Optimum conditions for ascorbic acid determination in three Iraqi citrus using HPLC technique

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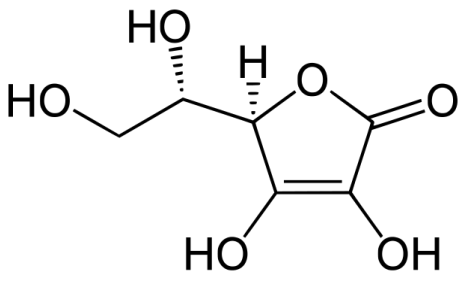
Abstract:

A high-performance liquid chromatography method was employed for the quantitative determination of ascorbic acid (AA) which called vitamin C in three types of Iraqi citrus (orange mandarin and aurantium) and to establish this goal , evaluation of ascorbic acid degradation is so important due to its significant criticality when exposure to ordinary atmospheric conditions. The chromatographic analysis of AA was carried out after their sequential elution with KH_2PO_4 (as mobile phase) by reverse-phase HPLC technique with C8 column and UV detection at 214 nm. .Bad resolutions was appeared clearly for C8 column , so another alternative condition were carried out to improve the resolution by replacement of C8 by C18 column .Statistical treatments were used to calculate relative standard deviation (RSD%) for the results to gain acceptable confidence to the present work , so the linearity of calibration curve, accuracy, and repeatability of this method are all satisfactory.

Key words: Citrus, Ascorbic acid, Antioxidant, degradation , HPLC.

Introduction:

Ascorbic acid (AA) $C_6H_8O_6$ is an organic compound with antioxidant properties , it dissolves in water to give mildly acidic solutions and can be easily oxidized as reducing agent[1].



Ascorbic acid is an essential nutrient in the diet , but it is easily reduced or destroyed by exposure to heat and oxygen[2].

It was shown that the higher the concentration, the better the stability. The stability was found to decrease significantly at concentration below 0.1 mg/l.

Stability studies at higher temperatures confirmed a large degree of degradation of Ascorbic acid[3]. The presence of heavy metal ions are of great importance. Metal-catalyzed destruction proceeds at a higher rate than noncatalyzed spontaneous autoxidation[4].

Its solution should be kept in air-tight containers and protected from heat, light and air so as to prevent the decomposition of its ascorbic acid content. Acidic pH around 2.1 was useful for sample preparation, ensuring sufficient stability and recovery of AA. Freshly squeezed fruit juices during storage showed colour changes as a result the degradation of vitamins and other components[5] , the degradation of ascorbic acid was investigated when 17 decomposition products were identified , also oxalic acid and 2,3-

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diketo-gulonic acid were determined[6].
Possible intermediates are 2-deoxyaldoteroses, 2-furoic acid , 2-furaldehyde , formic acid and oxalic acid. Oxidative degradation products such as 2,3-diketogulonic acid , 2,3 – dihydroxy-1,4-butanedioic acid also known as ascorbic acid degradation products[7,8].

Materials and Methods:

Equipment :

Centrifuge Heltich EBA20
Volumetric flasks (1000 and 25 ml);
pH meter TWT 7110
HPLC Shimadzu LC-20 AD
Detector UV-Vis Shimadzu
Reversible phase Column C8 –Si HiQ - 0.5 *25cm * 0.46cm
and column C18 tracer analytica - 0.5 *25cm * 0.46cm
HPLC syringe
Eppendorf micropipette
Samples : Iraqi citrus fruits (orange , mandarin , aurantium)

Reagents:

Ascorbic acid powder – fluka – 99.9% assay
KH_2PO_4 –Chem. supply
Deionized Water

HPLC conditions at first experiments:
Column: C8 - 0.5 *25cm * 0.46cm
Flow rate:1.5 ml/min.
Mobile phase: 3.4 g/l KH_2PO_4 – pH 2.5
UV detection : 214 nm.

Standard solutions

Stock solution of ascorbic acid (1 g/L) was prepared in deionized water and stored in darkness at room temp. Different working standard solutions were prepared daily by DI dilution . Another stock and standard solutions were prepared daily and freshly with highly precautionary requirements (mentioned below) to minimize degradation problems as possible.

