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DEVELOPMENT OF AN ANALYTICAL METHOD FOR DETERMINING VITAMIN C BY HPLC AND ITS APPLICATION TO CITRUS

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ABSTRACT: *HPLC method was developed and applied to the determination of Ascorbic acid (vitamin C) by separating Ascorbic acid from Citric acid interfering considerably with it, in Syrian Citrus fruits, in both Pulp and Peels. This study was applied on several Syrian fruits as: (Citrus sinesis (Navel, Jaffa), Grapefruit (Citrus paradisi) (ruby red-duncan), Citrus limon (Eureka-Hybrid), Citrus aurantium, Citrus reticulate (Clementine-Satsuma)) purchased from Aleppo markets. The maximum RDS% were not exceeded to 4.52% for all samples and 3.64% for the standards, with LOQ 0.942 and LOD 0.311. High performance liquid chromatography with UV-Vis detection were employed. The developed chromatographic method employed an C_{18} RP-column with dimension (250* x *4.6 mm; 5 µm). The diluted solution consists of buffer Na_2HPO_4 and NaH_2PO_4 with PH=2.5, adjusted by ortho-Phosphoric acid. The mobile phase was to be (methanol: water) HPLC grade with volume percentage (10:90) at PH =2.1, adjusting also by ortho- Phosphoric acid.*

Keywords: *HPLC, Separation, vitamin C, Citrus, peels or pulp*.

I. INTRODUCTION

Ascorbic acid is one of the important water soluble vitamins. It is essential for collagen, carnitine and neurotransmitters biosynthesis. Most plants and animals synthesize ascorbic acid for their own requirements. However, apes and humans cannot synthesize ascorbic acid due to lack of an enzyme gulonolactone oxidase. Hence, ascorbic acid has to be supplemented mainly through fruits, vegetables and tablets. The current US recommended daily allowance (RDA) for ascorbic acid ranges between 100-120 mg/per day for adults. Many health benefits have been attributed to ascorbic acid such as antioxidant, anti-atherogenic, anti-carcinogenic, immunomodulator and prevents cold, etc[1]. Fig. 1 represents the chemical structure of Ascorbic acid.

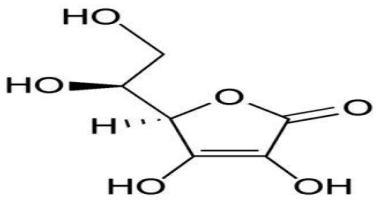

Fig. 1: Chemical structure of Ascorbic acid.

Numerous analytical techniques have been reported in the literature for the determination of Vitamin C in different matrixes. These include titrimetric[2 to 4], volumetric[4-5], and fluorescence[6-7]. Vitamin C has been separated and determined by gas chromatography[8], spectrophotometric[9 to 11], complexometric methods[12], and by enzymatic determination[13]. Also it has been separated and determined by high performance liquid

chromatography (HPLC) with several detections such as electrochemical detection[14], mass spectroscopy[15], fluorescence[16] and UV-Visible detection[17 to 19], and photo-diod array detection[20 to 23]. HPLC gives ability to determine vitamins mixtures in one step and with high accuracy and good sensitivity for components separation which helps us to save time, solvents and money.

Ascorbic and Citric Acids have been separated in the Citrus fruits by many columns: on C_{18}[16 to 23], and NH_2[16]. We tried in this study to separate vitamin C from Citric acid at the same time and to compare the amount of Vitamin C in the pulp and the peels in several fruits, and profiting from HPLC/UV-Vis detection to estimate Vitamin C content in different Syrian Citrus fruit, in both Peels and Pulp, after suggesting suitable HPLC conditions with UV-Vis detection.

