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A Simple and Rapid HPLC Method for Analysis of Vitamin-C in Local Packed Juices of Pakistan

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Abstract: The growing number of various tetra packed juices industries requires reliable quality control to insure the actual amount of ingredient and additives reported on the labels of packed juices by the companies. Here our research related to the development and validation of a simple and rapid RP-HPLC method to estimate the actual amount of Vitamin-C present in packed juices. It was estimated by Hitachi D-2000 Elite HPLC system manager using gradient pump system, separation was carried out by using C-18 column and detection by UV-Visible Detector. The retention time was within 1.63-1.65 minutes. Standard curves were linear over the concentration range of 0.1 to 2.5mg/ml. The extraction recovery was within 94 and 101%. The proposed method was found to be rapid, accurate, repeatable and consistent. This method was also compared with oxidation-reduction methods using standardized 2,6-Dichloroindophenol (DCP), to quantifying verifying ascorbic acid levels. We observed that the amounts of ascorbic acid reported by the two methods were mostly same and identical. In the result we found that Most of our local tetra packed juices companies may underestimate or overestimate the actual content of vitamin-C.

Key words: HPLC · Method Validation · Vitamin-C · Packed Juices

INTRODUCTION

Vitamin-C remains a popular topic in the scientific literature and there are many type of methods have been reported for the determination of vitamin C in many types of food. [1-3]. One of the most frequently used methods is based on the reduction of the blue dye 2,6-dichlorophenolindophenol by ascorbic acid (AOAC, 1999). The endpoint of the titration is indicated by the appearance of the pink acid form of the dye. This is a simple and fast method [4-6] and is usually used for validation of results. It is a suitable method to determine vitamin C not only in food or fruit juices but also in pharmaceutical products and biological samples [7]. However, this method is not valid for the samples having color and majority of packed juices have strong colors. So here we need to use the methods based on high-performance liquid chromatography (HPLC) various methods are available for determination of Vitamin-C by HPLC, [8-14] that allows the simultaneous determination of ascorbic acids, but having some disadvantages of being time consuming with poor recoveries and reproducibility, while the proposed method was found to be rapid, accurate, repeatable and consistent. It was successfully applied for the analysis of Vitamin –C in fruit and fruit juices.

The fruit juices in Packed form is one of the best source of fluids, having carbohydrates (sugars), vitamins and minerals [15, 16]. They are prepared synthetically or from fruits pulp such as orange, mango, guava, black currant, apple, apricot, pineapple, lemon, lime and peach, because The fruits plays an important role in human diet and food supplement. They are also considered as healthy food supplements because they contain carbohydrates, proteins, vitamins A, B1, B2, C, D and E; and minerals such as Ca, Mg, K, Zn and Fe. [17]. Besides their dietary importance, packaged juice drinks become more acceptable due to a number of factors such as convenience, low cost, environmental factors and manufacturers' competition style [18].

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However in the juices processing form the extraction to packing, the quality of the fresh products undergo remarkable modified that could reduce the nutritional value of the drinks [19]. Vitamin-C in The juices concentrates is one of the natural ingredient in the manufacture of many packaged fruit juices which is remarkably reduces by these process, because its highly sensitive to various modes of deterioration. The main factors that can e?ect ascorbic acid loss in juices include temperature, salt and sugar concentration, pH, oxygen, light, metal catalysts, initial concentration of ascorbic acid, microbial load and protection provided by the container [20]. Some primary symptoms of a lack of Vitamin C are: tiredness, physical and mental weakness and increased susceptibility for infections. Psychic disturbances like depressions or hysteria may also be possible due to the deficiency of Vitamin-C, it is also a strong reducing substance, That plays an important role in hydroxylation reactions, i.e. in the synthesis of collagen. So it is rather important for bone, cartilage tooth and for the healing of wounds. Another important role is that of antioxidants, that means it protects other substances from the oxidizing effects of oxygen. It also promotes the re-absorption of iron in the intestine and reduces the production of nitrosamines which might cause cancer [21, 22].

Vitamin-C cannot synthesize by Primates, including human beings and must rely on their diets to provide adequate amounts. The main sources of dietary vitamin C are these fresh and packed fruits and fruit juices. In addition, many packaged foods are “fortified” with additional vitamin C. our research is a development of new HPLC method to quantify the actual amount of Vitamin-C in Packed juices.