Statistical analysis

Statistical analysis was carried out using – (Statistical Data Treatment and Evaluation) [9] to calculate the mean and relative standard deviation (RSD%). Microsoft office was used to calculate coefficient of determination (R^2) and linear regression equation y= bx+ m

Results and Discussion:

Ascorbic acid degradation

In the present paper , time and light effects on the ascorbic acid degradation rate was studied especially the kind of solution container , Whether it is dark or transparent . The results shown in table -1 refer to the relation between the AA. concentration and degradation percent. Three prepared solution (25 , 75 and 100 ppm) were stored in transparent containers and the percentages of degradation after 24 hr. were found to be 77.8% , 59.6% and 42.2%) respectively .It clearly appears that increasing the concentration of AA. results in decreasing degradation% matching the results obtained in reference[3].

Method and optimum conditions

Due to high degradation in AA. (as shown in table-1) , it seems more difficult to determine the exact quantities of AA content in citrus juice , but with precautionary requirements , it has been possible to get confident results by avoiding errors resulting from AA degradation by carring out possible minimization of all factors that affects degradation under study.

Table-1: degradation% of ascorbic acid after 24 hr. storage in transparent containers

Sample and No. Of replicatios	Peaks area of AA. freshly prepared in transparent container	Peaks area of AA after 24 hours in transparent container	% degradation of AA
25 ppm			
1	316673	59838	
2	341934	89876	
3	329490	79803	
4	342420	60545	
5	300079	……	
Mean of areas	326119	72516	77.8
75 ppm			
1	1488400	571728	
2	1274346	520996	
3	1306813	599003	
4	1334025	501135	
5	1272333	503677	
Mean of areas	1335183	539307	59.6
100 ppm			
1	1852100	1079331	
2	1700492	1081615	
3	1651141	1040078	
4	1584454	902510	
5	1588946	948747	
Mean of areas	1675426	1010456	42.2

Preparation and determination of known standard samples must be established freshly to reduce time, air and light exposure. A stock solution of 1 g/L has been prepared and directly packaged with 2 colored bags (black and blue) to minimize light exposure as possible , while preparation of the standard solutions for calibration curve was carried out at once for each level of concentration (not as usual procedure by preparation all standards at the same time and determinates them respectively) to avoid time consuming problems , that means , the second standard was prepared after determination of the first one and so on. The same above procedure was carried out for the quantitative determination of citrus fruits and the black packaging started from sample filtration after squeezing process and then diluted 25 times before determination

Calibration curve and linearity

A calibration curve of A.A. (fig-1) was constructed by evaluation the peak area of four different concentrations of A.A. solutions (40,80,120,160 ppm) using HPLC chromatograms. Each measurement is a mean of four replicates.

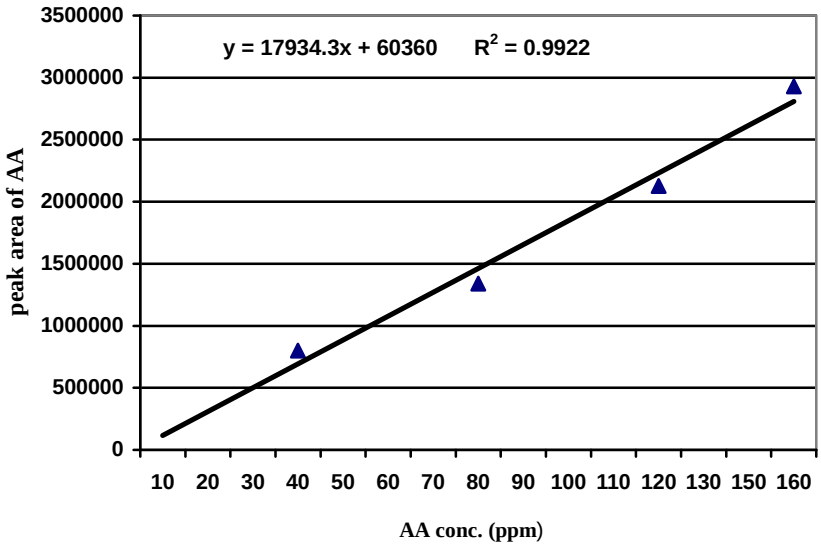


Fig-1:calibration curve of AA (C8 column)

As shown in (fig-2), HPLC chromatograms for these solutions refer to the high accuracy of retention time . A curve equation $y = bx + m$ with linear regression method to determine samples concentration was established. The equation was $y = 17934.3x + 60360$ and the R^2 value (0.992) shows acceptable linearity of the analytical method under study.

