# *A novel method for the spectrophotometric determination of L-arginine and tryptophan in different pharmaceutical formulations and pure form via homemade continuous flow injection analysis - Merging Zones Technique*

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## *Abstract*

An accurate and simple CFIA-Merging zones method for determination of two different amino acids ( L-arginine & tryptophan ) by using two different reactions .

The procedure for the estimation of arginine is based on the oxidation condensation reaction of arginine with α-naphthol in the presence of sodium hypobromite as an oxidizing agent in alkaline medium to produce a red colored product measured at $\lambda_{max}$ 501nm . While tryptophan procedure based on azo-coupling reaction of tryptophan with an azotized product which produced by treated of diphenyl amine sulphonate with sodium nitrate in acidic medium to yield a pink colored product measured at $\lambda_{max}$ 522 nm .

The ideal condition for the formation of the complexes was investigated and it was observed that the calibration curves were obeys with Beer's low at the range 3-1400µg.ml$^{-1}$and 5-100 µg.ml$^{-1}$ with detection limit 1 µg.ml$^{-1}$& 1 µg.ml$^{-1}$ for L-arginine and tryptophan respectively .

CFIA –Merging zones system was able to estimate L-arginine and tryptophan with sampling rate 45&52 sample /h respectively .

The novel method was found to be appropriate for the estimation of L-arginine and tryptophan in pharmaceutical formulations and in it's pure form .

Key words : L-arginine , tryptophan , azo-coupling reaction , oxidation condensation reaction , CFIA-Merging zones technique

**طريقة جديدة للتقدير الطيفي للارجنين و التربتوفان في مختلف المستحضرات الصيدلانية و في الشكل النقي باستخدام تقنية الحقن الجرياني المستمر – اندماج المناطق**

***بشرى بشير قاسم ، سارة فارس حميد**

**قسم الكيمياء/ كلية العلوم / جامعة بغداد / العراق**

**الخلاصة**

في هذا البحث تم استخدام طريقة دقيقة و سهلة وهي طريقة الحقن الجرياني – اندماج المناطق لتقدير حامضين امينيين مختلفين هما ( الارجنين و التربتوفان ) باستخدام تفاعلين مختلفين .

حيث إن الطريقة المتبعة لتقدير الارجنين تعتمد على تفاعل الأكسدة و التكاثف للارجنين مع الالفا نفثول بوجود الصوديوم هايبوبروميت كعامل مؤكسد في وسط قاعدي لتكوين معقد احمر اللون يقاس عند 501 نانومتر ، بينما

التربتوفان اعتمدت الطريقة على تفاعل الازوتة و الازدواج بين التربتوفان و ناتج الازوتة المتكون من معاملة الداي فنيل أمين سلفونات مع نتريت الصوديوم في وسط حامضي لتكوين معقد وردي اللون يقاس عند 522 نانومتر . الظروف المثلى لتكوين المعقدات تم دراستها و لوحظ ان منحنيات المعايرة تتلائم مع قانون بيير لامبرت عند المدى $1-3\mu g.ml^{-1}$ و $5-100\ \mu g.ml^{-1}$ مع حد كشف $1\ \mu g.ml^{-1}$ &$1\ \mu g.ml^{-1}$ للارجنين و التربتوفان بالتتابع .
نظام الحقن الجرياني المستمر – اندماج المناطق قادر على تقدير الارجنين و التربتوفان مع معدل نمذجة تصل إلى $45\mu g.ml^{-1}$ & $52\mu g.ml^{-1}$ نموذج في الساعة بالتتابع .
وجد إن الطريقة المستحدثة مناسبة لتقدير الارجنين و التربتوفان في المستحضرات الصيدلانية و في الصورة النقية.

## *Introduction*

Arginine and tryptophan are essential amino acids in human . The side chain of arginine consists of a 3-carbon aliphatic linear chain and its name according to IUPAC is 2-Amino -5-quanidinopentanoic acid ( as shown in figure )[1,2] . Tryptophan has the largest aromatic side chain in the alpha – amino acids and this chain is composed of two rings one hexagonal and the other pentagonal nitrogen form one of its corner as shown in figure (2) Tryptophan name according to IUPAC is (2S)-2-amino -3-(1H-indol-3-yl ) propanoic acid and its abbreviated as Try. , Trp. and W . Arginine and tryptophan are very important amino acids because of their importance in human body . Arginine is necessary for the body to make protein , the body needs it for the production of nitric oxide which produced by cracking arginine by the Citrulline enzyme inside the cell system , arginine works to organize and keep salt levels in the body at fixed levels [3-10 ] .

Tryptophan helps reduce the appetite and the anxiety symptoms . It's used to treat many diseases including depression and mood disorders , of used in some obesity drugs as a treatment , case of irritable bowel syndrome and colitis , chronic insomnia and sleep problems . To stimulate the mind and noise the efficiency of concentration and treatment of hepatitis psychotic [11-20] .

Tryptophan in turn is necessary for the production of serotonin which called happiness hormone ( a brain chemical necessary for the mood regulation and sleep ) . The lack of tryptophan and magnesium may led to cramps in coronary artery , as well as tryptophan works to improve peripheral nerves and resistance aging symptoms and governed by absorbing vitamin $B_3$[21-24] . The body can supply of tryptophan and arginine either part of excreted within the body or from eating food or supplements containing large proportions of these acids [25,26]

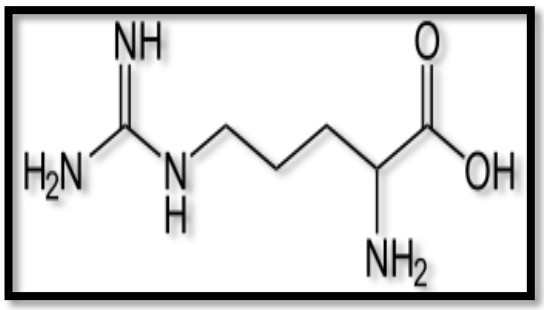



*Figure (1) : Structure of L-arginine*

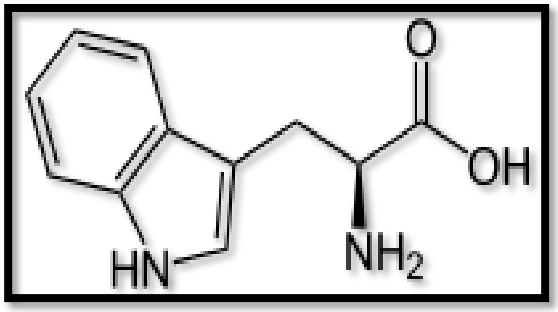



*Figure (2) : Structure of tryptophan*

***Experimental Apparatus & manifold variables***

Peristaltic pump ( two ways ) (Master flex Permer , USA) was used to transport reagents solution . Visible ( optima ) . Injection valve (six –three way , plastic , homemade ) which used to eliminate the amount of sample and reagents that used in this project , recorder which records the response as peak height ,class reaction coil with length 50 cm for arginine and tryptophan system and Flow cell 100 µl from quartz made specifically for flow injection system which contain input and output and 1 cm bath length which used for the absorbance measurements. Various types of tubes made of teflon were used for connection the portions of the system . The absorbance was measured at λmax 501 nm for arginine and 522 nm for tryptophan .

## *Chemicals and reagents*

All the chemicals used were of analytical grade and all the solutions were prepared with distilled water .

### *a/ for tryptophan reaction*

***Tryptophan Stock solution*** **(500µg.ml$^{-1}$ = 2.4 x10$^{-3}$M ) :** A 0.05 g amount of pure Tryptophan (BDH) was dissolved in deionized water then completed to 100 ml in volumetric flask with deionized water More dilute solutions were prepared by suitable dilution of the stock standard solution with deionized water .

***Diphenylamine sulphonate*****(1 x10$^{-3}$M ) :** An aliquot corresponding to 0.0271 g of DASA (BDH) was dissolved in 100ml volumetric flask with deionized water **.**

***Sodium nitrite*** **(2x10$^{-3}$ M) :** A (0.0138 g) amount of $NaNO_2$ (Merck) was dissolved in a 100 ml volumetric flask with deionized water

***Sulfuric acid*** **(BDH) (2M) :** Was prepared by diluting 10.9 ml of 18.4M of concentrated Sulfuric acid (BDH) with deionized water in 100ml volumetric flask .