MATERIALS AND METHODS

Chemicals and Reagents: Vitamin-C (Extra pure, Batch: 500074.100) was supplied by E Mark, Darmstadt, F.R Germany. methanol, acetonitril, of HPLC grade by (Sigma Aldrich Germany), potassium dihydrogen phosphate and meta phosphoric acid extra pure of (BDH England). Deionized water and Solvents of LC grade was obtained by filtering through 0.45µm filter membrane and degassed for 20 minutes by ultra sonic cleaner.

Chromatographic Conditions: The HPLC (Hitachi D-2000 Elite system manager) equipped with two pumps L-2130, auto injector / auto sampler L-2200 syringe loading sample injector valve’s fitted with 10µl sample loop of 200 vials and UV-VIS detector L-2420. The Chromatographic separation was achieved using Column oven L-2300 and column intersil ODS-3 C18 (GL Sciences Inc. Tokyo Japan 5µm, 250×4.6 mm). Flittering assembly (Model Rocker-300 Thaiwan) and ultrasonic cleaner Ceia (Model CP-104 Italy) were used for solvents filtration and degassing.

Titration Method: Titration methods is based on the reduction of the blue dye 2,6-dichlorophenolindophenol by ascorbic acid (AOAC, 1999). The endpoint of the titration is indicated by the appearance of the pink acid form of the dye. This official method is a simple and fast and were used in the determination of vitamin C content of the Packed Juices. 5ml of each sample was treated with 10 ml, 3% Meta phosphoric acid and filtered to remove possible protein interference. The filtrate was then titrated against freshly standardized 2, 6-Dichloroindophenol (DCP). The standardization was with 10 ml of standard ascorbic acid. Triplicate titration was conducted for all samples.

Method Validation: Method validation parameters studied were limit of detection (LOQ), limit of quantification (LOD), linearity, repeatability and accuracy. The LOQ was defined as the lowest concentration that could be determined with acceptable accuracy and precision. The LOD was determined by diluting solutions of known concentration until the response was three times the noise while LOQ was defined as the lowest concentration that could be determined with acceptable accuracy and precision. The LOQ was calculated on the basis of minimal accepted value of S/N 10. The Linearity was determined by calibration curves [23, 24]. For the construction of calibration curve, six calibration standard solutions were prepared and each standard solution was injected once. The repeatability was estimated by assaying six replicate samples on day-1 and day-2. The Accuracy was evaluated by the recovery determination.

Preparation of Solution

Solvents Preparation: (Potassium dihydrogen orthophosphate Buffer) 20 mM aqueous solution of KH_2PO_4 was dissolved in HPLC Deionized water to adjust the pH to 2.5, 3.0, 3.5 and 4.0±0.1 and meta phosphoric acid is also used instead of KH_2PO_4 for adjusting the same value of above pH for aqueous Phase. Methanol and acetonitirl used as directly as a Organic phase. All the solvents were filtered through 0.45µm filter membrane and degassed for 20 minutes by ultrasonic cleaner.

Standard Preparation: A standard stock solution of streptomycin (100mg/100mL) was prepared by dissolving the drug in Buffer. The concentration of standard was 1mg/ml solution was further diluted with mobile phase in same ratio to 0.1, 0.5, 1.0, 1.5, 0.2, 2.5 and 3.0µg/ml [25, 26]. All the solutions were refrigerated at 4°C and were brought to room temperature before use.

Sample Preparation: The sample solution was prepared and analyzed in the same manner as a standard solution. The sample solution was prepared by adding of five standard solutions of vitamin-c to made concentrations (1.0, 2.0, 3.0, 5.0 and 10.0 µg/ml) to a synthetic juice having no Vitamin C. The fortification is usually made by spiking. These fortified samples were allowed to stand at 4°C for 24 h after spiking.

Extraction of Vitamin-C: A volume of 5ml of juice were mixed with 5 ml Mobile phase. The mixture was centrifuged at 5,000 rpm for 5 min and filtered through PVDF Millipore filters (13 mm, 0.45 µm). 20ul sample uploaded to HPLC for analysis of vitamin-C. All samples were extracted in triplicate.