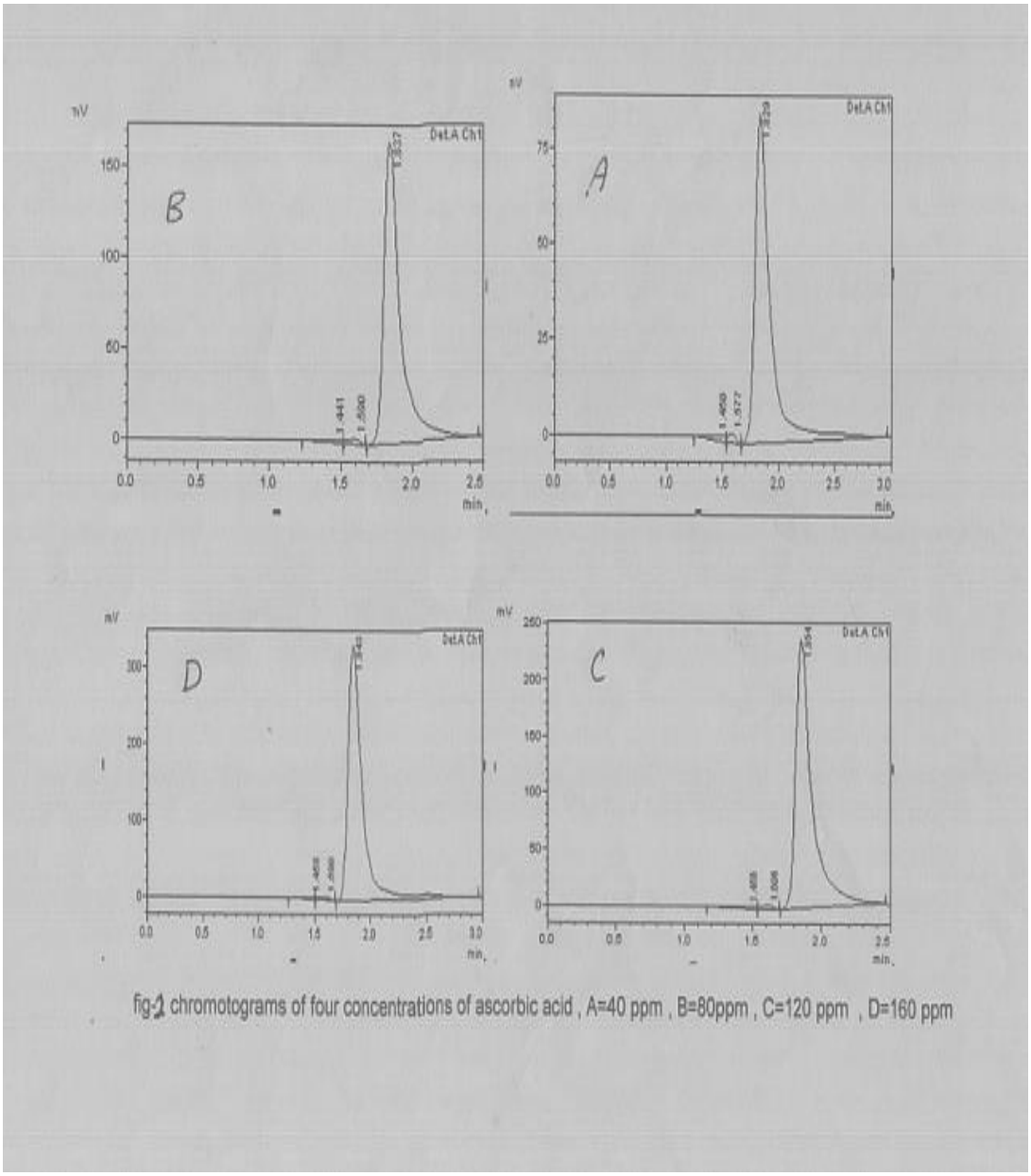


fig-2 chromotograms of four concentrations of ascorbic acid , A=40 ppm , B=80ppm , C=120 ppm , D=160 ppm

