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New FIA –spectrophotometric methods for determination of arginine in grape juice samples via sakaguchi reaction after separation with strong cation –exchange resin

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ABSTRACT

Simple and sensitive batch and FIA-spectrophotometric methods for determination of L- arginine in grape juice samples from different origins, these methods were based on oxidation – condensation of L – arginine with α – naphthol and urea in presence of sodium hydroxide as a medium for amino acid reaction. A red color developed when adding sodium hypobromite as oxidizing agent for amino acid. The colored product was soluble in water and stable for more than 1 hour and the absorbtion was measured at a maximum absorbance at 501 nm. A graphs of peak height versus concentration show that Beer, s law was obeyed over the concentration range of 1 – 45 and 3 – 1400 μg.ml^{-1} of arginine with detection limits of 0.468 and 0.12 μg. ml $^{-1}$ of arginine for batch and FIA methods respectively. The optimized FIA procedure sample with a throughput of 45 sample / hour. All different chemical and physical experimental conditions affecting on the development and stability of the colored product were studied and the proposed methods were applied successfully for the determination of arginine in grape juice samples.

KEYWORDS

Arginine;
Flow injection;
α – naphthol;
Oxidation – condensation reaction;
Spectrophotometric determination.

INTRODUCTION

Arginine is a conditionally indispensable amino acid[1]. Arginine is a nitric oxide precursor. Nitric oxide is formed from arginine via the enzyme nitric oxide syntheses (NOS)[2]. A significant nutritional problem in preterm in fants is a severe deficiency of arginine (hypoargininemia) which results in hypermmonemia, cardiovascular, pulmonary as well as neurological and intestinal dysfunction. Arginine deficiency may contribute to the high rate of infant morbidity and mortality associated with premature[3]. Arginine one of the most abundant amino acids in grape juice, is closely related to the levels of ethyl carbamate in wine[4-6]. Ethyl carbamate also known as urethane occurs in wines and some other fermented foods and beverages and is classified as a possible human carcinogen[7,8]. In 1988, the American wine industry has established a voluntary target for EC below 15 μg. L^{-1} in table wines and below 60 μg.L$^{-1.}$ Arginine is degraded to urea and ornithine by the enzyme arginase via urea. Circle pathway[5]. Some urea is assimilated by the yeast and some is released in to the fermentation medium with excess arginine in grape juice. Accumulated from ethyl carbamate[9-11]. Citrulline formed by arginine metabolism via argi-

nine deiminase pathway by wine malolactic bacteria, is the second significant precursor of ethyl carbamate in wine[12,13]. Urea and Citrulline accumulation in the fermentation medium mainly depends on the arginine level and the sort of yeast and malolactic bacteria strains[11], if arginine concentration in juici is higher than 1000 μg. $L^{-1,}$ ethyl carbamate concentration will be potentially above 15μg.L^{-1} in wine[14] the current voluntary limit in the United State. To avoid potential health hazard of ethyl carbamate in wine, we must determination of the arginine levels in grape juice ; is one of the most important steps for wine makers to realize hazard analysis critical control point (HACCP) control.

A simple and accurate method for determining the arginine levels in grape juice is the prerequisite for wine makers to take proper measures. Various methods for arginine analysis involve high performance liquid chromatography[15-17] amino acid analyzer and capillary electrophoresis[18], indirect determination by graphate furnace atomic absorption spectrometry[19], fluorimetry[20], isocratic RP – HPLC[21,22], Chemiluminescence[23], MS coupling with atmospheric pressure chemical ionization[24], LC / MS[25]. These methods are mostly slow, expensive or required ion pair reagent, derivatisation or laborious sample preparation procedures. The novelty of present method is analysis of arginine through the Sakaguchi reaction is specific, sensitive and has been used for determining trace amount of arginine in biological samples. In our experiment, α-naphthol with urea was selected as the Sakaguchi reagent because the chromogenic product formed in the presence of alkaline hypobromite by the reaction between Sakaguchi reagents with arginine and has been stabilized by the reaction occurs at 0^0C and excess urea was added with α-naphthol when the color developed via FIA / Merging zones system and its absorbance as peak heigh (mv) was measured at 501 nm.

However, the composition of grape juice is so complicated that arginine cannot be directly measure by Sakaguchi reagents due to interference of other compounds. Therefore separation of arginine from grape juice is a very important pretreatment for quantitative analysis. Ion exchange is commonly used to separate amino acids based on their isoelectric points. Amino acids are ampholytes with pH dependent net charges. Arginine has the highest isoelectric point (pI = 10.8) among amino acids commonly found in grape juice. In acidic solution, Arg^{2+} and Arg^{1+} can be adsorbed by strong cation – exchange resins and eluted from the resin by alkaline solution[26].

The purpose of this study was developed simple, accurate and rapid arginine separation method in three types of grape juices from a different origins with Sakaguchi reaction. The developed method could have the application potential in routine quantification of arginine in grape growers to monitor and control the level of ethyl carbamate.

EXPERIMENTAL

Chemicals

All chemicals used were of analytical reagent grade and all solution were prepared with distilled water, freshly prepared solutions were always used.

A standard solution of L- Arginine

($C_6H_{14}N_4O_2$, M.wt. 147.2 g.mol^{-1}, BDH)(2000 μg.ml^{-1} = 1.15x10^{-2} M) was prepared (0.4 g) amount of pure arginine was dissolved in a mount of distilled water then completed to 200 ml in a volumetric flask with distilled water, more dilution were made when it were necessary.