***Sulphamic acid*** **(SDI) (2x10$^{-3}$ M ) :**Prepared by dissolving 0.0194 gm of sulphamic acid in deionized water and completed the volume to a 100ml in volumetric flask with deionized water, the solution stored in refrigerator avoiding direct light and used within one week .

***b/ for arginine reaction***

***standard solution of L-arginine*** (174.2 g.mol-1, BDH) (2000 µg.ml-1 = $1.15\times10^{-2}$ $g.mol^{-1}$) was transferred to a 200 mL volumetric flask and was dissolved and completed to the mark with distilled water, more dilution were made when it were necessary.

***stock solution of α-naphthol*** (M.w. = 144.17 g.mol-1, BDH) ($1.4\times10^{-3}$M) was prepared by dissolving 0.02 g of α-naphthol in 95 mL of ethanol, shacked well and completed to 100 mL in volumetric flask with distilled water. Taking 20 mL of this standard solution and diluted with distilled water in 100 mL volumetric flask.

***stock solution of sodium hydroxide*** (40 $g.mol^{-1}$, BDH) (2.5 M) was prepared by dissolving 10 g of NaOH in 100 mL volumetric flask in distilled water.

***10% urea*** was prepared by dissolving 10 g of urea (BDH) in 100 mL of distilled water in calibrated flask to prepare 1.67 M urea.

***Sodium hypobromite*** (0.25 M NaOBr): was prepared by dissolving 5 g of NaOH (1.25 M) in 100 mL distilled water, then added 0.64 mL Br2 (wt = 2 g, density = 3.103 $g.cm^{-3}$) .

***Pharmaceutical preparations of Tryptophan* (500µg.ml $^{-1}$ )**

Pharmaceutical preparations were obtained from commercial sources .

**1.** Sundown Naturals, 5-HTP ( L-5-hydroxy tryptophan), Dietary supplement 200mg, Supports a calm and Relaxed mode USA.

**2.** Noxidrim(5-HTP) Complement Alimentire 100mg (SOLGAR ) USA.

**3.** Natural (5-HTP) TR Time Release USA 200mg , Dietary supplement.

***Pharmaceutical preparations of L-arginine (500µg.ml $^{-1}$)***

Dosage forms were obtained from commercial sources available tablet by selecting thirteen tablets from three types companies were analyzed by the developed methods. The names of the different suppliers, these included:

1- Argi power (1500 mg of L-arginine HCl), OLIMP sport nutrition , 120 caps., EU .
2- Arginine POWER (800 mg of USP L-arginine supplement facts), ULTIMATE NUTRITION, 100 caps., USA .
3- Ezerex, (2500 mg of L-arginine HCl), 30X6.4 g sachets, Italy.

To determine the content of 5-hydroxy tryptophan and L-arginine in capsules , the average weight were determined by selecting 13 tablets randomly from different packets. The tablets were weighted and the Hard gelatin capsules were removed and the contents were finely powdered then weighing an amount equivalent to 0.05g for each drug . The powder was dissolved in deionized water for tryptophan and in distilled water for L-arginine drugs , transferred into a 100ml volumetric flask , and completed to the mark with the same solvent . Then the solution was filtered to remove any insoluble residue affecting on the response . A further appropriate diluted solution were made up by dilution with deionized water for tryptophan and distilled water for arginine to allocate the concentration within the linearity of the calibration curve .

***Methodology for L-arginine system***

The manifold system for estimation of L-arginine via spectrophotometric reaction with α-

naphthol to obtain red coloured product was composed from one line as shown in figure (3) .

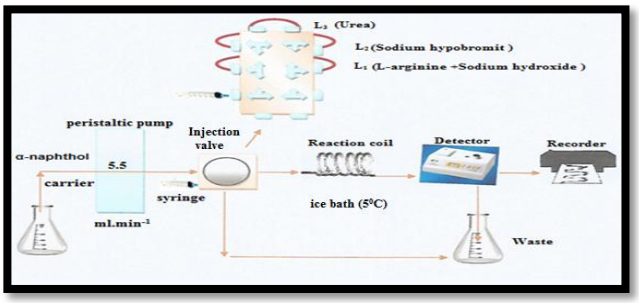
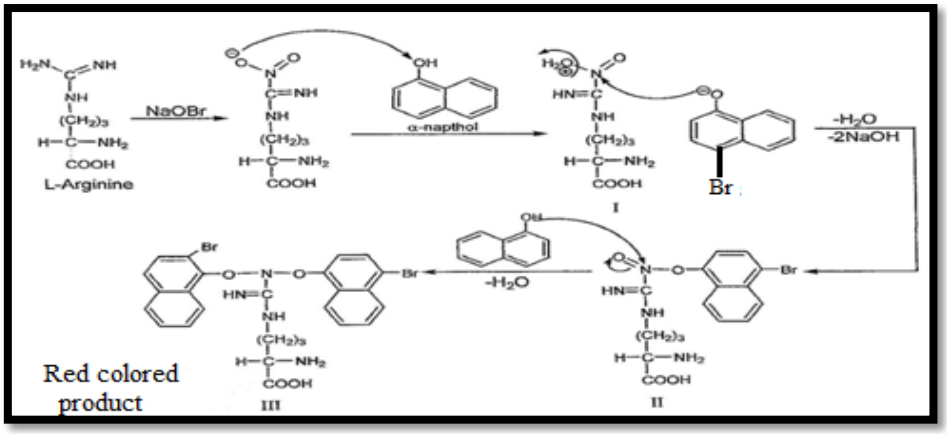



***Figure (3) :The manifold system for determination of L-arginine by CFIA- merging zones technique***

The proposed system of CFIA was consist of the carrier stream (distilled water ) leading to injection valve which contain three loop (different loop length with 0.5mm I.D.) that fills by the sample and reagents according to the order (L-arginine with few drops of sodium hydroxide , L1) , ( α-naphthol and urea , L2) and ( sodium hypobromite , L3) . The flow rate of the system was 5.5 ml.min-1 and the injection sample was 42.19µl .

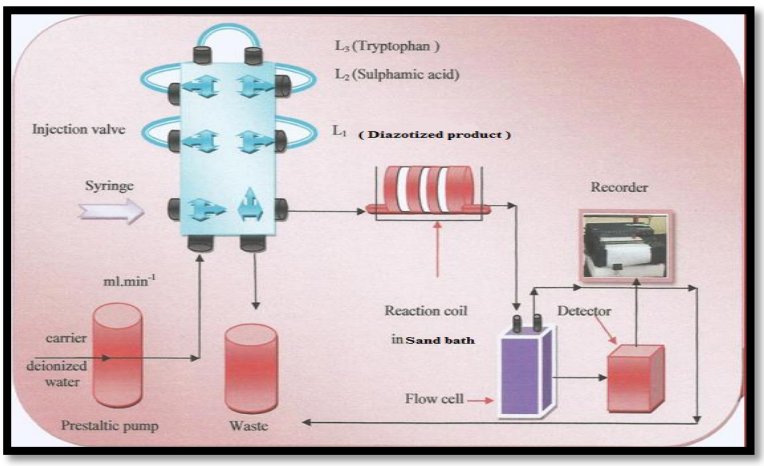
The response was observed by using spectrophotometer which was sensitive for any changed in absorbance and by recorder which record the response as peak height in mV . Each solution was measured for three time . The proposed mechanism was as follows in scheme (1).