Table -2 refers to the accuracy of calibration curve results of A.A. and their relative standard deviation (RSD%) , it shown that for peaks area ranging from 2.3% to 4.49% for the four levels , while for the retention time , RSD% ranging from 0.15% to 0.33% and the later values indicate specific stability of several operation conditions of HPLC system such as pump pressure and flow rate .

Table – 2: Peaks areas and retention times of four different concentration of A.A. using HPLC (C8 column).

Sample and No. of replicates	Peak area	Retention time Min.
40 ppm		
1	818048	1.836
2	797087	1.846
3	839806	1.829
4	759148	1.830
5	792162	1.832
Mean	801250	1.8346
RSD%	3.3%	0.33%
80 ppm		
1	1364091	1.837
2	1358449	1.835
3	1358997	1.842
4	1274656	1.829
5	1339457	1.847
Mean	1339130	1.838
RSD%	2.5%	0.33%
120 ppm		
1	2213328	1.848
2	2025759	1.848
3	2046485	1.854
4	2226179	1.846
Mean	2127937	1.849
RSD%	2.3%	0.16%
160 ppm		
1	2803659	1.846
2	3147806	1.849
3	2856635	1.847
4	2910135	1.853
Mean	2929558	1.848
RSD%	4.49%	0.15%

Column: C8 - 0.5 *25cm * 0.46cm
Flow rate : 1.5 ml/min.
Mobile phase: 3.4 g/l KH_2PO_4 – pH 2.5
UV detection : 214 nm.

Determination of AA. in Iraqi citrus juice:-

Quantitative analyses of three types of Iraqi citrus (orange, mandarin, aurantium) have been established using C8 column as shown in table-3 , their A.A. concentration value obtained were : 491.8 , 511.8 and 244.2 ppm respectively (take into consideration dilution factor of 25 times for each origin sample of citrus) and their

Table–3: Ascorbic acid concentrations of Iraqi citrus juice

Citrus type	Retention time	AA. Peak area	Conc. of AA. ppm
Orange			491.8
1	1.83	247854	
2	1.837	237668	
3	1.823	224709	
Mean	1.83	236743	
RSD%	0.31%	4%	
Mandarin			511.8
1	1.836	257709	
2	1.829	236993	
3	1.83	237087	
Mean	1.831	243929	
RSD%	0.17%	4%	
Autumn			244.2
1	1.825	148640	
2	1.834	141194	
3	1.831	153983	
Mean	1.83	147939	
RSD%	0.2%	3.5%	

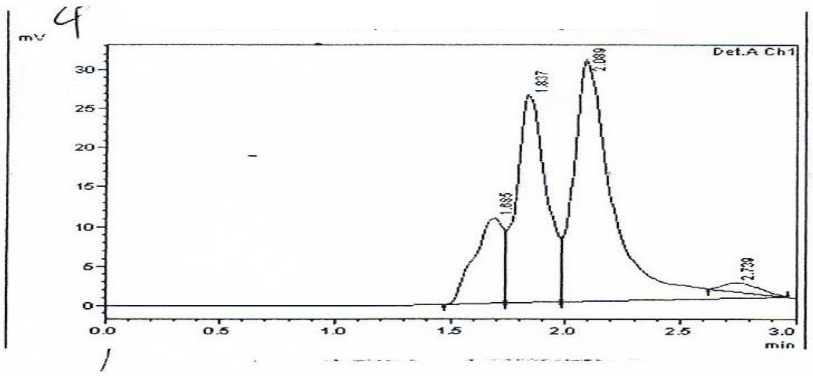
chromatograms shown in (fig 3,4,5).To diagnose other peaks shown in the chromatograms of citrus juices , it is known from references that citric acid accompanied ascorbic acid as significant convergent peak in citrus juice . Obtained RSD% values indicate that the results refer to acceptable accuracy .