A 10% urea

(CH_4N_2O, M.wt. 60.06 g·$mol^{"1}$, BDH) prepared by dissolving (10 g) of urea in 100 ml of distilled water in volumetric flask to prepare 1.67 M of urea.

A stock solution of α- naphthol

($C_{10}H_8O$, M.wt 144.17g. mol^{-1}, BDH) (1.4x10^{-3} M) 0.02g of α- naphthol are dissolved in 95 ml ethanol, and completed to 100 ml in a volumetric flask with distilled water, then take 20 ml of stock solution and completed to 100 ml in a volumetric flask with D.W.

A stock solution of sodium hydroxide

(NaOH, M.wt 40 g.$mol^{-1,}$ BDH) (2.5 M) was prepared by dissolving 10 g of pure sodium hydroxide in 100 ml distilled water in a volumetric flask.

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Sodium hypobromite

(NaOBr, 0.25M) prepared by dissolving (5 g) of pure sodium hydroxide (1.25M) in 100 ml distilled water, then added (0.64 ml) of Br_2 (Bromine). the solution stored in cold and dark.

Juice samples preparation

Grape samples it has been selected from imported samples in the domestic market sample (1) product of Ammerica importer by (farm – pik) company, sample (2) product of Australia imported by (SPT)(Southern Produce Traders), sample (3) product of Australia imported by (Happy Valley Fruits) company. The samples were cleaned by distilled water, crushed and the juice was clarified by centrifugation at 1100 rpm for 20 min at room temperature, then filtered, the filtrate was passed through filter paper to remove any precipitant in the juice.

Apparatus & manifold

All spectral and absorbance measurements were performed on optima visible SP-300 digital single beam recording spectrophotometer (Japan), for the absorbance measurements as peak height by kompensograph (Siemens) or absorbance with digital millimeter. A quartz flow cell with 75 µl internal volume and 1 cm bath length was inside the detection unit used for absorbance measurements. A one channel manifold was employed for the FIA/margining zones spectrophotometric determination of L-arginine. A peristaltic pump (Master flex C/L, USA) with power supply (Yaxun, 1501 AD, china) was used for to transport the solution, In addition, injection valve (six – three ways homemade which including three loops made of Teflon) were loaded with chemicals and the reagents solutions, injection valve was employed to provide appropriate volumes of standard solutions and samples. Flexible vinyl tubes of 0.25 mm internal diameter were used for the peristaltic pump. Reaction coil was made of glass with internal diameter of 2mm. Distilled water as carrier (ml. min^{-1}) was combined with injected sample (grape sample L_1), and they merged with the reagent(α- naphthol with urea L_2) in the presence of (sodium hypobromite solution L_3), then mixed in reaction coil with length 50 cm was placed in ice path, injection samples 42.19 µl, flow cell of carrier of 5.5 $ml.min^{-1}$, the absorbance as high peak was measured at 501nm.

For batch procedure, was carried out on shimadzu UV- 1800 (Japan) double beam spectrophotometer and quartz cuvette with an optical path length of 1 cm.

For separation of arginine from grape juice we used strong cation exchange resin, Amberlite IR120 ($4.2mmol.Kg^{-1}$)resin (Na^+), particle size 0.4-1.2mm, 46-52% were selected on a preselect ion of different resins provided by several producers. New resins were pretreated with 2 M HCl for 2 hour and then washed with distilled water. Pretreated resin were packed in a column (1.0 cm i.d. x30 cm). Sample (50.0 ml, pH 2.0) was loaded to the column at the rate of 0.5 $ml.min^{-1}$, then rinsed with distilled water, Finally the sample was eluted with (1 M)NaOH solution at the rate of 2 $ml.min^{-1.}$

Reaction mechanism of the proposed method

The proposed mechanism that sodium hypobromite oxidizes the guanidine group of arginine and the resulting compound condenses with α- naphthol. a colored solution was obtained as shown in scheme (1) :