II. MATERIALS AND METHODS

2.1 Apparatus

HPLC analysis was performed on an YL 9100 HPLC system equipped with binary pump YL9111, vacuum degasser series YL9101, YL9130, column compartment and YL9120 UV-Vis detector. Chromatographic separations were obtained by using 5 µm SGE HPH 125 C_{18} column (250 x 4.6 mm; 5 µm). Ultrasonic bath (Daihan), analytical balance TE64 Sartorius sensitivity 0.01 mg, Germany digital pipettes (Isolab), UK centrifuge (Centurion Scientific Ltd).

2.2 Chemical regents

Standard Vitamin C was purchased from PROLABO (EEC), Citric acid from Srlchem (India), HPLC-grade water, di-Sodium hydrogen-o-phosphate anhydrous were purchased from QUALIKEMS (India). Methanol HPLC was purchased from SHAMLAP (Syria). Sodium dihydrogen orthophosphate dehydrate AR was purchased from SD fine (India). Ortho-phosphoric acid was purchased from POCH (Poland).

2.3 Standard preparation

Standards stocks solutions of Vitamin C in concentration of 1 mg/mL and 3 mg/mL for Citric acid were prepared by dissolving a required amount of each Vitamin C and Citric acid in diluted solution (buffer solution). The working standard solutions were between (0.4-500 mg/L) for vitamin C, prepared by diluting the standards stocks solutions with diluted solution.

2.4 Calibration Curve

To construct the calibration curve, five replications (25 µL) for each standard solution were injected immediately after preparation into the column and peak areas of chromatograms were measured.

2.5 Sample preparation

Peels: The fruits were washed with tap water then peeled carefully. Weigh a required amount of collected peels between 2 to 7 g, then put it in an electric mixer with an appropriate volume of buffer and mix them till homogenization for 5 minutes then transferred it to 50 mL volumetric flask and diluted up to the volume with buffer. The samples were degassed by ultrasonication for 2 minutes, and were centrifuged at 5000 rpm/min for 20 min. 1 mL of solution was transferred to 10 mL volumetric flask and the obtained volume was reached. The obtained solution was filtrated through a 45 µm nylon syringe filter and then the samples were injected into the column immediately**.**

Pulp: Weigh required amount of Pulp between 5 to 15 g, and put it in an electric mixer with an appropriate volume of buffer, and mix them till homogenization for 5 minutes then transferred it to 50 mL volumetric flask and diluted up to the volume with buffer. The samples were degassed by ultrasonication for 2

minutes and were centrifuged at 5000 rpm/min for 20 min. 1 mL of solution was transferred to 10 mL volumetric flask and the obtained volume was reached. The obtained solution was filtrated through a 45 µm nylon syringe filter, and then the samples were injected into the column. The concentrations of Vitamin C in the samples were then calculated using peak data and standard curve.

2.6 Chromatographic conditions

Column C18 (250 x 4.6 mm; 5 µm) was used. Mobile phase was methanol: water HPLC grade with volume percentage (10:90) at PH = 2.1, adjusting by ortho-phosphoric acid. Flow rate 1 mL/min. Detection wavelength 242 nm for Ascorbic acid and 212 nm for Citric acid. Diluted solution is buffer. Injection volume: 25 µL.

III. RESULTS AND DISCUSSION

Chromatograms of the Standards solutions at five different standard concentrations, which every concentration was injected five times under our conditions showed that Vitamin C was well separated from Citric acid with very good resolution, and the separation of these two components in our methods was achieved in less than 5 min, the linearity was from 1.2 to 400 mg/L. The linearity curve is presented in Fig. 2, with LOQ 0.942 and LOD 0.311, and maximum RSD% was 3.64. Standard chromatogram is presented in Fig.3.

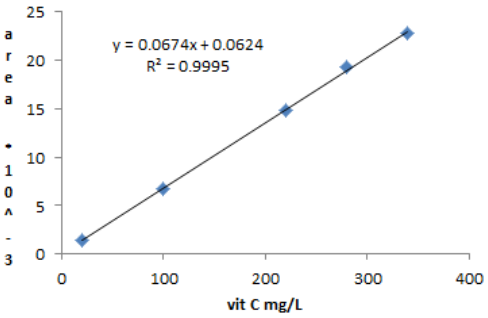

Fig.2: Vitamin C Standard curve.