The chromatograms shown (fig 3,4,5) indicate weak peaks resolution (Rs) between convergent peaks . Additional work required to improve the separation. Several attempts were done due to alteration of some parameters such as flow rate and pH of mobile phase, but no significant improvement obtained .Replacement the type of column from C8 to C18 was one of the available choices as an attempt to achieve this goal.

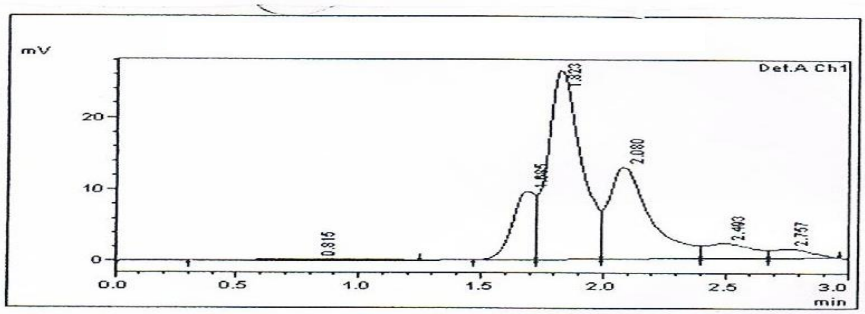
C18 Calibration curve and linearity

The results shown in (fig-6) refer to good calibration curve linearity was obtained with C18 column (R^2=0.9988) and (fig-7) indicates that very close retention time was obtained. Acceptable precision and accuracy were established (table -4) due to statistical calculation of RSD% .

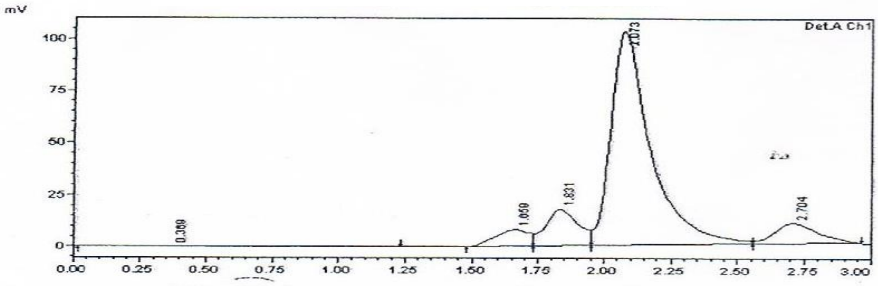
Column : C8 - 0.5 *25cm * 0.46cm
Flow rate :1.5 ml/min.
UV detection : 214nm.
Mobile phase : 3.4 g/l KH_2PO_4 _ pH 2.5