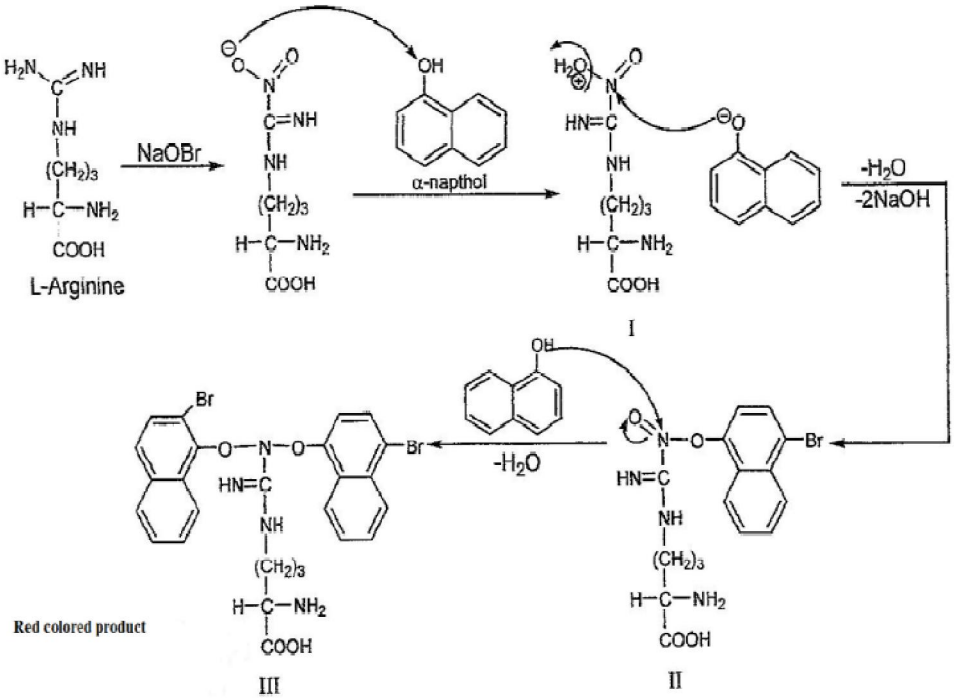
procedures

A/ General batch procedure

An increasing concentration (1-45)$\mu g.ml^{-1}$ of L-arginine was prepared in to a series of 25 ml volumetric flask, put in ice bath, then added 1ml of sodium hydroxide (2.5M) and 1ml of α-naphthol ($1.4x10^{-3}$M), mix for (2min), then put 0.1ml of cooled sodium hypobromite (0.25M).The standard solutions were shaken for (4second), 1ml of urea (1.67M) was added and mixed. After 10 min, the absorbance of colored product was measured at λ_{max} = 501 nm against the reagent blank.

B/General FIA procedure

A L-arginine solution (3-1400)$\mu g.ml^{-1}$ was prepared from stock solution of 2000 $\mu g.ml^{-1.}$The injection volumes of [42.19 µl (L_1), 43.175 µl (L_2), and 54.95 µl (L_3)] are consist of grape samples was injected in loop 1, while of 1ml of urea (1.67M)



Scheme 1 : The proposed mechanism of the reaction between arginine and α-naphthol in alkaline medium

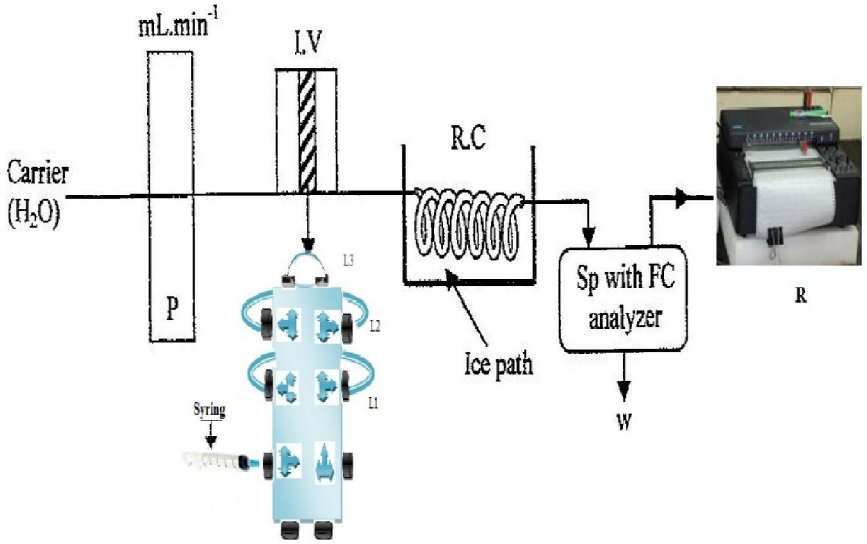


Figure 1 : Schematic diagram of flow injection / merging zones –spectrophotometric analysis P, Prestaltic pump ; I.V, Injection valve ; R.C, Reaction coil ; F.C, Flow cell ; D, Detector (Vis – spectrophotometric) ; W, Waste ; R, Recorder, (L_1= grape sample, L_2 = α- naphthol with urea, L_3 = Sodiumhypobromite)

with 1ml of α-naphthol ($1.4x10^{-3}$M) was injected in loop 2. and sodium hypobromite (0.25M) was injected in loop 3. Distilled water as carrier of the sample and other chemicals of each loop were carried out with flow rate of 5.5 ml. min^{-1} and reaction coil length of 50cm, as shown in Figure (1). The resulting absorbance of the red product was measured at 501 nm and a calibration curve were constructed. Optimization of conditions were performed on 35μg.ml^{-1} of L–arginine.

RESULTS AND DISCUSSION

Absorption spectra

L-Arginine forms a red colored product (λ_{max} of 501 nm) with α – naphthol in alkaline medium. The absorption spectra of the colored product are given in Figure (2).

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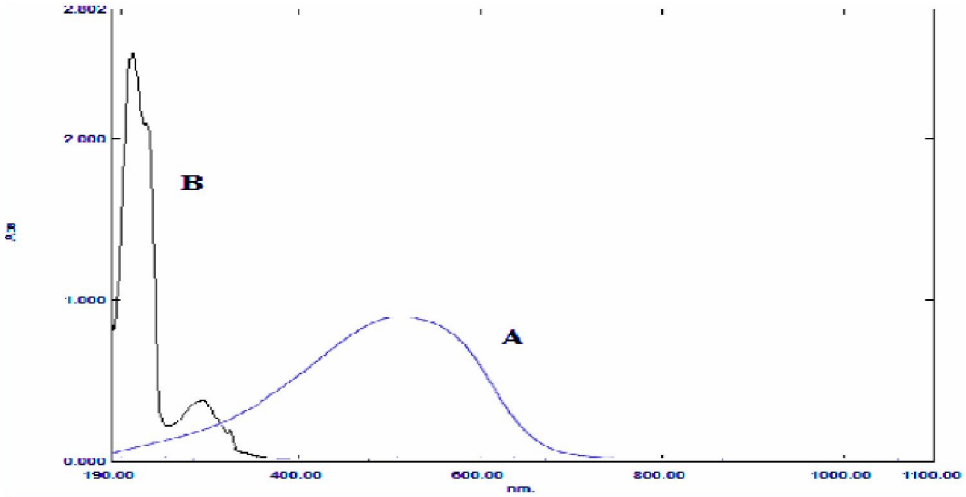