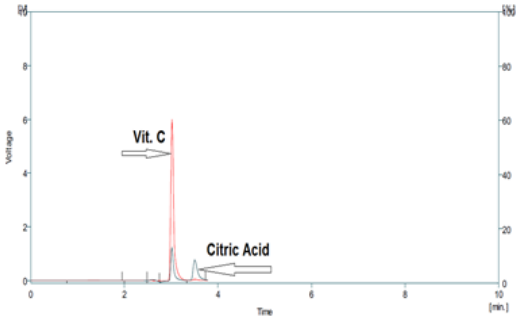

Fig.3: Chromatogram of Ascorbic acid and Citric acid.

3.1 Vitamin C content in each kind of Citrus

The amounts of Vitamin C in the pulp and the peels in several studied Syrian fruit of presented for five different samples for each, in tables from (1 to 9) respectively and All the separation chromatograms obtained for Vitamin C in pulps and peels for studied fruits (Navel, Jaffa, Grapefruit (ruby red), Grapefruit (duncan), Eureka, Hybrid, Citrus aurantium, Clementine, Satsuma) were presented in Figs. (4 to 21). And the contents amounts of Vitamin C in the pulp and the peels in several studied Syrian fruit were presented for five different samples for each, in tables (1 to 9) respectively.

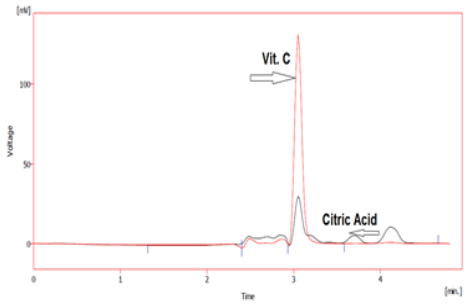

Fig.4: Vit. Content in Navel pulp.

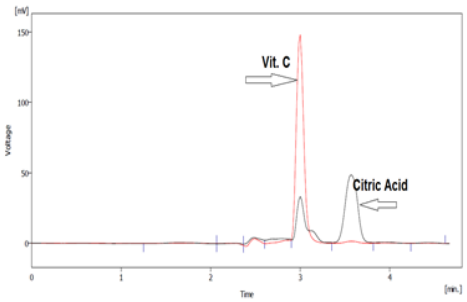

Fig.5: Vit. C content in Navel peels.

Table 1: Vit. C contents in pulp and peels in Navel fruit.

Pulp			Peels		
($\overline{X}$)*, mg/100 g	SD	RSD%	($\overline{X}$)*, mg/100 g	SD	RSD%
70.31	2.40	3.41	90.03	0.72	0.80
48.33	0.40	0.83	82.73	2.54	3.07
39.67	0.35	0.88	100.17	1.77	1.77
71.71	2.38	3.32	96.37	3.33	3.45
76.16	1.17	1.53	159.89	1.73	1.08

* n=3

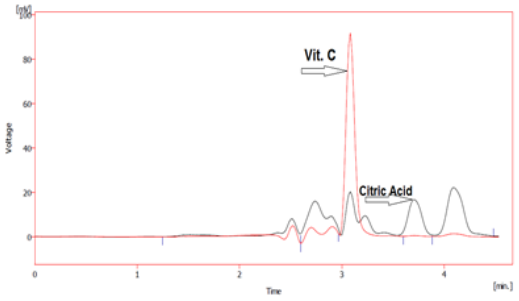

Fig.6: Vit.C content in Jaffa pulp.

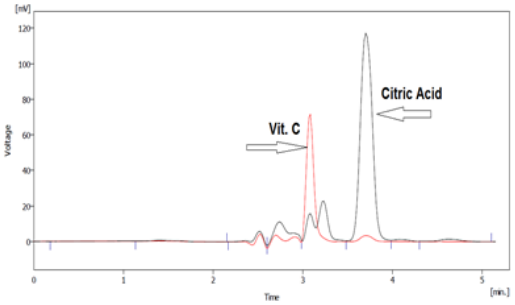

Fig.7: Vit. C content in Jaffa peels.