Optimaization of Mobile Phase Composition: The mobile phase composition was optimized by different ratios. The aqueous phase was composed of Potassium dihydrogen orthophosphate and meta phosphoric acid buffer while the organic phase was methanol and acetonitrile. Many efforts were made on the adjustment of the ratios of the components of mobile phases, pH, flow rate and wavelength of UV absorbance. The best separation and recovery were made by using 20% methanol with 80% buffer at pH 3.0±0.1 by 300nm wavelength [27].

Chromatography: The optimized mobile phase was a methanol and Buffer. Pump A was adjusted at flow rate 20% for Methanol while pump B at 80% flow rate for Buffer. The flow rate was set at 1 ml/min.

The injection volume was 10µL for samples and standards, which is injected to the column by auto sampler. The separation was achieved using Column oven L-2300 at 40°C and column Intersil ODS-3 C18 (GL Sciences Inc. Tokyo Japan 5um, 250×4.6 mm) and the detection wavelength was set at 240 nm. The UV absorbance of the effluent was scanned by UV Spectrophotometer (optima S-3000 Kyoto, Japan) over the range of 200-400nm and was obtained by measuring the absorption of 0.1µg/ml solution, prepared from stock solution. This showed a maximum absorbance on 240nm.

RESULTS

LOD and LOQ: Limit of detection (LOD) and limit of quantification (LOQ) of the assay method was determined. Results showed that the detection limit of Vitamin-C was 0.05 mg/ml and was a good improvement of before reported methods. For measurement, consideration was given only when the first condition was satisfied for ascertaining the presence of target compound with a signal/noise ratio of 3 (S/N = 3). The LOQ calculated was 0.1mg/ml**.** So, LOQ was started from this concentration. The LOQ was calculated on the basis of minimal accepted value of S/N =10.

Linearity: Intra-day and inter-day precision was determined by injecting 20µl six standard spiked sample. (n = 6). The mean of the recorded peak area of inter day and intra day is taken for calibration curve. (Table 1) The peak areas which were automatically measured by an integrator of HPLC instrument. The calibration curve obtained by plotting peak area against concentration of the standard and spiked samples in (Graph 1) which showed linearity in accordance to Beer's law over this range and the linearity equation was y = 10926x - 6445 for standard and y = 10825x + 1474 for sample. The regression coefficient r^2 were same. 9999(n=6) [28, 29].

Table1:Peak Areas of Vitamin-C std. and Spiked sample.

		Peak Areas of Standerd			Peak Area of Spiked sample		
S.No	Conc. µg/ml	Inter Day	Intra Day	Mean	Inter Day	Intra Day	Mean
1	0.1	113546	112057	112801.5	105346	110643	107994.5
2	0.5	226765	220917	223841	215632	214365	214998.5
3	1.0	325462	320023	322742.5	326402	329824	328113
4	1.5	435622	432167	433894.5	438541	431654	435097.5
5	2.0	546232	542765	544498.5	512765	517656	515210.5
6	2.5	658742	654321	656531.5	655436	657654	656545

Each 10µl Injection of Streptomycin Sulphate Peak Area (automatically measured by an integrator of HPLC instrument)

Table 2: Recovery and Precision of Vitamin-C

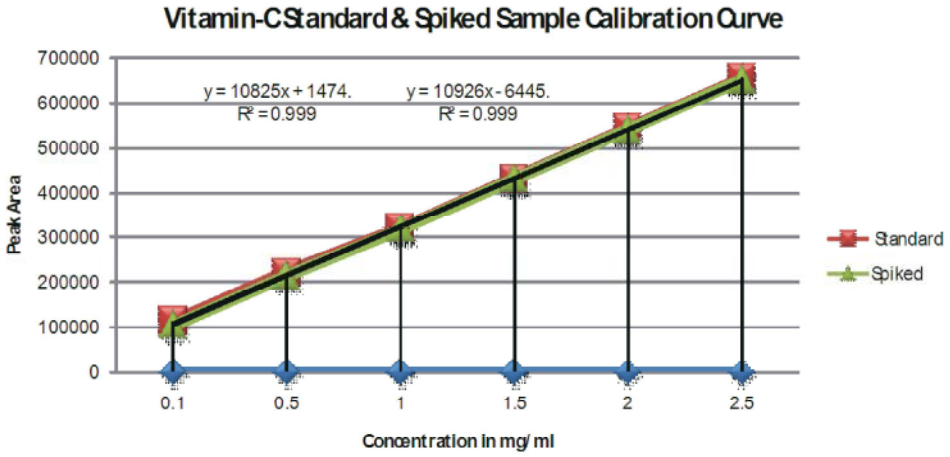
	Recovery			Precision		
S.No	Conc. (µg/ml)	Conc. Day 1 (µg/ml)	Conc. Day 1 (µg/ml)	Mean (µg/ml)	Recovery (%)	RSD%
1	0.1	0.0941	0.0965	0.0953	95.30	1.780752
2	0.5	0.522	0.443	0.4825	96.50	11.5775
3	1.0	1.016	0.972	0.994	99.40	3.13005
4	1.5	1.523	1.438	1.4805	98.70	4.059715
5	2.0	1.971	1.818	1.8945	94.72	5.710601
6	2.5	2.512	2.492	2.502	100.08	0.565233