Figure 2 : (A) Absorption spectra of the colored product, 20µg.ml^{-1} of Arginine against reagent blank and (B) blank against distilled water

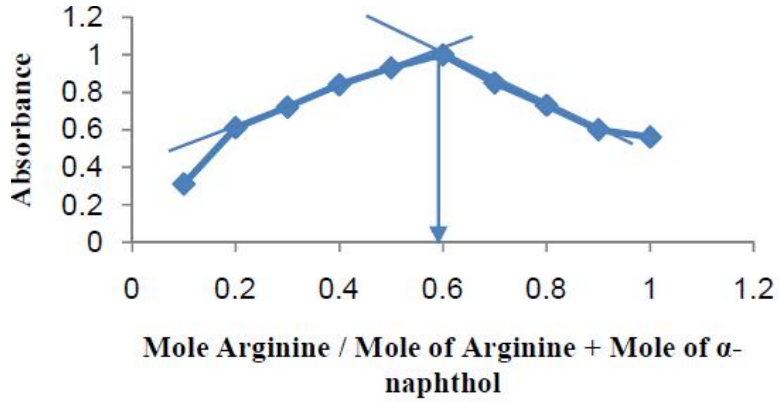

Figure 3 : Continuous variation plot of the reaction between Arginine and α- naphthol using batch procedure

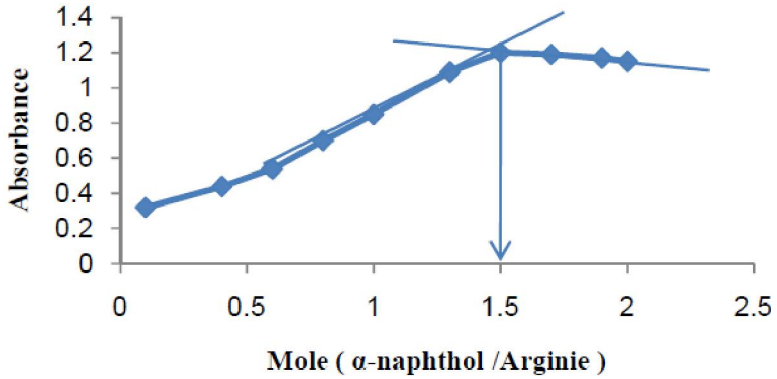

Figure 4 : The molar ratio plot for the reaction of Arginine with α-naphthol in an alkaline medium

The stoichiometry of the reaction between arginine and α-naphthol was investigated under the recommended optimum conditions by(Continuous variation) Job's method[27] according to the following procedure : in to a series of 25 ml volumetric flasks, increasing volumes (9-1ml) of arginine 20µg.ml^{-1} were added,followed by putting it in ice then adding 1ml of sodium hydroxide (2.5M) and decreasing volumes (1-9 ml) of (1.4x10^{-3}M) α-naphthol mix well for (2 min), then added 0.1 ml of sodium hypobromite (0.25M), the solution was shaken for 4 sec. then putting 1ml of urea (1.67M) and mixed. After 10 min the absorbance was measured versus reagent blank at λ_{max} 501 nm as shown in Figure (3).

And also a mole ratio method was performed of the reaction, an increased volumes (0.1-2) ml of $1.4x10^{-3}$M, α-naphthol were added to a 1 ml of 20 µg. ml $^{-1}$ L- arginine which was oxidation by 0.1ml sodium hypobromite (0.25 M), and reacted with α-naphthol ($1.4x10^{-3}$M) and the colored product was stabilized by adding urea (1.67M). as shown in Figure (4).

The result obtained (Figure 3&4) indicate that a (1:2) colored product was formed.

Batch spectrophotometric determination

The factors affecting on the sensitivity and stability of the colored product which resulting from the reaction between L-arginine and α-naphthol in alkaline medium were investigated.In the subsequent experimental the concentration 20µg.ml^{-1} was chosen as the optimum concentration of arginine to obtain the highest absorbance. The effect of different concentration of sodium hydroxide (0.1-1.2M) was carefully studied for the reaction between L-arginine and α-naphthol in alkaline medium in the presence of urea and in ice path (5^0C) to increase the time of Complex stability using sodium hypobromite as oxidizing the guanidine group of L- arginine and resulting compound which condenses with α-naphthol which described above procedure, 1M NaOH seems to be optimum as shown in Figure (5).

The effect of divers concentration of urea on the reaction was investigated, the highest absorbance at 0.3M of urea was found necessary for complete the reaction and it was appropriate for the optimum as shown in Figure (6).

The effect of various concentration of α- naphthol was tested on the maximum formation of the colored product, Figure (7) shown that $8x10^{-4}$M was adequate to gain the maximum absorbance

The effect of different concentration of sodium hypobromite was studied.A 0.1M sodium hypobromite gave the highest absorbance and was chosen for subsequent experimental, as shown in Figure (8).