Table 2: Vit. C contents in pulp and peels in Jaffa fruit.

Pulp			Peels		
($\overline{X}$)*, mg/100 g	SD	RSD%	($\overline{X}$)*, mg/100 g	SD	RSD%
30.53	0.21	0.69	60.85	2.54	4.17
32.61	0.68	2.08	76.96	2.84	3.69
40.46	1.50	3.71	100.09	2.44	2.44
39.35	0.95	2.41	99.52	3.82	3.84
47.25	0.39	0.82	146.84	0.67	0.46

***** n=3

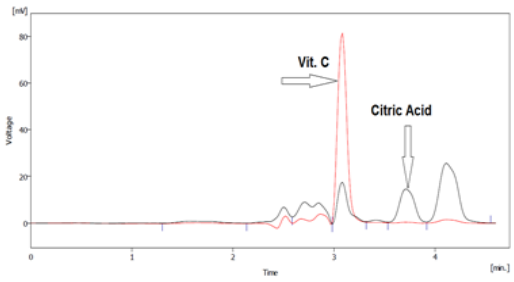

Fig.8: Vit. C content in ruby red pulp.

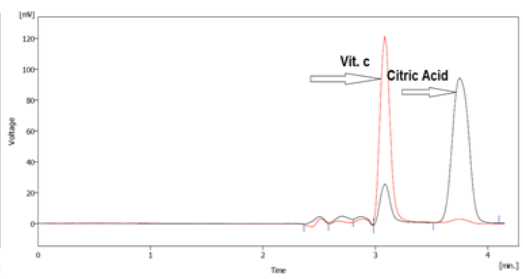

Fig.9: Vit. C content in ruby red peels.

Table 3: Vit. C contents for pulp and peels in Grapefruit (ruby red) fruit.

Pulp			Peels		
($\overline{X}$)*, mg/100 g	SD	RSD%	($\overline{X}$)*, mg/100 g	SD	RSD%
34.57	0.28	0.81	82.02	2.82	3.44
39.97	0.20	0.50	123.55	1.71	1.38
35.76	1.45	4.05	140.00	3.16	2.26
36.51	0.49	1.34	151.40	1.21	0.80
51.99	0.79	1.52	145.44	1.27	0.87

* n=3

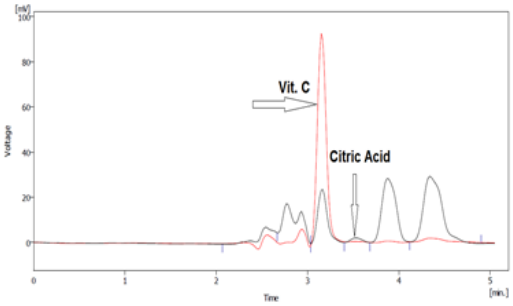

Fig. 10: Vit. content in duncan pulp.

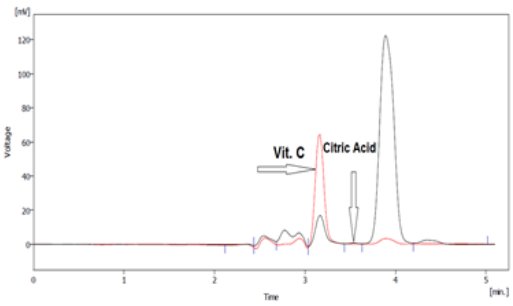

Fig. 11: Vit. C content in duncan peels.

Table 4: Vit. C contents for pulp and peels in Grapefruit (duncan) fruit.