***Scheme(1) The proposed mechanism of Sakachuci reaction for determination of L-arginine***

***Methodology for tryptophan system***

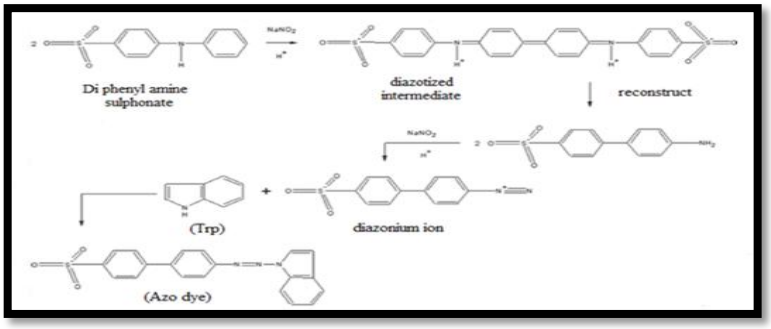
The manifold system for estimation of tryptophan via spectrophotometric condenses with diazotization product to obtain pink coloured product was composed from one line as shown in figure (4) .




***Figure(4) :The manifold system for the estimation of tryptophan by CFIA – merging zones technique***

The proposed system of CFIA was consist of the carrier stream (deionized water) leading to injection valve which contain an azotized product ( Diphenyl amine sulphonate with sulphuric acid & sodium nitrite ) ($L_1$ ) , sulphamic acid ( $2x10^{-3}$ M, $L_2$ ) ,and Tryptophan solution ( 5-100 µg .ml $^{-1}$ , $L_3$ ) respectively. The flow rate of the system was 2.7 $ml.min^{-1}$ and the injection sample was 54.95µl .

The response was observed by using spectrophotometer which was sensitive for any changed in absorbance and by recorder which record the response as peak height in mV . Each solution was measured for three time . The proposed mechanism was as follows in scheme (2) .




***Scheme (2) Reaction sequence mechanism of tryptophan***

***Result and Discussion Spectroscopic study***

***For L-arginine system***

Using optimum chemical as well as physical conditions achieved in previous experiments . A series of volumetric flask capacity (25mL) containing increasing volumes (0.25 – 11.25mL) standard solution of arginine 100µg.mL-1 with few drops of sodium hydroxide(1M) , add 1mL of α-naphthol($8x10^{-4}$M ) & mixed well .

Then put the volumetric flasks in ice bath for 4-6 min . Add 0.1mL of sodium hypobromite (0.1M) and the flask was shaken , for 5 min , then added 1mL of urea( 0.3M) and thoroughly mixed . The solutions were diluted to mark with distilled water and mixed well . The colored complex of arginine was developed and measured at λmax501nm against the reagent blank prepared in the same way without arginine [27] . Each measurement was repeated three times .The standard curve was constructed for the estimation of arginine and found that the extent of the concentrations which obeys Beer's law over the concentration range of 1–45µg.mL$^{-1}$

***For Tryptophan system***

Using the optimum physical as well as chemical parameters achieved in previous experiments in to a set of calibration flasks (25ml ) added (1.1ml) of diphenyl amine sulphonate(9x10$^{-4}$M) and put the flasks in ice bath to get a fixed temperature about 50C , then added 10.7ml of sulphuric acid (3.5M) into all flasks and mixed gently . After 5 min were added 1.25ml of sodium nitrite (4x10$^{-3}$M) and the mixture was put in ice bath for 5 min , 0.15 ml of sulphamic acid (0.5M ) solution were added and the mixture remain in ice bath with occasional shaking for 5 min , then were added appropriate volume of tryptophan solution(1000 µg.ml$^{-1}$) to the flasks to gate 3-40 µg.ml $^{-1}$ after dilution and made the volume up to 25 ml by adding deionized water , mixed completely & incubated for 15min in water bath at 30$^{0}$C .The pink colored product was measured in the range of 100-1100 nm by UV-visible spectrophotometer. The product showed higher absorbance at $\lambda_{max}$ 522 nm [28]

***Optimization of Experimental Parameters for L-arginine system***

***Effect of α-naphthol concentration***

A series of solutions (1x10$^{-4}$ – 1.5x10$^{-3}$ M ) were prepared of α-naphthol . Using flow rate 4 mL min-1 ,with 54.95µl of 35 ppm arginine as injected sample volume . All measurements were repeated for three successive times . figure (5) shows that 1.4x10$^{-3}$M of α-naphthol is the optimum concentration and was used in subsequent experiments .

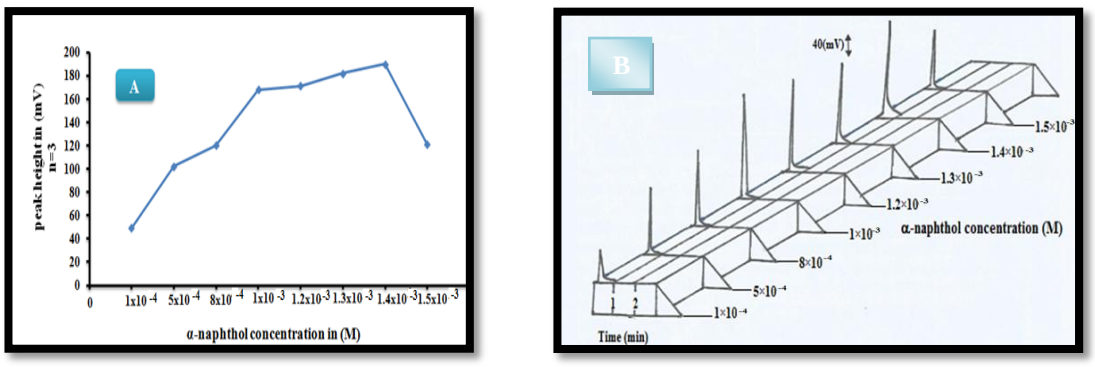



***Figure (5) ) (A)Effect of α-naphthol concentration on peak height using Sakaguchi reaction for determination of L-arginine , (B) Response profile .***

### *Effect of sodium hydroxide concentration*

The effect of sodium hydroxide concentration on the sensitivity was investigated by using optimum concentration of α-naphthol $1.4x10^{-3}M$ series of diluted solution of sodium hydroxide (0.1-3M) were prepared , 54.95 µl sample volume used . The data obtained were plotted as shown in Figure (6) . A 2.5M was chosen as the best concentration .

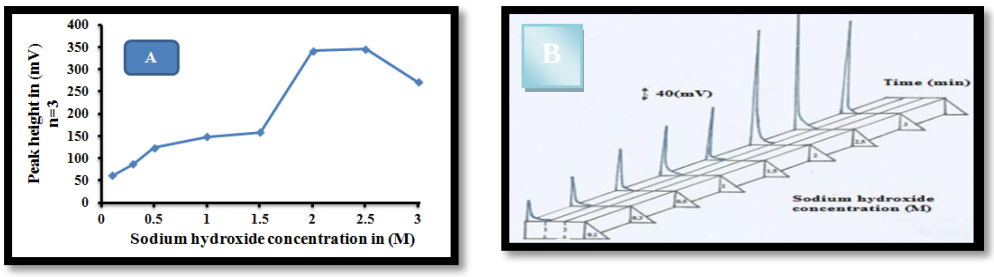



*Figure (6) (A)Effect of sodium hydroxide concentration on peak height using Sakaguchi reaction for determination of L-arginine , (B) Response profile*

## *Effect of urea concentration*

The effect of urea concentration was investigated , within range of concentration (0.1-1.72M ) of urea was used in the experiment . Figure (7a&b)which shows that an increase on the peak height with an increase in urea concentration which was added in order to destroy the excess of hypobromite and to increase the time of stability of the complex formed [28] . It can be seen that a 1.67M of urea was the optimum concentration and was chosen for further use .

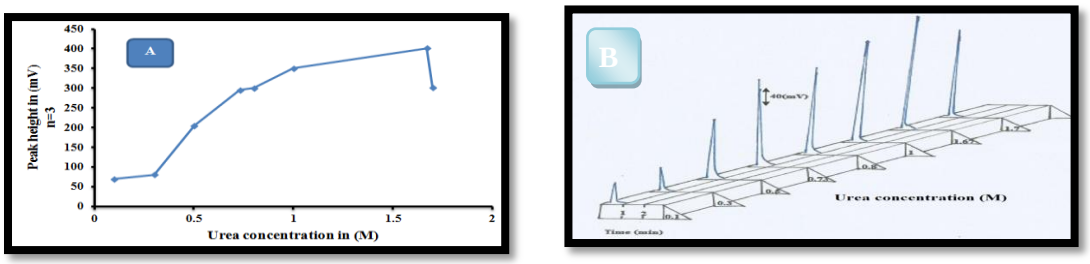



*Figure (7) (A) Effect of urea concentration on the reaction of L-arginine with α-naphthol using NaOBr as oxidizing agent (B) response profile .*

## *Effect of sodium hypobromite*

Various concentrations (0.01-0.4M) of sodium hypobromite used as oxidizing agent on the analytical signal was investigated and the result indicated that the best concentration of sodium hypobromite is 0.25M, the optimum sodium hypobromite concentration was selected to be 0.25M as shown in figure (8a&b) .