Table 3: Recovery of Vitamin-C by HPLC method in different Packed Juice samples

Brands	Flavors	Mfg.Date/ Exp. Date	Remains (M)in Exp.	Labeled mg/Pack	Recovery mg/pack	Diff. In mg	Diff. %age
Country Lahore	Guava	06-01-2012					
		06-01-2013	9	100	106.65	+06.35	+06.35
	Grapes	06-01-2012					
		06-01-2013	9	100	86.56	-14.44	-14.44
	Apple	06-01-2012					
		06-01-2013	9	100	80.21	-19.79	-19.79
Fresh Lahore Pakistan	Apple	11-02-2012					
		10-02-2013	10	40	28.43	-11.57	-27.50
	Mango	10-01-2012					
		10-01-2013	9	32	21.11	-10.89	-34.03
Popular Karachi Pakistan	Apple	25-01-2012					
		25-01-2013	9	50	16.65	-33.35	-66.70
	Mango	01-01-2012					
		01-01-2013	9	50	15.98	-34.02	-68.04
Shizan, Lahore Pakistan	Apple	16-02-2012					
		16-02-2013	10	42	36.20	-05.80	-13.80
	Mango	01-03-2012					
		01-03-2013	11	42	36.21	-05.79	-14.47
	Guava	01-02-2012					
		31-01-2013	9	60	48.12	-11.88	-19.80
	Grapes	13-12-2011					
		12-07-2012	8	42	39.43	-06.11	-14.54
Tops Lahore Pakistan	Apple	02-03-2012					
		02-03-2013	11	60	53.23	-06.77	-11.28
	Mango	01-02-2012					
		09-02-2013	10	64	49.41	-14.59	-22.79
	Grapes	07-12-2011					
		06-12-2012	8	30	31.51	01.51	+05.03
Mezan Lahore Pakistan	Apple	12-03-2012					
		12-03-2013	11	40	19.12	-20.88	-52.70
	Mango	13-03-2012					
		12-03-2013	11	40	19.52	-20.48	-53.70
Orla Lahore Pakistan	Apple	06-03-2012					
		06-03-2013	11	40	29.11	-10.89	-27.22
	Mango	07-01-2012					
		06-01-2013	11	40	27.98	-12.02	-30.05

+ve value indicate higher value from labeled -ve sign indicate low value from labed

Recovery and Precision: The current method is valid and accurate. The Accuracy was evaluated by the recovery determination of spiked samples [30-32]. Our results showed the amount obtained by this method were between 94% and 99 %. (Table 2) the absolute recoveries of streptomycin Sulphate were determined in triplicate by direct comparison of peak area of standard versus sample. (Figure 4,5). The data was analyzed statistically by calculating average mean.



■ For standard y = 10825x + 1474. R^2 = 0.999
▲For spiked sample y = 10926x - 6445. R^2 = 0.999

Graph 1:

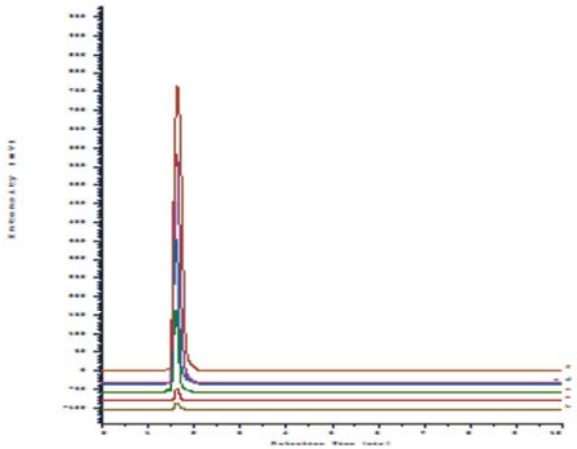


Fig. 1: Chromatograph of Standards

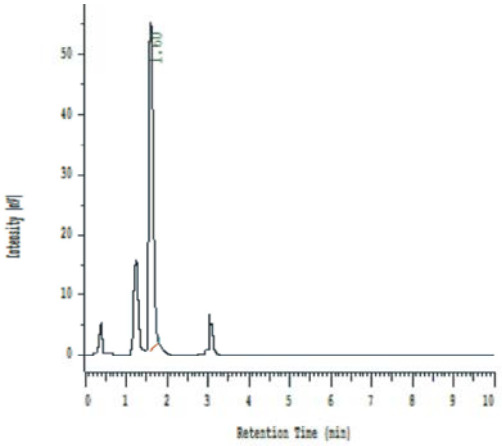


Fig. 2: Chromatogram of Spiked Vitamin-C.10ul Spot of 1mg/ml

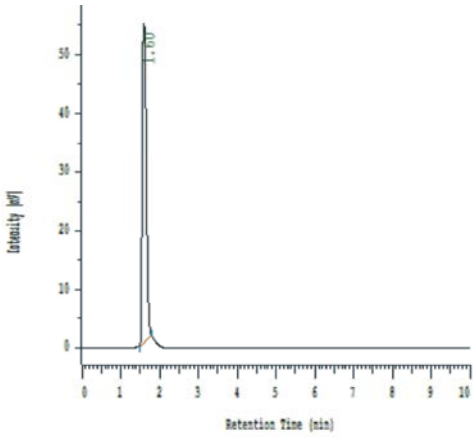


Fig. 3: Chromatogram of Standard Vitamin-C.10ul Spot of 1mg/ml

Specificity: The specificity of the method was ascertained by analyzing standard drug and sample. The retention time (RT) of streptomycin confirmed by comparing the RT with that of the standard, which was within 1.67-1.70 minutes. The presence of other ingredients in the formulation did not cause any interface with Vitamin-C peak so specific for analysis of Vitamin-C.

DISCUSSION

Several analytical methods have been developed in the last decades to determine the vitamins content in fruits and juices. However these analytical methods of Vitamin-C in most cases are based on outdated procedures, which are complicated, time consuming,

inaccurate and do not allow the simultaneous determination of the vitamins. Here the current chromatographic technique was the modification of many techniques reported by many authors [32-34] which found to be rapid, valid, accurate, time-saving, low cost and modern method to present a comparative evaluation of vitamin-C composition fruit juices. The method was also validated with the official Titration method of dye (AOAC-2000). We found this HPLC method is valid for quantifying the actual amount of vitamin-C present in packed juices, because the official method may over estimated the Vitamin-C content of all juices. For this purpose different brands of packed juices were purchased from retail shops in Peshawar Pakistan and were categorized on the bases of fruit juice manufacturing companies. Eighteen (18) different types of Packed Juices were collected. Sampling was carried out between October 2011 and February 2012. From the result of each metrics analyzed there were small significant difference in the value obtained by the chromatographic and official titrimatric method. The results become very shocked for us that no one of our local companies had a setup to monitor such an ingredient in the amount as he labeled it on juices pack. The maximum deficiency were found in popular juices, (Table 3) where the vitamin-C value were 66-68% lower then the labeled, While 52-53% deficiency were found in the samples of Mezan juice. Almost all the samples are underestimated Fresh, Orla and Tops also showed 27-34%, 27-30% and 11-22% lower then labeled. The minimum deficiency was found in the samples of Shezan and Country juice samples i.e. 13-19% and 14-19%. Only the two samples of Country and Tops which were found a little overestimate 05 and 06% from labeled value of Vitamin-C. Its mean the addition of vitamin-C was very roughly added or there was a lack of monitoring ingredients.

CONCLUSION

The aim of this study was to develop a selective and sensitive HPLC method for the rapid detection and quantification of Vitamin-C and found that this method was a valid, rapid and accurate for the actual estimation of Vitamin-C in Packed juices and can be used for a variety of beverages samples.

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