(Fig-3)chromatogram of orange



(Fig-4)chromatogram of mandarin



(Fig-5)chromatogram of aurantium

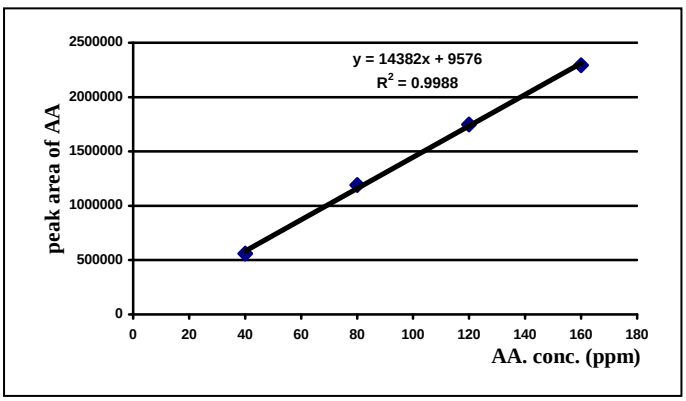
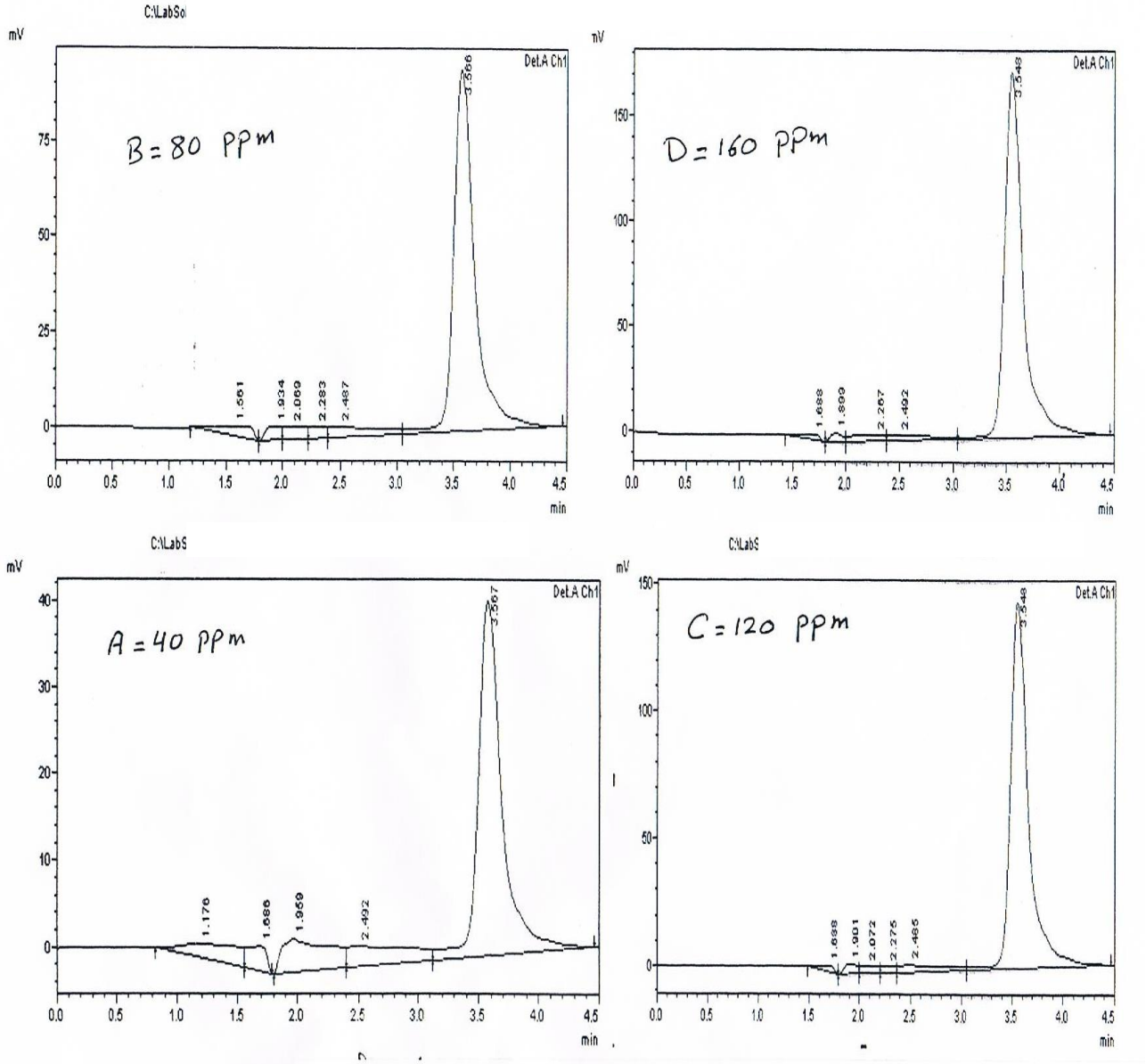


Fig-6: calibration curve of AA (C18 column)

Column : C18 - 0.5 *25cm * 0.46cm
Flow rate :1.5 ml/min.
UV detection : 214nm.
Mobile phase : 3.4 g/l KH_2PO_4 – pH 2.5



(Fig-7) chromatograms of different A.A. concentration (ppm) for calibration curve study using C18 column