Pulp			Peels		
($\overline{X}$)*, mg/100 g	SD	RSD%	($\overline{X}$)*, mg/100 g	SD	RSD%
32.76	1.01	3.08	74.68	1.30	1.74
21.92	0.41	1.87	51.39	0.26	0.50
44.99	0.34	0.75	107.72	1.85	1.72
34.30	1.55	4.52	87.23	2.86	3.28
42.41	0.46	1.08	93.74	3.14	3.35

* n=3

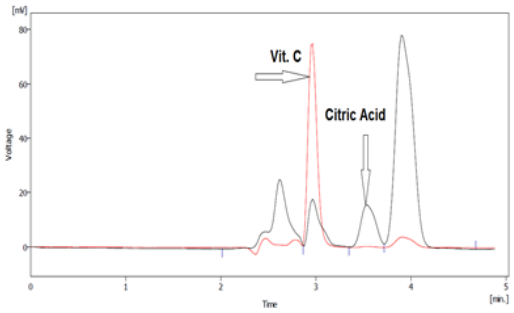

Fig. 12: Vit. C content in pulp of Eureka.

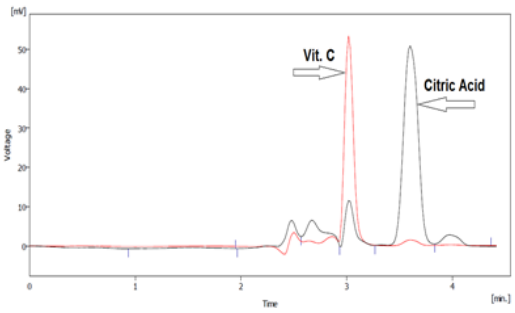

Fig. 13: Vit. C and content in Eureka peels.

Table 5: Vit. C contents for pulp and peels in Eureka fruit.

Pulp			Peels		
($\overline{X}$)*, mg/100 g	SD	RSD%	($\overline{X}$)*, mg/100 g	SD	RSD%
49.16	0.54	1.10	68.54	2.86	4.17
29.91	0.49	1.64	37.25	1.48	3.97
35.45	0.61	1.72	76.93	0.14	0.18
45.99	1.03	2.24	62.73	2.49	3.97
52.10	0.21	0.40	78.86	1.46	1.85

***** n=3

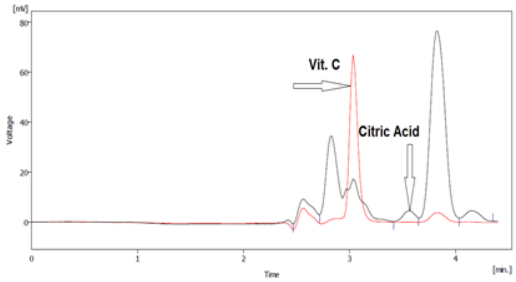

Fig. 14: Vit. content in Hybrid pulp.

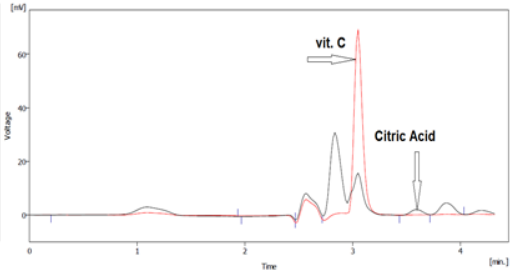

Fig. 15: Vit. C content in Hybrid peels.

Table 6: Vit. C contents for pulp and peels in Hybrid fruits

Pulp			Peels		
($\overline{X}$)*, mg/100 g	SD	RSD%	($\overline{X}$)*, mg/100 g	SD	RSD%
24.10	0.29	1.20	99.30	2.69	2.71
15.57	0.30	1.93	83.82	3.79	4.52
18.30	0.43	2.35	58.86	0.79	1.34
24.34	0.06	0.25	42.47	1.53	3.60
24.40	0.18	0.74	31.48	0.04	0.13

***** n=3

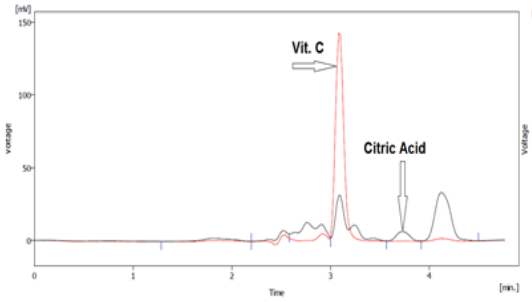

Fig. 16: Vit. C content in aurantuim pulp.