FIA- spectrophotometric determination

The batch method for the determination of L-arginine was adopted as a basis to develop a FIA / merging zones system.

Optimization of chemical conditions

The influence of sodium hydroxide concentration (0.1-3M) was investigated,the result indicate that the best concentration of NaOH is 2.5M at maximum peak height (n=3) mv, as shown in Figure (9)

The experiment was done at various α-naphthol concentration of ($1x10^{-4}$ – $1.5x10^{-3}$ M) Figure (10) shows the maximum peak height at $1.4x10^{-3}$M which soluble in ethanol (99%), and was selected as the optimum concentration of reagent as shown in Figure (10).

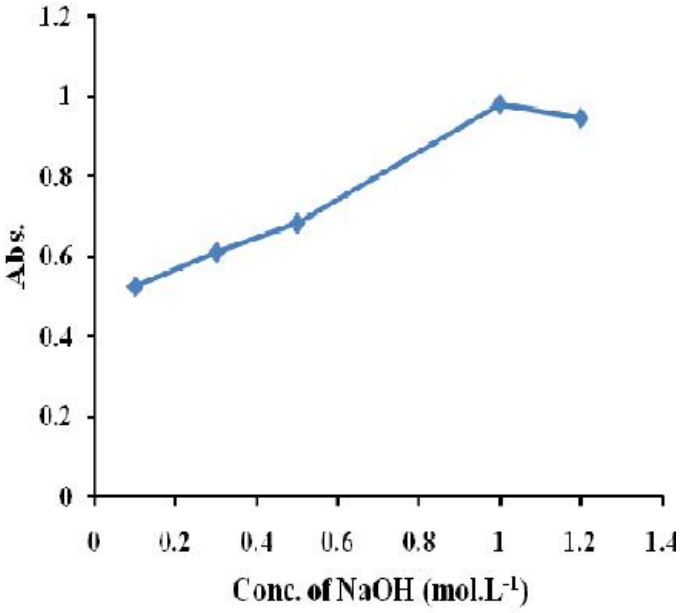

Figure 5 : Effect of the concentration of NaOH in (M)

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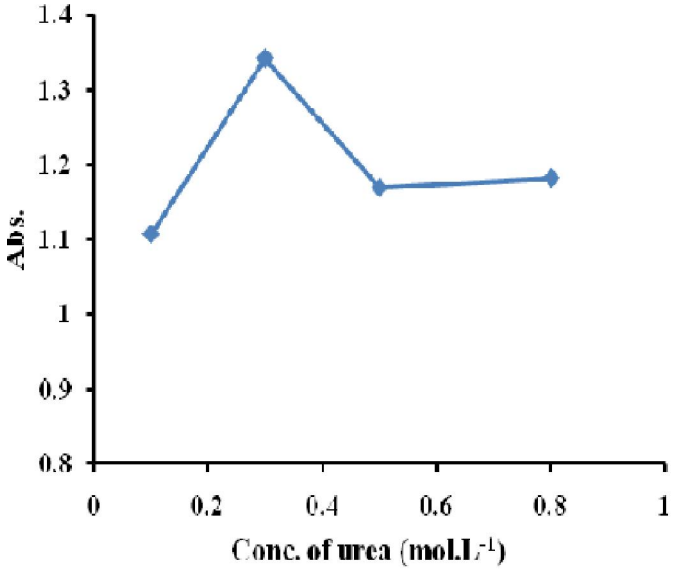

Figure 6 : Effect of urea concentration in (M)

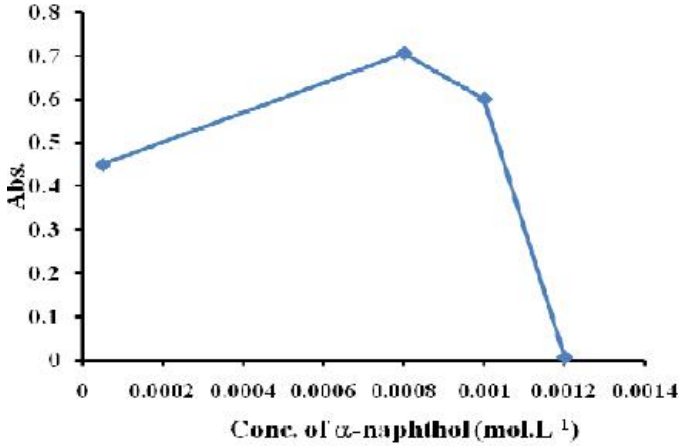

Figure 7 : Effect of α-naphthol concentration in (M)

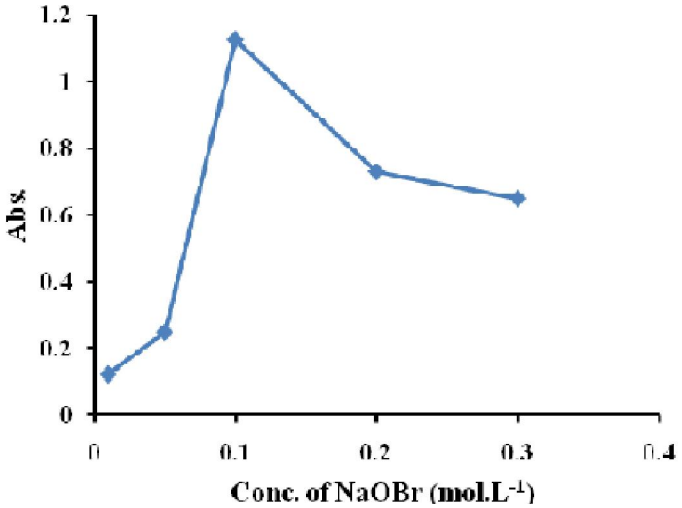

Figure 8 : Effect of concentration of oxidizing agent in (M)

