}.Therefore; The calibration curve in the concentration range of 8.10–53.47 µg mL^{-1} is linear, as shown if Fig. 9. The data of the determination of tryptophan in pure form using ninhydrin are listed in Table 3.

Table 3: Tryptophan micro-determination in pure form using [ninhydrin] = 8.028x 10^{-4} M at λ_{max} = 570 nm.

Weight taken of tryptophan µg mL^{-1}	Weight found of tryptophan µg mL^{-1}(*)	Recovery (%)	SD	RSD (%)
8.26	8.10	98.06	0.0021	0.81
16.53	16.30	98.61	0.0035	0.42
20.67	20.61	99.71	0.0067	0.58
41.34	40.58	98.16	0.0071	0.39
49.6	49.97	100.7	0.0036	0.16
53.74	53.47	99.50	0.0046	0.19

(*) average of five replicates

The limits of detection (LOD) and quantitation (LOQ) are calculated to be 0.0760 and 0.2304 µg mL^{-1}, respectively. The standard deviation (SD) values are in the range from 0.0021 to 0.0071 and the relative standard deviation values (RSD %) are 0.16–0.81%. The calculated standard deviation and relative standard deviation are both low, implying that the suggested method is accurate and precise. This is also verified by the calculated Sandell sensitivity value, 1.108 x 10^{-8} µg cm^{-2}; which illustrates the method's high sensitivity.

All of the above data refer to a final conclusion that both glycine and tryptophan react with ninhydrin to form 1:1 Ruhemann's purple (RP). The mechanism

of reaction may be given by the proposed scheme 1, via condensation under the properly

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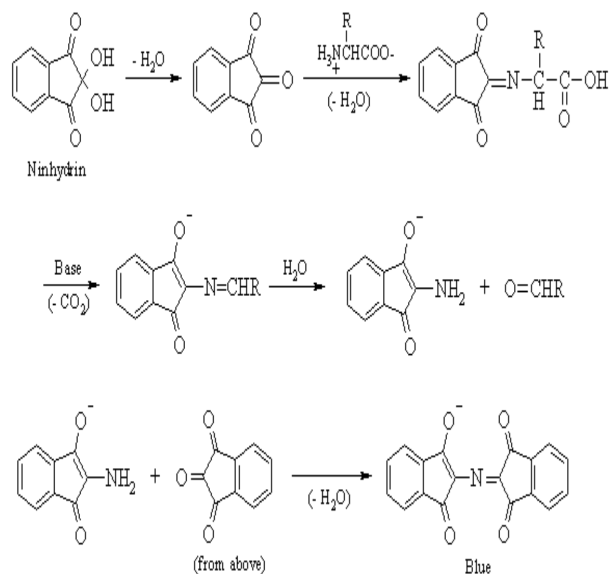
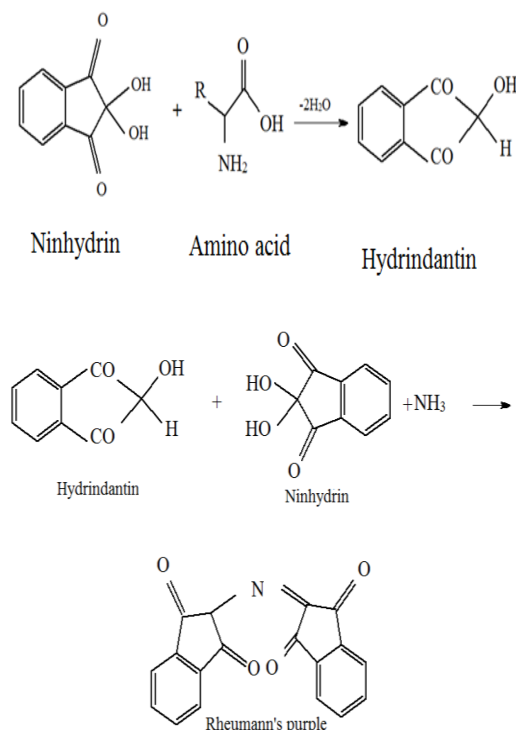


Scheme 1. Glycine‒ninhydrin condensation

Generally it can take the following scheme 2



Scheme 2. Amino acid‒ninhydrin condensation

Therefore the determination of glycine and tryptophan using ninhydrin individually is so difficult; but the total amino acids in a mixture can be obtained.