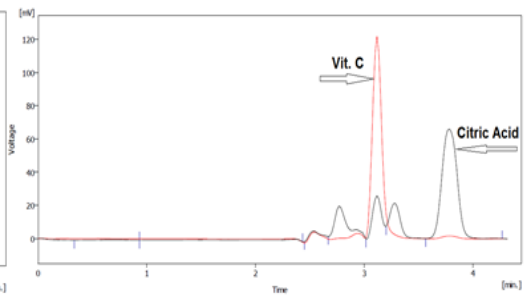

Fig. 17: Vit. C content in aurantium peels.

Table 7: Vit. C content for pulp and peels in Citrus aurantium fruit.

Pulp			Peels		
($\overline{X}$)*, mg/100 g	SD	RSD%	($\overline{X}$)*, mg/100 g	SD	RSD%
49.56	0.77	1.55	160.74	2.35	1.46
60.27	0.22	0.36	156.69	1.61	1.03
56.08	0.83	1.48	122.99	0.81	0.66
60.42	0.44	0.73	112.85	2.52	2.23
49.83	0.16	0.32	196.53	0.82	0.42

***** n=3

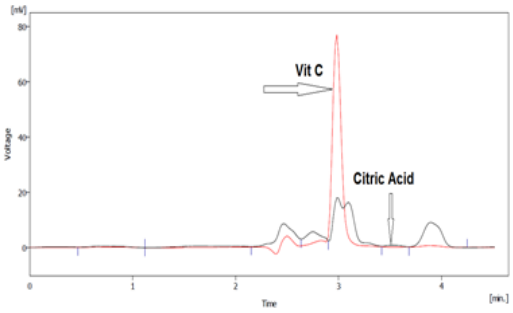

Fig.18: Vit. content in Clementine pulp.

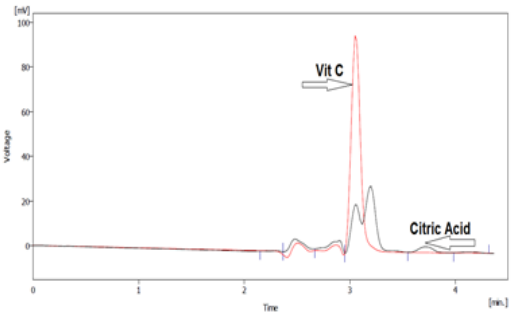

Fig. 19: Vit. C content in Clementine peels.

Table 8: Vit. C content for pulp and peels in Clementine fruit.

Pulp			Peels		
($\overline{X}$)*, mg/100 g	SD	RSD%	($\overline{X}$)*, mg/100 g	SD	RSD%
46.73	1.85	3.96	114.29	2.68	2.34
46.99	0.49	1.04	136.84	1.31	0.96
52.00	0.51	0.98	135.88	4.31	3.17
47.53	1.41	2.97	123.49	3.49	2.83
57.98	0.66	1.14	148.86	1.09	0.73

***** n=3

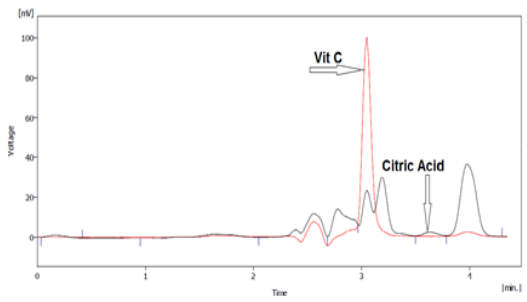

Fig. 20: Vit. C content in Satsuma pulp.