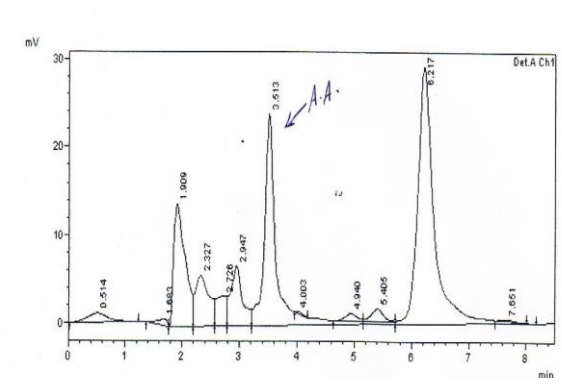
Table –4: AA. results at different Concentrations (C18 column)

Sample and No. of replicates	Ascorbic acid Peaks area	Retention time (Min.)
40 ppm		
1	578575	3.564
2	541136	3.555
3	554250	3.556
4	559854	3.567
5	571283	3.568
Mean	561019	3.562
RSD%	1.8%	0.15%
80 ppm		
1	1222340	3.556
2	1190992	3.571
3	1206941	3.566
4	1138078	3.564
5	1190505	3.557
Mean	1189771	3.562
RSD%	4.38%	0.16%
120 ppm		
1	1750039	3.548
2	1761844	3.551
3	1708617	3.604
4	1770914	3.548
Mean	1747853	3.56
RSD%	1.36%	0.55%
160 ppm		
1	2411218	3.532
2	2315200	3.526
3	2350575	3.523
4	2282867	3.531
5	2103426	3.548
Mean	2292657	3.532
RSD%	4.5%	0.24%

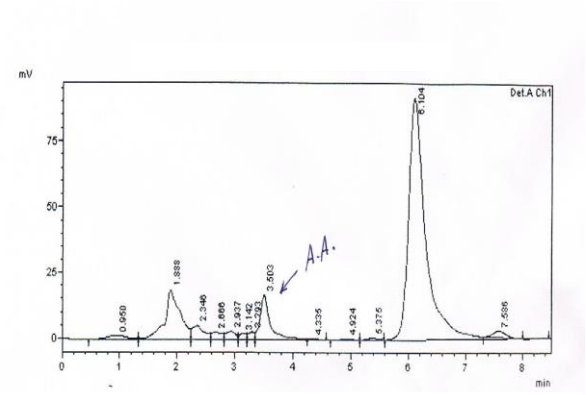
Column : C18 - 0.5 *25cm * 0.46cm
Flow rate :1.5 ml/min.
UV detection : 214nm.
Mobile phase : 3.4 g/l KH_2PO_4 – pH 2.5

Determination of A.A. in Iraqi citrus juice using C18

From the first three type of Iraqi citrus juice , unfortunately , mandarin is not included in the second experiment due to the end of the fruit season during this work , so only orange and aurantium were determinated (fig-8,9) , ***three replicate measurements*** for each type were carried out .



(Fig-8)Chromatogram of orange (C18 column)



(Fig-9) Chromatogram of aurantium (C18 column)

Column : C18 - 0.5 *25cm * 0.46cm
Flow rate :1.5 ml/min.
UV detection : 214nm.
Mobile phase : 3.4 g/l KH_2PO_4 – pH 2.5

Results obtained for orange peaks area with mean value equal 355409, while aurantium peak area record mean value equal 218543 . According to curve equation obtained from C18 column, the quantity of AA. in orange = 601 ppm and for aurantium =363ppm after multiplied by 25 times which represents dilution factor.

Comparing between results from this study and another one obtained from Adana provinces of Turkey ref.[10] show that the quantity of AA. dependent not only the type of fruit but the geography region of it, for example AA. conc. of six varieties of orange ranging from 855 ppm to 248 ppm (fig-10) while our obtained values were 601 ppm for Iraqi orange and 363 ppm for aurantium .