From Figure (10) shows that an increase the α-naphthol concentration might be cause decrease in oxidizing reaction of L- arginine. Urea was added to the reaction, it mixed with α- naphthol and loaded

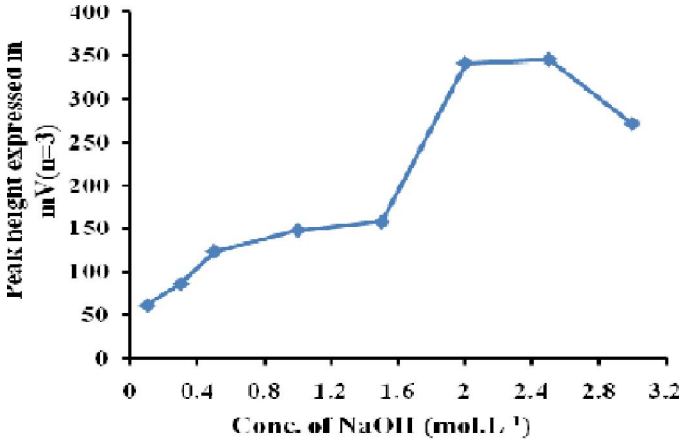

Figure 9 : Effect of NaOH conc. in (M)

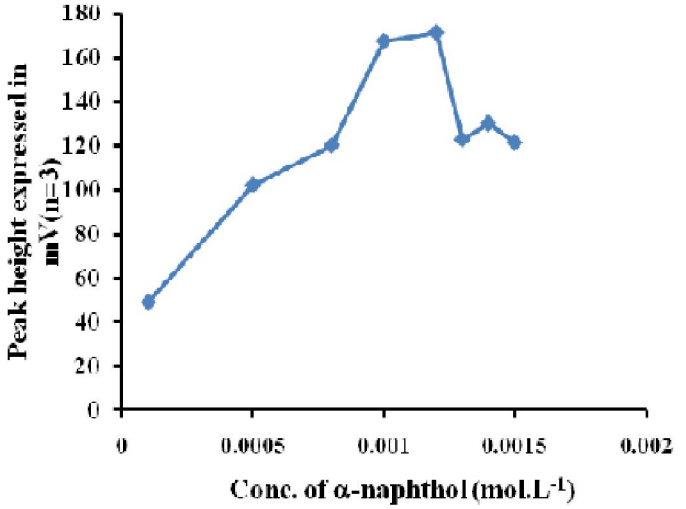

Figure 10 : Effect of α-naphthol concentration in (M)

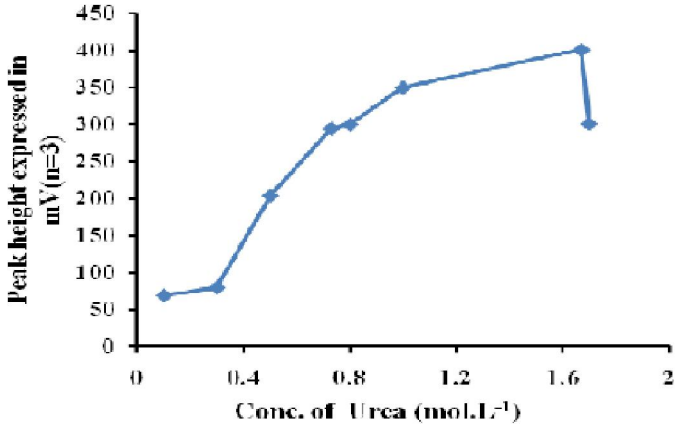

Figure 11 : Effect of Urea concentration in (M)

in loop2 to increase the time of stability of the complex formed, therefore the influence of various concentration was investigated and 1.67M seems to be the perfect absorbance as peak height (n=3) mv as shown in Figure (11).

The influence of different concentration of sodium hypobromite was studied, the concentration of 0.25M was found to be the ideal value as oxidizing agent of L-arginine with α-naphthol in the existence of urea in ice path (5^0C), as shown in Figure (12).

A preliminary physical condition was used 42.19µl as sample volume (L_1) which loaded by L-

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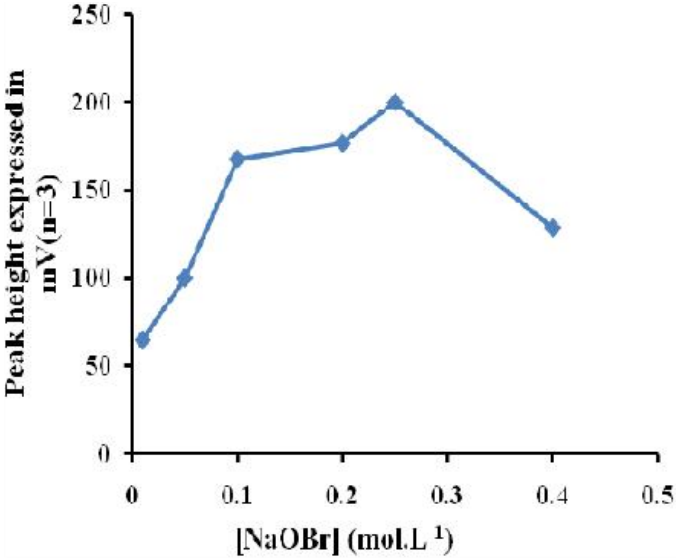