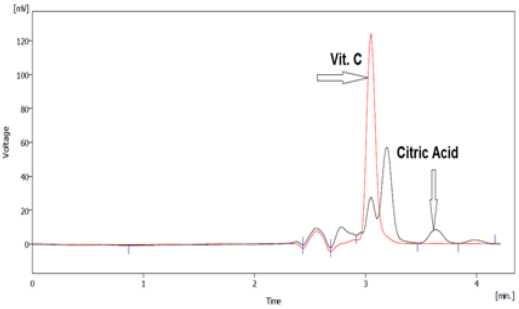

Fig. 21: Vit. C content in Satsuma peels.

Table 9: Vit. C content for pulp and peels in Satsuma fruit.

Pulp			Peels		
$(\overline{X})$*, mg/100 g	SD	RSD%	$(\overline{X})$*, mg/100 g	SD	RSD%
43.70	0.99	2.26	125.24	2.99	2.39
45.40	1.77	3.90	168.68	7.57	4.49
45.26	0.81	1.79	184.91	3.55	1.92
34.53	0.96	2.78	87.35	2.75	3.15
32.90	0.67	2.04	102.83	1.88	1.83

* n=3

3.2 Results Comparison

We resume the obtained results in table 10 by comparing between the intervals of Vitamin C content in mg/100 g in both pulp and peels for each studied fruits by our suggested HPLC method with C_{18} column.

Tabel 10: The content of Vit. C in 100 g of each pulp and peels of Citrus.

Pulp (mg/100 g)			Peels (mg/100 g)		
Citrus sinesis	Navel	39.67 – 76.16	Citrus sinesis	Navel	82.73 –159.89
	Jaffa	30.53 – 47.25		Jaffa	60.85 –146.84
Citrus limon	Eureka	29.91 – 52.10	Citrus limon	Eureka	37.25 – 78.86
	Hybrid	15.57 – 24.40		Hybrid	31.48 – 99.30
Citrus reticulate	Clementine	46.73 – 57.98	Citrus reticulate	Clementine	114.29 – 148.86
	Satsuma	32.90 – 45.40		Satsuma	88.35 –184.91
Citrus paradise	Grapefruit(duncan)	21.92 – 44.99	Citrus paradisi	Grapefruit(duncan)	51.39 –107.72
	Grapefruit(ruby red)	34.57 – 51.99		Grapefruit(ruby red)	82.02 –151.40
Citrus aurantium	Citrus aurantium	49.56 – 60.42	Citrus aurantium	Citrus aurantium	112.85 – 196.53

IV. CONCLUSION

In this work, we developed a new method with a new conditions to determine Vitamin C in some Syrian Citrus fruit without interventions with matrix components especially citric acid in the same fruit. Under the suggested chromatographic conditions Vitamin C and Citric acid could be separated and determined in less than 5 min. It was possible to separate and determine Vitamin C in the pulp and the peels of the Citrus fruit.

By the comparison between Vitamin C content in both pulp and peels. It was found the content of Vitamin C in peels is 1.52 – 3.31 times bigger than the pulp in studied different citrus fruits. And it was the biggest in Citrus aurantium peels and the smallest in hybrid pulp. So, it is recommended to eat both pulp and peels of the fruit for benefiting of more quantity of Vitamin C. Our obtained results are comparable to the results reported by other investigators in different literatures with very little differences, due to several factors that affect ascorbic acid levels in fruits. These factors include: climate, temperature and amount of nitrogen fertilizers used in growing the plants. Climatic conditions such as light and temperature have been reported to affect the chemical composition of horticultural crops. The literature has been reported that fruits which are exposed to maximum sunlight have been shown to contain higher amount of ascorbic acid than those shaded on the same plant[4]. Under the recent study we could have very good separation and fine peaks in a short time. So we can save a lot of solvents and chemical regents and a time of analysis.

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