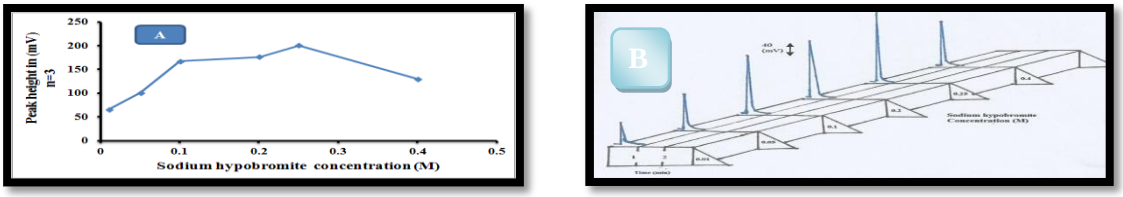



*Figure (8) (A) Effect of sodium hypobromite concentration on the reaction of arginine with α-naphthol , (B) Response profile*

## *Effect of manifold variables for L-arginine system*

### *Effect of Flow Rate*

The effect of flow rate was studied under the optimum chemical parameters . The results obtained shows that the ideal flow rate of pump ( the extreme height of peak and least dispersion ) will be in 5.5 mL .min$^{-1}$ . At lower flow rate, the dispersion will be the highest level whereas at greater flow rate, the reaction may be not complete as shown in figure (9a&b) .

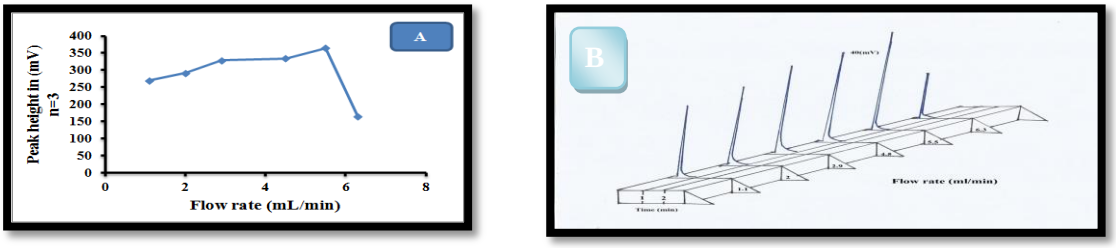



***Figure (9) (A) Effect of flow rate on the reaction between arginine with α-naphthol using sodium hypobromite as oxidizing agent ,(B) Response profile .***

### *Effect of sample and reagents volumes*

The injected volume of sample and reagents were studied by using different sample and reagents volumes (70.25, 54.95, 43.175, 42.19) µl ; using open valve mode .The results obtained show that injected volumes of 42.19 , 43.175 and 54.95µl for sample volume (35µg.mL$^{-1}$)with 2.5M NaOH [$L_1$] , 1.4x10$^{-3}$M α-naphthol in 1.67 urea[$L_2$] and 0.25M NaOBr [$L_3$] , respectively were optimum volumes to gave the highest response as shown in figure (10 a&b) .

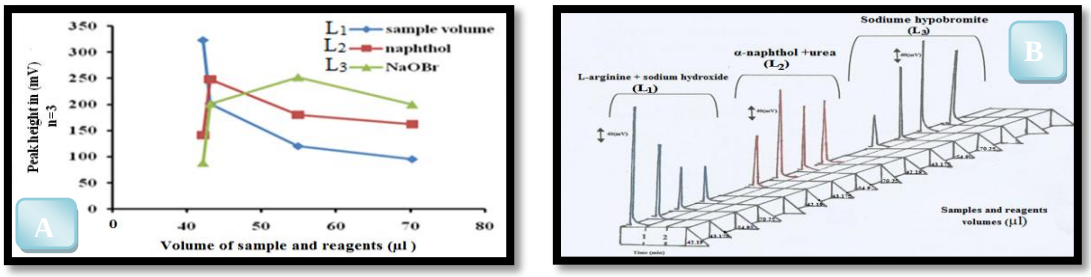



***Figure (10) (A) Effect of sample and reagents loops on the response of arginine complex , (B) Response profile.***

### *Effect of reaction coil*

The effect of different reaction coil lengths ( 50 , 100 , 150 , 200 ) with (i.d. 2 mm) on the reaction of arginine was examined . It was found that the peak height was decreased with the reaction coil length up to 50 cm as shown in figure (11 A&B) . a sharp decline in the peak height was observed above this value because of the dispersion phenomena . There for , a 50 cm gave the maximum peak height and was chosen as optimum .

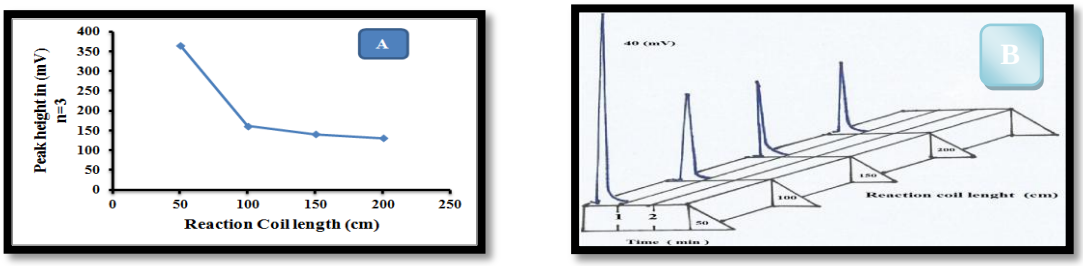



*Figure (11) (A) Effect of Reaction coil on the response expressed as peak height in mV of the reaction of arginine , (B) Response profile*

## *Effect of Temperature*

For studying the reaction between L- arginine and α-naphthol at different temperatures ( 0 – 45$^0$C ) to observe the effect of this factor on the progress and behavior of the chemical reaction by using ice bath . As shown in figure (12 A&B) , the appropriate temperature was (5$^0$C) which gave the highest signal which represented by peak height in (mV).

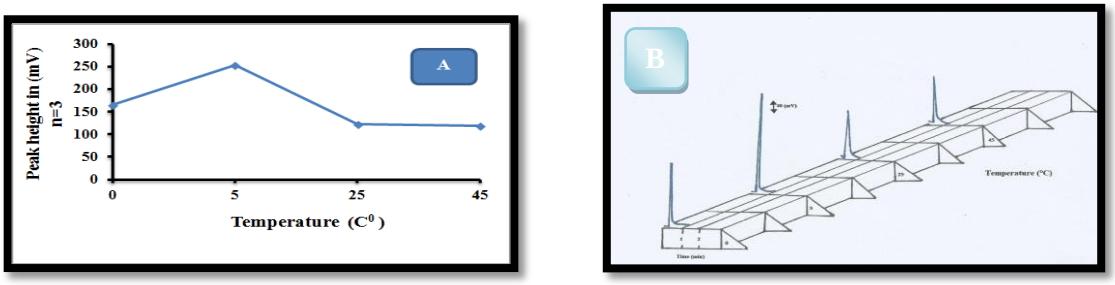



*Figure (12) (A) Effect of Temperature (C$^0$) , (B)Response profile*

## *Purge Time*

Purge time for the sample segment to be injected via the carrier stream (distilled water ) was studied , using the ideal chemical and physical parameters were studied previously , (5,10,15,20,25,30,35) sec and open valve ( injected mode) ) were used for this study . Figure (13 A&B ) shows that the purge time more than 35 sec giving a highest response intensity . For this reason open valve was selected as optimum purge time to complete transportation of sample from sample loop to flow cell .