3.3. Selection of the Suitable Wavelength (λ_{max}) in order to Determine the Total Amino Acid Content in a Binary Mixture of Glycine and Tryptophan

The absorption spectrum of the product of reaction of ninhydrin with a mixture of glycine and tryptophan was scanned against water in the wavelength region 400– 675 nm (Fig. 10).

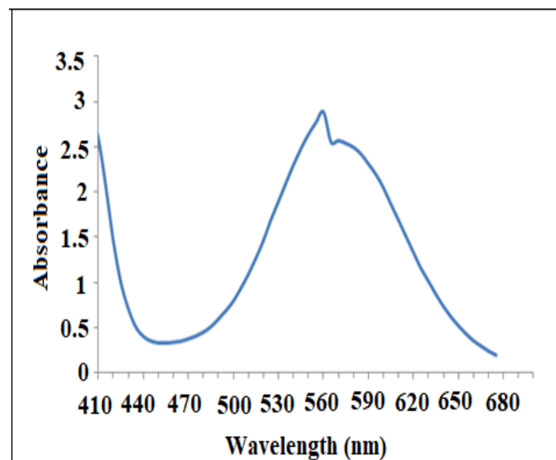


Fig.10. Absorption spectrum of ninhydrin reaction products with tryptophan and glycine in a binary mixture ($\varepsilon = 2.824x\ 10^4$ L mol^{-1} cm^{-1}).

These data show that the formed product manifests two maxima at 560 and 570 nm as a result of reaction between ninhydrin and both glycine and tryptophan (Scheme 2). The inspection of the above results refers to the possible interference between tryptophan and glycine in determination of anyone of them via reaction with ninhydrin in a mixture. Therefore, the use of ninhydrin in a synthetic embryo media will give the total amino acids. It is also possible to use either determined amount of tryptophan or glycine to estimate the total amino acids in actual human embryos' culture medium in terms of anyone of them.

Before going to determine amino acids in the embryos' culture medium, we should check the interfering possibility of the medium components. This will start with interference study of some components on amino acids determination using ninhydrin.

3.4. Interference Effect

The influence of different components on the total amino acid determination is studied. This is done by observing the change in the absorbance reading, at λ_{max}= 570 nm, due to the presence of interfering compounds. During each measurement, the concentration of amino acid was kept constant [glycine] = [tryptophan] = 1.0×10^{-4} M. The measured absorbance due to (glycine + tryptophan) was 1.781, average of five replicate measurements,

was not changed by the presence of glucose, sodium pyruvate, $NaHCO_3$ and KCl (Average absorbance of five replicates= 1.784). These results are given in Table 4 and encourage the use of ninhydrin in micro-determination of total amino acids in terms of tryptophan or glycine.

Table 4: Interference of embryo culture medium components on glycine and tryptophan quantitation in a binary mixture using ninhydrin indicator.

Component	Molarity x 10^{-4} M	Absorbance	Interference effect
Glucose	4.0	1.706	±0.007
Sodium pyruvate	2.0	1.719	±0.026
$NaHCO_3$	2.0	1.555	±0.211
KCl	2.0	1.814	±0.016

3.5. Validity of Beer's Law for Micro-determination of the Total Amino Acids Content (Glycine and Tryptophan) in the Synthetic Mixture

The total amino acid content is determined spectrophotometrically under ideal conditions of pH, reagent concentration, temperature, stoichiometric ratios, and wavelength. The absorbance-concentration curve is shown in Fig 11.

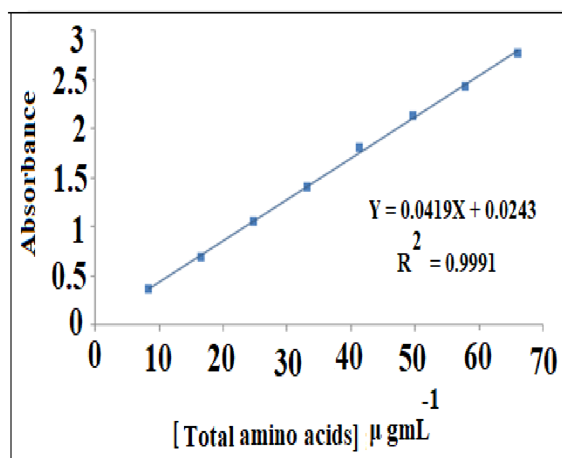


Fig. 11. Beer's law's applicability for determining total amino acids using ninhydrin at λ_{max} = 570 nm.