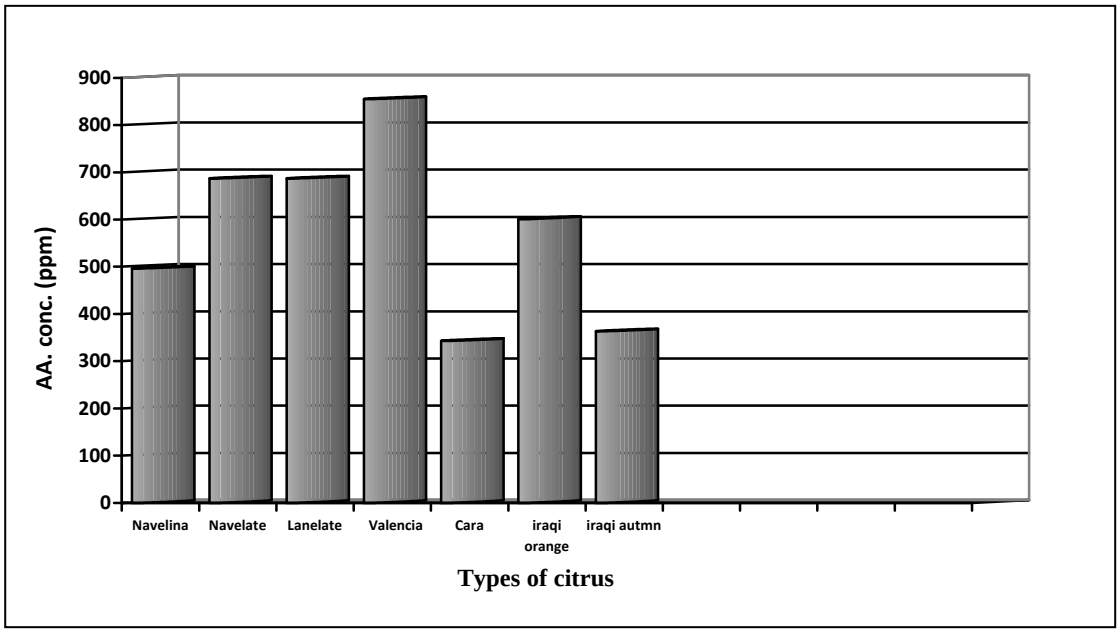


Fig-10: comparison of AA conc. between iraqi citrus juice and turkish types.

Conclusion:

Optimum conditions were established in this work by

1-Monitoring and minimizing AA. degradation to reach accurate quantitative determination of ascorbic acid in any citrus juice .

2-Peaks resolution is so important to reach optimum method for whole HPLC analysis , and results obtained from this work proves that good resolution and separation was establishd by using C18 instead of C8.

3- Also , increasing the number of theoretical plates for ascorbic acid from 729 in C8 column to 1611 in C18, confirm the benefit of this column in such work.

Acknowledgment

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الظروف المثلى لقياس حامض الاسكوربيك في ثلاثة انواع من الحمضيات العراقية باستخدام تقنية HPLC

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الخلاصة:

تم استخدام تقنية (HPLC) لقياس كمية فيتامين C في ثلاث انواع من الحمضيات العراقية (البرتقال ، اللانكي والنارنج) ، ولغرض تحقيق ذلك فلابد من تقييم حالة التكسر والتجزأ المهمة التي تحصل في حامض الاسكوربيك عند تعرضه للظروف الجوية الاعتيادية ، تم انجاز التحليل الكروماتغرافي لفيتامين C باستخدام KH_2PO_4 (كطور متحرك) والعمود C8 كطور ثابت وبتحسس مقداره 214 nm. كطول موجي لمقياس طيف UV . تداخل القمم كان واضحا باستخدام العمود C8 لذا كان التوجه لاستخدام ظروف بديلة تعززت باعتماد العمود C18 بغية الحصول على نتائج اكثر واقعية بازالة تداخل القمم .تم معاملة النتائج المستخرجة بالمعالجات الإحصائية لاحتساب نسبة الحيود النسبي (RSD%) وهي ضرورية لإضفاء مصداقية مقبولة على هذه النتائج والذي انعكس بدوره لبلوغ دقة مقبولة الى حد كبير على معاملة الخط المستقيم لمنحنى التوازن.