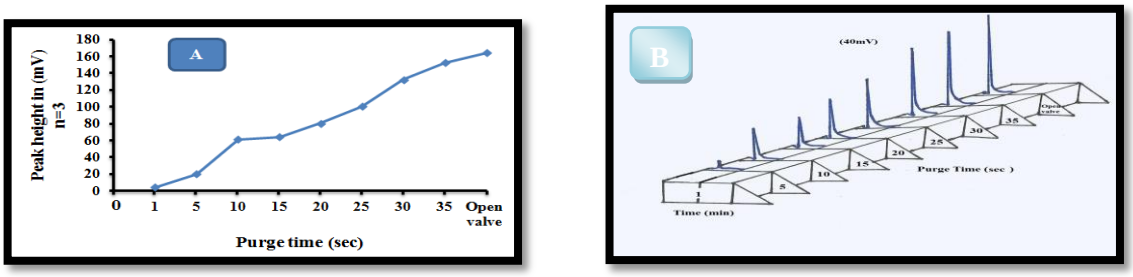



*Figure (13) (A) Effect of purge time , (B) Response profile*

***Optimization of Experimental Parameters for tryptophan system***

***Effect of chemical variables***

***Effect of Sulphuric Acid Concentration***

The influence of sulphuric acid concentration ( 0.1-3 M) which was used as a medium for the reaction on the response intensity was studied . The results shows that the optimum concentration for sulphuric acid is 2M as shown in Figure (14 A&B ) .

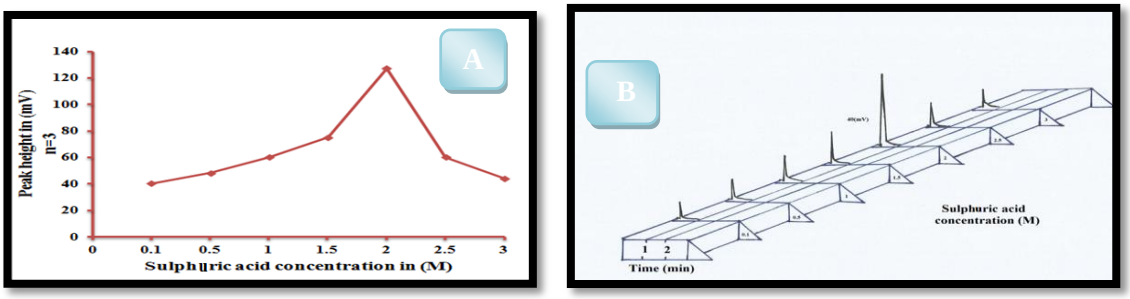



*Figure (14) (A) Effect of Sulphuric acid concentration , (B) Response profile*

***Effect of Sodium nitrite concentration***

Influence of sodium nitrite concentration was studied . Variable concentration (2.5 $x10^{4-}$ – $4x10^{3-}$M ) of sodium nitrite was used in the experiment . The results obtained are shown in Figure (15A&B)which shows that an increase on the peak height with an increase in the sodium nitrite concentration which was added in order to oxidize the reagent DASA in the presence sulphuric acid as a medium . It can be seen that a $2x10^{-3}$M of sodium nitrite was the optimum concentration and was chosen for subsequent experiment .

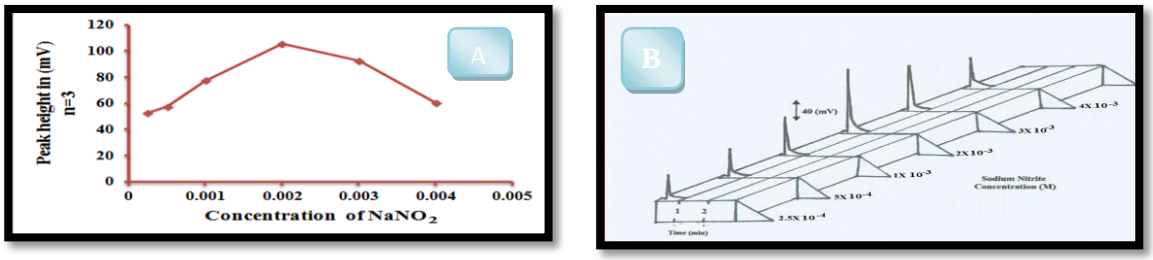



*Figure (15) (A)Effect of sodium nitrite concentration on peak height for determination of tryptophan , (B) Response profile*

***Effect of DASA concentration***

A series of solutions ($6.25x10^{-5}$- $8x10^{-4}$ M ) were prepared of DASA , all measurements were repeated for three successive times . The results obtained are shown in Figure (16A&B) shows that $5x10^{-4}$ M DASA is the optimum concentration . An increase in concentration up to $5x10^{-4}$ leads to decrease in response intensity ; this causes a decrease in peak height , due to increased dispersion which leads to a significant decrease in peak height .

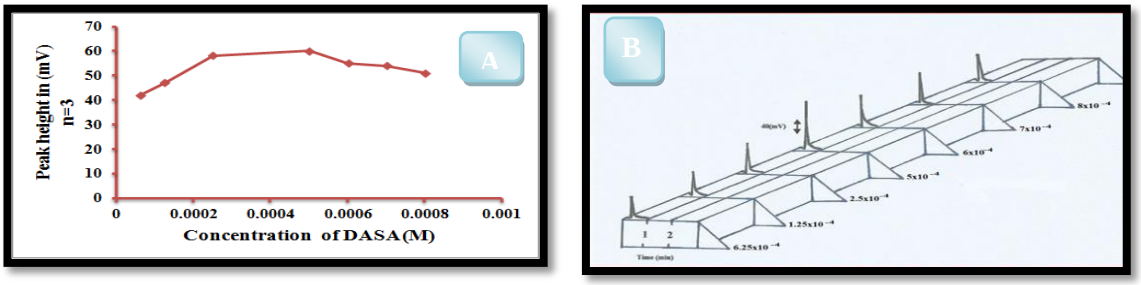



*Figure (16) (A) Effect of DASA concentration on the response intensity ,(B) response profile*

**Effectof Sulphamic Acid Concentration**

The influence of sulphamic acid concentration on the sensitivity generally was studied , series of diluted solution of sulphamic acid ($2.5x10^{-4}$ – $6x10^{-3}$ M ) were prepared . The data obtained were plotted as shown in figure (17 A&B) . A $1x10^{-3}$ M was selected as the ideal concentration of sulphamic acid for the work conducted in the research .

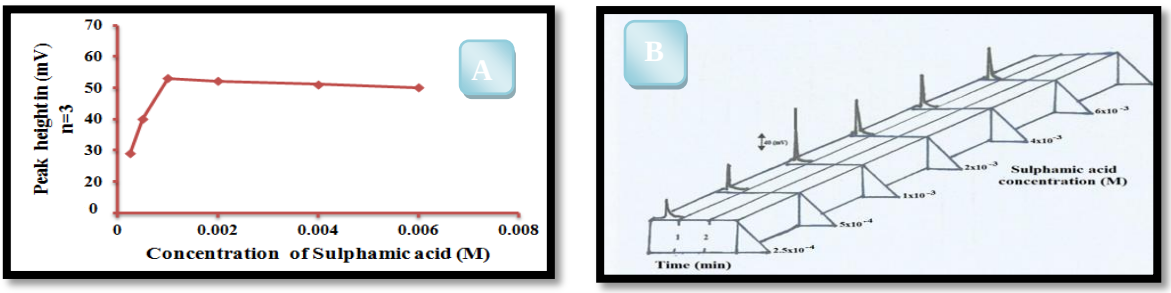



**Figure (17) (A)Effect of sulphamic acid concentration on peak height for the determination of tryptophan , (B) Response profile.**

**Effect of manifold variables**

**Effect of Flow Rate**

The effect of flow rate was studied under ideal chemical parameters . The results of the study shows that the perfect pump flow rate which gave the higher value of peak height and lower value of dispersion will be in 2.7 ml $.min^{-1}$ . At low flow rates , the dispersion will be the highest level whereas at a high flow rates, increase dilution occurs and then heterogeneity in the merging zones of the solution for carrier which hold sample , high speed affect in the process of mixing where it is irregularly spread and consists regions which the mixing high and the other which the mixing low, leading to a lack of sensitivity and this leads to a decrease in peaks height as shown in figure (18 A&B) .