The calibration curve in the concentration range of total amino acids 8.356−65.64 µg mL^{-1} is linear, as shown in Fig. 11. for the determination of the total amino acids using ninhydrin at λ_{max}= 570 nm. The analytical parameters deduced from this calibration curve are shown in Table 5.

The average recovery values obtained vary from 99.05 to 102.9 %. Table 6 lists the analytical parameters for determining the total amino acids under study. The limits of detection (LOD) and quantitation (LOQ) have been determined to 0.2588 and 2.0744 µg mL^{-1}, respectively. The standard deviations (SD) are 0.0011−0.0099 and the relative standard deviation values (RSD %) are 0.1098−0.8603. The calculated standard deviation and relative standard deviation are both low, indicating that the suggested method is accurate and precise. This is further reinforced by the calculated Sandell sensitivity of 1.170 x 10^{-7}µg cm^{-2} which confirms the method's strong sensitivity. The molar absorptivity and regression equation for the total amino acids are tabulated in Table 5. The obtained data has a correlation coefficient of 0.999.

Table 5: Analytical parameters for the determination of total amino acids (tryptophan and glycine) by the proposed method using ninhydrin reagent λ_{max} = 570 nm.

Parameters	The value
λ_{max} (nm)	560
[Total amino acids] µg mL^{-1}	8.356−65.64
ε (L mol^{-1} cm^{-1})	0.0855x 10^5
% Recovery	99.05−102.9
SD	0.0011−0.0099
RSD (%)	0.1098−0.8603
LOD (µg mL^{-1})	0.2588
LOQ (µg mL^{-1})	2.0744
Y= aX+ b, a b	 0.085 0.024
R^2	0.999
S (µg cm^{-2})	1.170 x 10^{-7}

From these data it is clear that, the proposed method can be applied for micro-determination of total amino acids in a binary mixture without interference due to the compounds involved in the interference study.

Table 6: Micro-determination of the total amino acids in a binary mixture using ninhydrin as a reagent

[Total amino acids] (µg mL^{-1})	Weight found (µg mL^{-1})(*)	Recovery (%)	SD	RSD (%)
8.267	8.356	101.1	0.0032	0.8603
24.80	24.56	99.05	0.0011	0.1098
33.06	33.07	100.0	0.0099	0.7007
41.34	42.56	102.9	0.0046	0.2559
49.61	50.44	101.7	0.0036	0.1689
57.88	57.61	99.53	0.0047	0.1941
66.13	65.64	99.26	0.0085	0.3069

(*) [glycine + tryptophan] average of five replicates

Finally, as indicated by the SD and RSD values, the spectrophotometric method can be effectively utilized for the determination of total amino acids in the concentration range given above with high accuracy, precision, and sensitivity. This consistent application of the suggested method encouraged to apply this method on micro-determination of total amino acids may be present in actual human embryos' culture medium collected from in vitro fertilization hospital.

3.6. Determination of Total Free Amino Acids in the Actual Culture Medium of Human Embryos by Using the Proposed Ninhydrin Reagent Method

The ninhydrin test was used to determine total amino acids in the culture medium of human embryos. The absorbance of the reaction product with the culture medium at λ_{max} was 0.516. The regression equation for the calibration line of standard glycine was $Y = 0.0546\ X + 0.0023$ and ε value was found to be 0.04116 x 10^5 L mol^{-1} cm^{-1}. The total amino acid content in the culture medium was determined to be 510.98 μg mL^{-1}.

3.7. Determination of the Total Free Amino Acids Present in Culture Medium by Using Ninhydrin-pyridine Method

Determination of total free amino acids following the ninhydrin method (at Agriculture Research Center; Soils, Water and Environment Res. Ins. (SWERI) gave total amino acids concentration= 481 μg mL^{-1} which compares good with that obtained by analysis of the same medium using the proposed ninhydrin method which is 510.98 μg mL^{-1} .