Figure 12 : Effect of different concentration of sodium hypobromite on peak height intensity(mv)

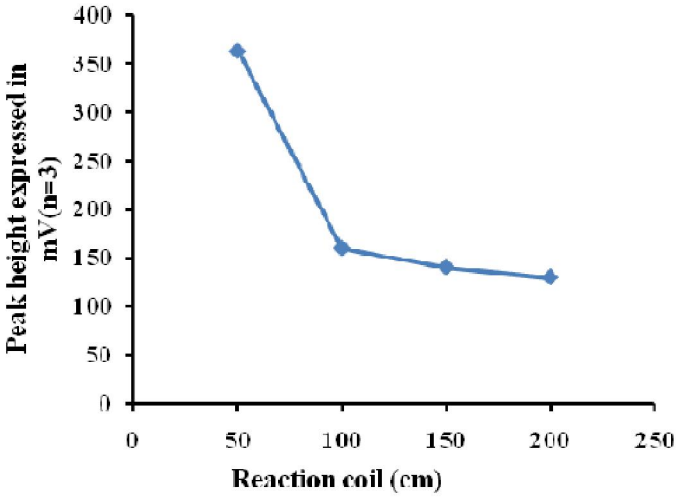

Figure 13 : Effect of reaction coil length in (cm)

arginine 35µg.ml $^{-1}$ with adding some drops of sodium hydroxide 2.5M, 43.175µl of α-naphthol($1.4x10^{-3}$M) in 1.67M urea (L_2) and 54.95 µl of 0.25M sodium hypobromite (L_3). It was concluded that distilled water can be used as carrier compared with the base was used. Distilled water was preferred as a carrier stream (ml.min $^{-1)}$ with high sensitivity for determination.

Optimization of physical conditions

The effect of variables such as reaction coil, injection volume of reagents and sample and flow rate on the analytical response was investigated. The peak height depends on the residence time of the sample in the system that is affected by flow rate and reaction coil lengths, the results during the study of the effect of reaction coil lengths (50,100,150,200) show that by increasing the reaction coil lengths up to 50 cm the sensitivity decrease. At longer distance there was increase in dispersion and it will decrease the peak height. as shown in Figure (13).

The effect of variation of sample and reagent volumes were investigated under optimum conditions. L-arginine concentration was 35µg.ml$^{-1,}$ α-naphthol ($1.4x10^{-3}$M) with urea(10%), and sodium hypobromite(0.25M). the results obtained indicated that a(42.19,43.175 & 70.25) µl was suitable volumes for sample and reagent respectively using different lengths of loops and using open valve mode gave the best response as shown in Figure(14).

The influence of flow rate on the response of the

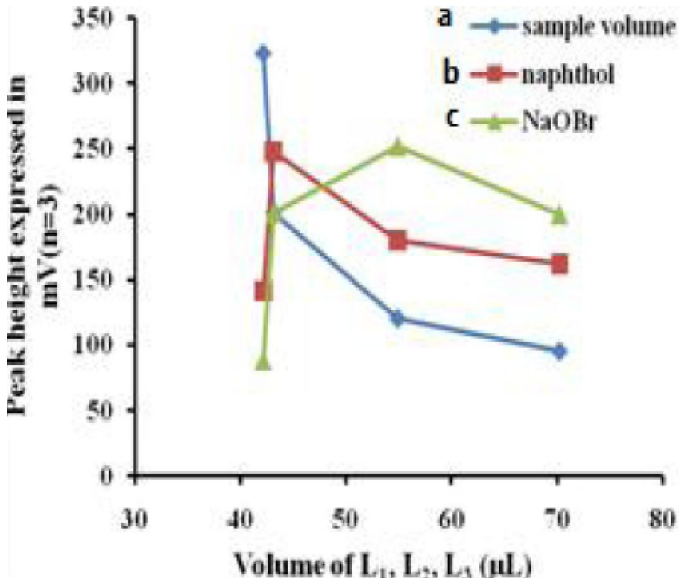

Figure 14 : Effect of variation of sample and reagent volumes of a/ L-arginine in NaOH. b/α-naphthol with urea. c/ sodium hypobromite

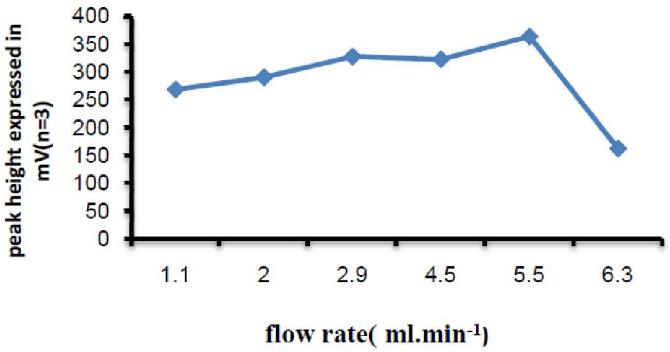

Figure 15 : Effect of flow rate (ml/min)

reaction was studied in the range of 1.1- 6.3 ml.min^{-1}. The result obtained indicated that a flow rate of 5.5 ml.min^{-1} of distilled water as carrier gave the highest response as shown in Figure (15).