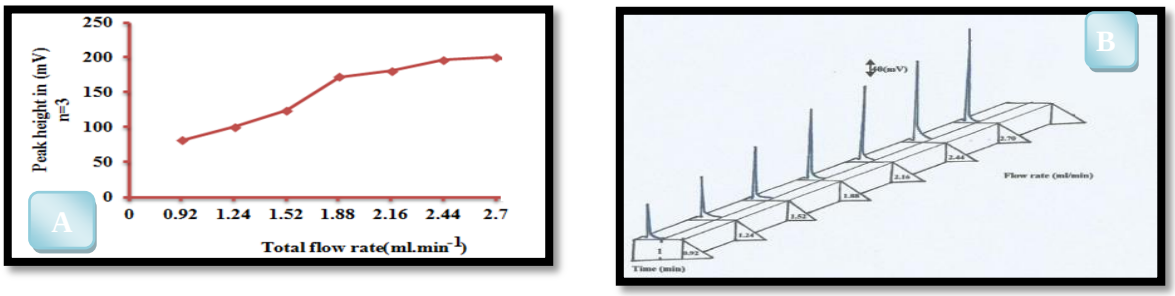



**Figure (18) (A) Effect of flow rate on the response intensity , (B) Response profile**

**Effect of sample & reagents volumes**

The injected volume of sample and reagents were investigated by using various sample and reagents volumes (70.25, 54.95, 43.175, 42.19) µl ; using open valve mode .The results obtained showed that injected volumes of 42.19, 43.175, and 54.95µl for azotized product [$L_1$] , sulphamic acid ($1x10^{3-}$ M) [$L_2$] and 20µg.ml$^{-1}$ sample of tryptophan [$L_3$] respectively were ideal volumes to gave the highest response as described in figure (19 A&B) .

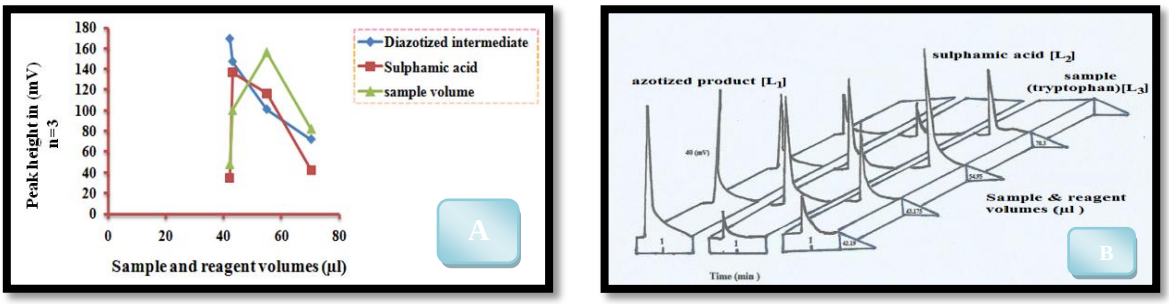



**Figure (19) (A) Effect of sample and reagents volumes on the response expressed as peak height in mV , (B) Response profile**

**Effect of reaction coil *length***

The effect of reaction coil length on the response intensity of the colored product , using various lengths of reaction coil ranging (50-200 cm ) .The results revealed in figure (20 A&B ) that 50cm produced the highest absorbance and used in subsequent experiments.

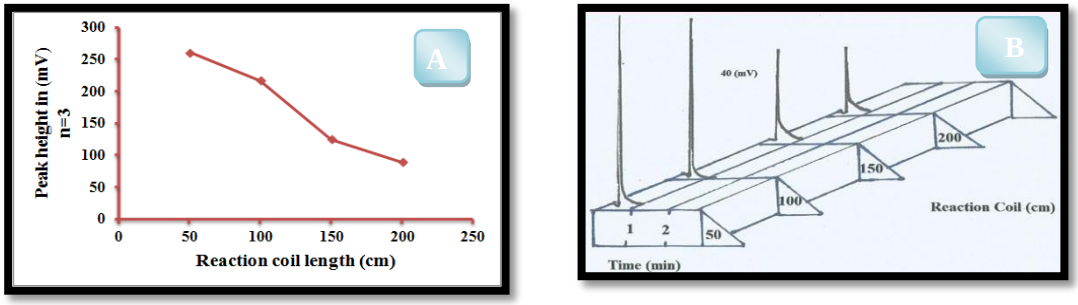
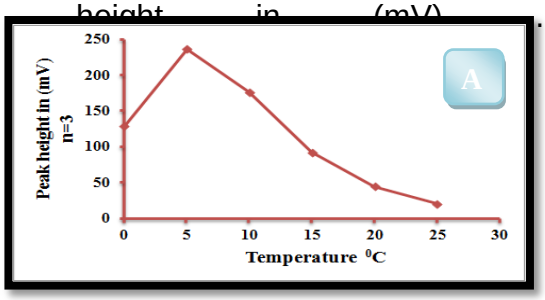



**Figure (20) (A) Effect of reaction coil length on the response intensity of the reaction 0f tryptophan (B) Response profile**

## *Effect of Temperature*

The study of the reaction between DASA with sodium nitrite to obtain diazotized intermediate in a range of temperatures (0 –25$^0$C ) was observed the impact of temperature on the progress of the chemical reaction. As referred in figure (21 A&B) , the appropriate temperature was 5$^0$C which granted the highest response expressed as peak height in (mV)



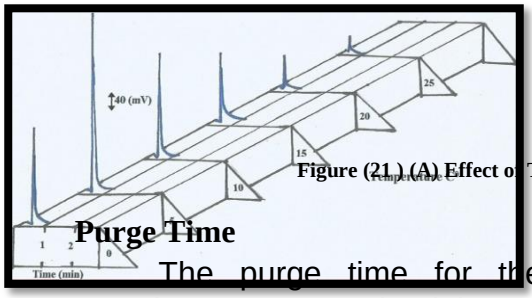
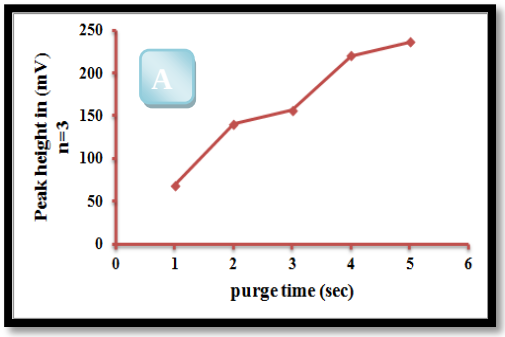



**Figure (21 ) (A) Effect of Temperature ($C^0$) , (B)Response profile**

## Purge Time

The purge time for the sample segment to be injected via the carrier stream (deionized water ) was studied , using the ideal chemical and physical parameters were previously studied , (35 – 50) sec and open valve ( injected mode) were used for this study . Figure (22 A&B ) shows that the purge time was more than 50 sec gave a highest response intensity . For this reason open valve was selected as the optimum purge time to complete transmission of sample from sample loop to flow cell .



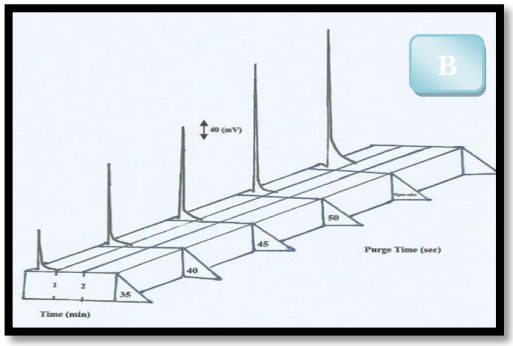




***Figure (22) (A) Effect of purge time , (B) Response profile***

## *Calibration graph*

A set of arginine and tryptophan solutions ( 3-1400µg.mL$^{-1}$) and (5-100 µg.mL$^{-1}$ ) respectively were prepared by a suitable dilution of stock solution . All chemical and physical parameters were fixed at their optimum values . Each measurement was recurrent three times .