3.8. Using Amino Acid Analyzer to Identify and Determine the Free Amino Acids Present in Culture Medium of Actual Human Embryos.

Data of analysis of individual amino acids present in the culture medium of human embryos are represented in Fig. 12. This figure shows the characteristic peaks for each amino acid present in the culture medium detected at λ= 570 nm except proline which is detected at λ= 440 nm .

A Comparison between the results obtained from analysis of the actual human embryos' culture medium by using the ion chromatographic method and the results in literature [36] is shown in Table 7.

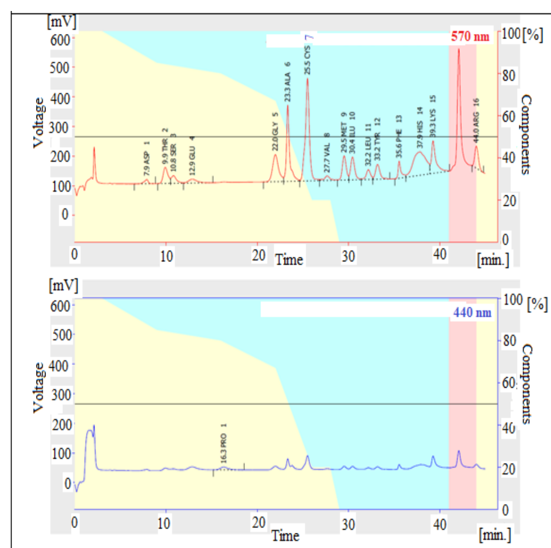


Fig. 12. Ion chromatogram of the actual human embryos' culture medium using amino acid analyzer.

Table 7: Determined concentration of free amino acids present in actual culture medium by using amino acid analyzer and the results reported in literature

Amino acid	Concentration (μg mL^{-1}) from literature [36]	Concentration (μg mL^{-1}) from amino acids analyzer
Alanine	5.791	21.32
Aspartic acid	7.321	2.31
Asparagine	6.870	Standard was not present
Arginine	57.14	14.93
Cysteine	12.50	88.05
Glutamic acid	7.945	8.01
Glycine	4.729	13.12
Histidine	17.22	49.9
Isoleucine	28.99	13.04
Leucine	30.17	5.69
Lysine	33.92	21.41
Methionine	7.610	14.25
Phenylalanine	18.50	8.84
Proline	5.872	9.06
Serine	6.095	3.68
Threonine	25.73	11.43
Tryptophan	5.718	Decomposed
Tyrosine	18.12	13.30
Valine	27.30	3.09
Total	327.54	301.41

The difference in the total concentration of amino acids determined by the amino acid analyzer (301.41 μg mL^{-1}) and that in literature (327.54 μg mL^{-1}) is due to the decomposition of some amino acids like tryptophan and the absence of the standards of some other amino acids like asparagine which made the determination of them not possible and subsequently lowered the total amino acids content which was detected by the amino acids analyzer.

4. Conclusions:

The key objective of this manuscript is to determine the total and individual free amino acids in the culture medium of human embryos. Determination of the total amino acids was done by two spectrophotometric and one chromatographic methods and the results were compared with that reported in literature. The total amino acid concentration in the culture medium under investigation by the proposed ninhyrin method was 510.98 μg mL^{-1} and that obtained by the ninhydrin/pyridine method was 481 μg mL^{-1}. The amino acids analyzer which utilizes the ion chromatographic method gave a total concentration of amino acids in the medium of 301.41 μg mL^{-1} which compares well with the results in literature which is 327.54 μg mL^{-1}. Some components of the

culture medium for human embryos were used to check the interference possibility and no interference was found. This means that successful application of the suggested procedures to the spectrophotometric micro-determination of total amino acids content in actual human embryos' culture medium is possible.

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Role of Authors:
Prof. Mohamed A. Zayed: Supervised the whole work presented in this manuscript, revised its whole content and followed its submission to the journal. **The Ph.D. student Mohamed Mostafa Taha:** Did the whole work in lab, tabulate the results and wrote the draft of the whole text.

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