The injection time was also an important conditions that effect on the sample throughput and was investigated by calculating the interval time between the sample injection and the appearance of the end of the signal. the reaction time of each samples was 80 sec., therefore the sample through put was 45 sample/hour.

Analytical parameters

Analytical characteristics such as detection limit, linear range, relative standard deviation and correlation coefficient of each method were determined[28] and also explained in TABLE (1), results proof the high sensitivity of the developed procedure.

These small points were referred to high repeatability and reproducibility of the developed FIA compared with the batch procedure. The FIA-merging zone technique is more convenient than the former method because of its speed (sample through put of 45 injection h^{-1}), wider linear range of calibration graph and good recovery were obtained.

Analysis of grape juice samples

The suggested methods were applied for the quantitative determination of L-arginine in grape juice samples[29]. Three types from different sources were pretreatment as shown in chemicals, TABLE (2) summarizes the results obtained by the proposed methods. They gave a good accuracy & precision, the proposed methods were compared successfully with the official method[30], since F-test and T- test TABLE (3) indicate that there was no significant differences between the proposed method and the official method. The calculated valued for F- test were

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TABLE 1 : Analytical characteristics of the procedures developed for the determination of L- arginine

Parameters	Batch method	FIA method
Linear range ($\mu g.mL^{-1}$)	1-45	3-1400
Regression equation	y=0.025x+0.243	y=0.468x+152.5
Correlation coefficient (r)	0.9869	0.9966
Linearity (r^2%)	97.78	99.3
Relative standard deviation (RSD%)	0.0145 (at 15 ppm)	0.057 (at 60 ppm)
Slope (b), ($ml.\mu g^{-1}$)	0.025	0.468
Intercept (a)	0.243	152.5
Standard deviation of slope (S_b)	3.9×10^{-3}	0.0185
Standard deviation of intercept (S_a)	0.019	25.39
Limit of detection (LOD)	0.468	0.12
Limit of quantification (LOQ)	4.68	1.186
Sample through put (hr^{-1})	4	45

TABLE 2 : Application of the proposed and official methods for the determination of L- arginine in grape juice

Grape juice samples	Proposed methods						Official method recovery%
	Batch			FIA-Merging zones			
	Present conc. ($\mu g.ml^{-1}$)	Rec.* %	RSD* %	Present conc. ($\mu g.ml^{-1}$)	Rec.* %	RSD* %	
Sample (1) product of America Importer by (farm – pik) company	5	100	0.2	20	100.05	0.084	100.1
	12	99.5	0.64	40	100	0.025	
	30	100.6	0.99	80	100	1.25	
Sample (2) product of Australia imported by (SPT) (Southern Produce Traders)	5	98.8	3.2	20	100.4	0.54	99.87
	12	99.91	0.083	40	100	0.00	
	30	100	0.023	80	99.98	0.012	
sample (3) product of Australia imported by (Happy Valley Fruits) company	5	99.2	0.72	20	100	0.00	100
	12	100.08	1.49	40	99.325	0.729	
	30	100	0.00	80	100.01	0.021	

*Average of three determination

TABLE 3 : The comparison of the proposed batch and FIA methods with standard method using T- and F- statical tests

Grape juice samples	Proposed methods				Official method	
	Batch		FIA			
	Rec%	$(x_i-x^-)^2_1$	Rec%	$(x_i-x^-)^2_1$	Rec%	$(x_i-x^-)^2_2$
Sample (1) product of America Importer by (farm – pik) company	100.03	0.062	100.01	$2.5x10^{-3}$	100.1	$1x10^{-4}$
Sample (2) product of Australia imported by (SPT) (Southern Produce Traders	99.57	0.044	100.1	0.019	99.87	0.012
sample (3) product of Australia imported by (Happy Valley Fruits) company	99.76	$4x10^{-4}$	99.77	0.036	100	0.014
	$(x_i)_1$= 99.78	$\Sigma(x_i-x^-)^2_{1=}$ 0.106	$(x_i)_1$= 99.96	$\Sigma(x_i-x)^2_{1=}$ 0.057	$(x_i)_2$= 99.99	$\Sigma(x_i-x^-)^2_{2=}$ 0.026

$F_{calculated(Batch)}=S_1^2/S_2^2=0.052/0.012=4.33$, $F_{calculated\ (FIA)}=S_1^2/S_2^2=0.025/0.012=2.08$; $F_{theoretical}=19.01$, $F_{theoretical}>F_{calculated}$; at 95% confidence level, $T_{calculated\ (Batch)}=0.178$, $T_{calculated\ (FIA)}=0.36$, $T_{theoretical}=2.770$, $T_{theoretical}>T_{calculated}$; at 95% confidence level.

(4.33) & (2.08), T-test values were (0.178) & (0.36) for the batch and FIA methods respectively, did not

exceed the critical values of F- test = 19.01 and T-test = 2.770 ($n_1+n_2 - 2 = 4$). These confirming that there are no significant differences between the proposed methods and the official method with respect to precision and accuracy in determination of L – arginine in grape juice samples as shown in TABLE (3).

CONCLUSION

A batch and FIA methods were described for the determination of L- arginine. Although very few methods are available for the determination of L-arginine by spectrophotometric analysis. The proposed method are rapid, simple & offers the advantages of sensitivity more than all reported spectrophotometric methods, which needed a difficult conditions, expensive material & the method that obeyed Beer's law gave a good application for the grape juice than the other methods which have low linear range[31].

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