The response which represented by peak height in (mV) plotted against the concentrations of arginine and tryptophan . The results obtained were displayed in figure (23,24) which offers the contrast of response with concentration of arginine . Data were processed mathematically[29,30] and will clarify the method was used to calculate the linear equation of the class y = a + bx

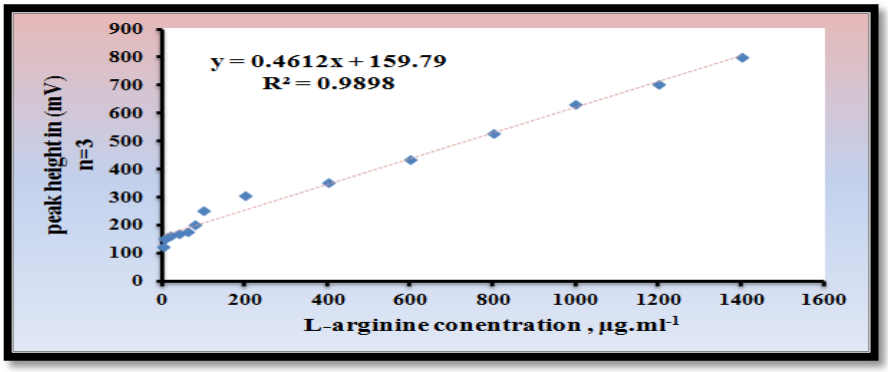



**Figure (23) Linear calibration graph for estimation of L- arginine using CFIA –Merging zones system**

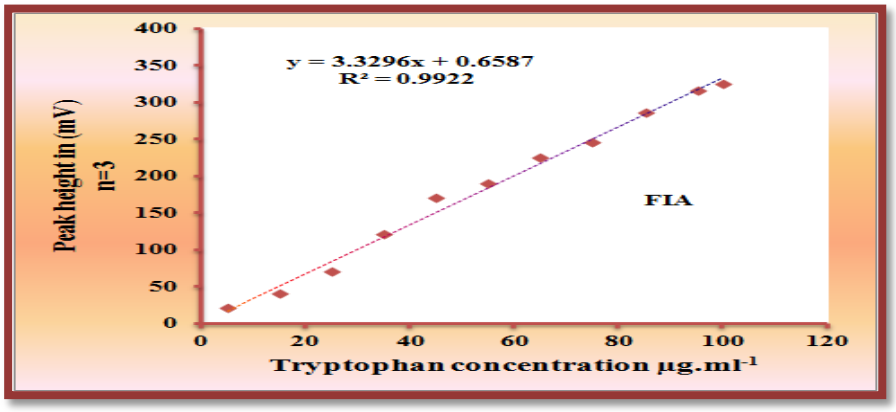



**Figure (24) : Linear calibration graph for estimation of tryptophan by CFIA / Merging zones system**

The data obtained from calibration graph treated statistically by using ANOVA and the result obtained shown that there is an important relation between the concentration of arginine or tryptophan and the response obtained [31,32].As shown in table (19,20 )

**Table (19) ANOVA for linear equation results for L-arginine system**

| Source | Sum of squares | $D_f$ | Mean square | $F_{stat.} = S_1^2/S_2^2$ |
|---|---|---|---|---|
| Regression | $\sum(\hat{y}_i-\bar{y})^2$ = 699086.1 | $V_1$ = 1 | 699086.1 | 1263.845 |
| Error | $\sum(\bar{y}_i-\hat{y}_i)^2$ =7190.849 | $V_2$ = 13 | 533.1422308 | |
| Total | 706276.949 | 14 | | |

$F^{v_1}_{v_2} = F^{v_1}_{v_{13}} = 4.667 << F_{stat.} = 1263.845.$

Table (20) ANOVA for linear equation results for tryptophan system

| Source | Sum of squares | $D_f$ | Mean square | $F_{stat.} = S_1^2/S_2^2$ |
|---|---|---|---|---|
| Regression | $\sum(\hat{y}_i-\bar{y})^2$ = 116656.7 | $V_1$ = 1 | 116656.7 | 1151.889 |
| Error | $\sum(y_i-\hat{y}_i)^2$ = 911.4687 | $V_2$ = 9 | 101.2743 | |
| Total | 117525.411 | 11 | | |

$F^{v_1}_{v_2} = F^{v_1}_{v_9} = 5.117 << F_{stat.} = 1151.48$ .

The limit of detection was calculated by using three different methods and the resulting obtained were tabulated in table (21,22 )

**Table (21) summarized all calculation value of detection limit for L-arginine system .**

| Gradual dilution for the minimum concentration in calibration graph | Based on the value of slope $x=\frac{3S_B}{slope}$ | Linear equation $\hat{y}(mV) = y_B + 3S_B$ |
|---|---|---|
| 1 µg.mL$^{-1}$<br>1x10$^{-3}$µg/µl<br>1ng/µl | 521.48 µg.mL$^{-1}$<br>0.52148µg/µl<br>521.48ng/µl | 399.402 µg.mL$^{-1}$<br>0.339402 µg/µl<br>339.402 ng/µl |

$y_B$ = average response for the blank solution (equivalent to intercept in straight line equation , $S_B$= standard deviation of blank solution , x = value of L.O.D. based on slope.

**Table (22) Summarized all calculation value of detection limit for tryptophan system .**

| Gradual dilution for the minimum concentration in calibration graph | Based on the value of slope $x=\frac{3S_B}{slope}$ | Linear equation $\hat{y}(mV) = y_B + 3S_B$ |
|---|---|---|
| 1 µg.ml$^{-1}$<br>1x10$^{-3}$µg/µl<br>1 ng/µl | 0.0198 µg.ml$^{-1}$<br>1.98x10$^{-5}$µg/µl<br>0.0198 ng/µl | 91.23 µg.ml$^{-1}$<br>0.09123µg/µl<br>91.23 ng/µl |

## Repeatability

The repeatability of the proposed methods were examined by carrying out ten injection sample measurements for two concentration of L- arginine and tryptophan ,the accuracy and efficiency of the developed systems to determine the amino acid (l-arginine and tryptophan ) under

consideration .As indicated in figures (25,26) .

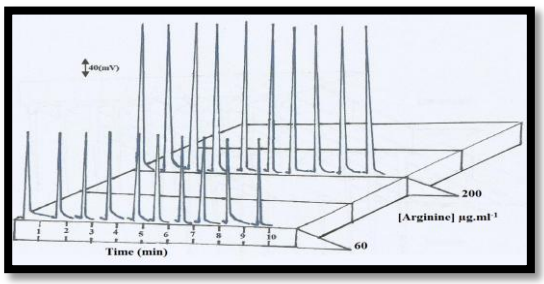



**Figure (25) Repeatability profile for determination of L-arginine by Sakachuci reaction using CFIA / Merging Zones system**

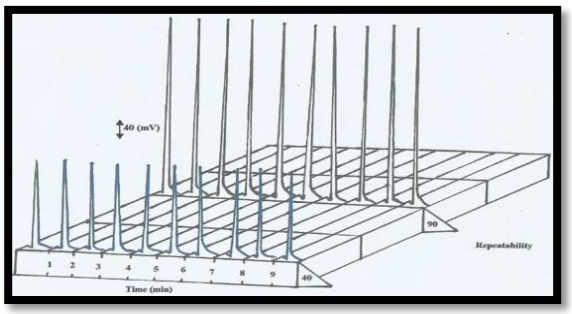



**Figure (26) Repeatability measurements profile for determination of tryptophan by azo-coupling reaction using CFIA/Merging zones system .**

## Pharmaceutical Applications for L-arginine and tryptophan

The proposed method was used to determination of L-arginine and tryptophan in pharmaceutical formulations (tablets ) available in the markets . the standard addition method was applied to the analysis of each amino acid (L-arginine and tryptophan ) in pharmaceutical formulations . Results were mathematically treated for standard additions method . The results were tabulated in Tables (25,26)

**Table (25) Application of the developed method for estimation of L-arginine in pharmaceutical preparations**

| Pharmaceutical preparations | Conc. of L-arginine µg.mL$^{-1}$ | | *$E_{rel}$ % | *Rec% | *RSD% |
|---|---|---|---|---|---|
| | Present | Found | | | |
| **Arginine Power (1.5g L-arginine HCl) MEGA caps , EU** | 20 | 19.99 | -0.05 | 99.95 | 1.00 |
| | 40 | 40.10 | 0.25 | 100.03 | 0.25 |
| **Arginine Power (0.8g L-arginine supplements facts), USA** | 20 | 20.12 | 0.6 | 100.6 | 0.07 |
| | 40 | 40.16 | 0.4 | 100.4 | 0.02 |
| **Ezerex(6.4g L-arginine /sachets) (Sigma-Tau), Italy** | 20 | 20.019 | 0.095 | 100.1 | 1.40 |
| | 40 | 40.2 | 0.5 | 100.5 | 0.02 |

*Average of three determination

**Table (26) Applications of the developed method for the determination of tryptophan in pharmaceutical preparations**

| Pharmaceutical preparation | Conc. of tryptophan µg.ml$^{-1}$ | | *$E_{rel}$ % | *Rec% | *RSD% |
|---|---|---|---|---|---|
| | Present | Found | | | |
| Sundown Naturals 5-HTP ( L-5-hydroxy tryptophan) Dietary supplement 200mg Supports a calm and Relaxed mode (USA) | 20 | 20.15 | +0.75 | 100.75 | 0.297 |
| | 50 | 49.91 | -0.18 | 99.82 | 0.082 |
| Noxidrim(5-HTP) Complement Aliment ire 100mg (Solcar ) (USA) | 20 | 19.99 | -0.05 | 99.95 | 0.05 |
| | 50 | 50 | 0.0 | 100 | 0.014 |
| Natural (5-HTP) TR Time Release (USA) , 200mg , Dietary supplement | 20 | 19.99 | -0.05 | 99.95 | 0.1 |
| | 50 | 50.16 | +0.32 | 100.32 | 0.558 |

*Average of three determination

## Assessment of developed methods

For assessment the success and the accuracy and precision of the developed methods , the result s obtained by the CFIA method were compared with those obtained by standard method [33, 34] .The results obtain by two various methods were statistically compared , using the variance ratio F-test & the t student – test at confidence limit 95% in all cases [35] . The calculated F- and t- values did not exceed the theoretical values which showed that there was no significant variations between the two methods in precision and accuracy for estimation of L- arginine and tryptophan in pharmaceutical preparations . The results are tabulated in tables (27,28) .

**Table (27) The comparison of the Merging –zones / CFIA method with standard method using F- and t- statistical tests**

| Pharmaceutical preparations | Proposed methods<br>Merging Zones /CFIA | | Official method | | S | Value | |
|---|---|---|---|---|---|---|---|
| | *Rec% | $(x_i-\bar{x})^2_1$ | *Rec% | $(x_i-\bar{x})^2_2$ | | $t_{cal}$* (critical.) | $F_{cal}$** (critical.) |
| Arginine – pure | 100 | 0.04 | 100.00 | 0.031 | 0.05 | 0.704<br>2.447 | 1.09<br>9.28 |
| Arginine Power (2.5g L-arginine HCl) MEGA CAPS, EU | 99.99 | 0.0441 | 100.20 | $6.25x10^{-4}$ | | | |
| Arginine Power (0.8g L-arginine supplements facts), USA | 100.5 | 0.09 | 100.50 | 0.106 | | | |
| Ezerex (2.5g L-arginine, sachets) (Sigma-Tau), Italy | 100.3 | 0.01 | 100.00 | 0.031 | | | |
| | $(\bar{x}_1)$ = 100.2 | $\sum(x_i-\bar{x})^2_1$= 0.1841 | $(\bar{x}_2)$ = 100.175 | $\sum(x_i-\bar{x})^2_2$= 0.169 | $n_1+n_2-2=6$ | $n_1-1=3$<br>$n_2-1=3$ | |

*$t_{calculate}$ , ** $F_{calculate}$

S = Pooled standard deviation = $\sqrt{\frac{(n_1-1)s_1^2-(n_2-1)s_2^2}{n_1+n_2-2}}$ : $(n_1+n_2-2)$ = number of degrees of freedom . $s_1^2$ = variation = $\frac{\Sigma(x_{i-}\bar{x})^2_1}{n_1-1}$ . $s_2^2$ = variation = $\frac{\Sigma(x_{i-}\bar{x})^2_2}{n_2-1}$

$(n_1-1)$& $(n_2-1)$ = number of degrees of freedom of proposed method (Merging Zones /CFIA method ) and standard method respectively

$t = \frac{|\bar{x}_1-\bar{x}_2|}{s\sqrt{\frac{1}{n_1}+\frac{1}{n_2}}}$ , $F=\frac{s_1^2}{s_2^2}$ or $\frac{s_2^2}{s_1^2}$ , F> 1 , $s_1^2 = 0.061$ , $s_2^2 = 0.056$

**Table (28) Comparison of the FIA/merging zones method with standard method using F- & t- statistical tests**

| Pharmaceutical preparations | Proposed methods<br>FIA | | Official method | | S | Value | |
|---|---|---|---|---|---|---|---|
| | *Rec% | $(x_i-\bar{x})^2_1$ | *Rec% | $(x_i-\bar{x})^2_2$ | | $*t_{cal}$ (critical.) | $**F_{cal}$ (critical.) |
| **Pure tryptophan** | 99.98 | 0.012 | 100.05 | $2.5x10^{-3}$ | 0.069 | 0.256<br>2.447 | 1.40<br>9.28 |
| **Sundown Naturals 5-HTP ( L-5-hydroxy tryptophan) Dietary supplement 200mg, (USA) Supports a calm and Relaxed mode** | 100.28 | 0.036 | 99.95 | 0.022 | | | |
| **Noxidrim(5-HTP) Complement Aliment ire , (USA) 100mg (Solcar )** | 99.97 | 0.014 | 100 | 0.01 | | | |
| **Natural (5-HTP) TR Time Release USA 200mg , Dietary supplement** | 100.13 | $1.6x10^{-3}$ | 100.2 | 0.01 | | | |
| | $(\bar{x}_1)$ = 100.09 | $\sum(x_i-\bar{x})^2_1$= 0.063 | $(\bar{x}_2)$ = 100.1 | $\sum(x_i-\bar{x})^2_2$ = 0.044 | $n_1+n_2-2=6$ | $n_1-1=3$<br>$n_2-1=3$ | |

*t calculate , **F calculate ;

S = Pooled standard deviation = $\sqrt{\frac{(n_1-1)s_1^2-(n_2-1)s_2^2}{n_1+n_2-2}}$ : $(n_1+n_2-2)$ = number of degrees of freedom . $s_1^2$ = variation = $\frac{\Sigma(x_{i-}\bar{x})^2_1}{n_1-1}$ . $s_2^2$ = variation = $\frac{\Sigma(x_{i-}\bar{x})^2_2}{n_2-1}$ , $(n_1-1)$& $(n_2-1)$ = number of degrees of freedom of proposed method (spectrophotometric classical method ) and standard method respectively

$t = \frac{|\bar{x}_1-\bar{x}_2|}{s\sqrt{\frac{1}{n_1}+\frac{1}{n_2}}}$ , $F=\frac{s_1^2}{s_2^2}$ or $\frac{s_2^2}{s_1^2}$ , F> 1 , $s_1^2 = 0.021$ , $s_2^2 = 0.015$

## *conclusion*

Efficient and Simple flow injection analysis-merging zones method with spectrophotometric detection was proposed for the estimation of arginine and tryptophan in pharmaceutical formulation samples. In this FIA system, it is the reagent that is injected into the flowing system which is made up of the sample and some other solutions. This makes it possible to automate analytical systems This technique was also successfully adopted to eliminate background absorption from the sample matrix.

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