

VITAMIN C IN ORANGE JUICES DETERMINED BY HPLC: INFLUENCE OF THE WAVELENGTH OF DETECTION

DOSAGGIO DELLA VITAMINA C IN SUCCHI DI ARANCIA MEDIANTE
HPLC: INFLUENZA DELLA LUNGHEZZA D'ONDA DI MONITORAGGIO

A.J. MELÉNDEZ, E. BEJINES, I.M. VICARIO and F.J. HEREDIA

Área de Nutrición y Bromatología, Facultad de Farmacia, Universidad de Sevilla,
41012 Sevilla, España

Tel. +34 954556761, Fax +34 954557017, e-mail heredia@us.es

ABSTRACT

Two different types of orange juice, ultrafrozen (UFOJ) and from concentrate (OJFC), were analysed to evaluate the applicability of a chromatographic method for analysing organic acids to quantify vitamin C. The 2,6-dichlorophenolindophenol titration method was used as reference. Detection at 214 nm allowed vitamin C to be quantified accurately in OJFC. For UFOJ, the levels of vitamin C obtained by means of the selected wavelengths were significantly different than those

RIASSUNTO

Sono stati analizzati due tipi di succhi d'arancia (da prodotto surgelati o da concentrato) con l'obiettivo di valutare la validità di un metodo cromatografico per la determinazione di acidi organici per la quantificazione della vitamina C. Prendendo come riferimento il metodo di valutazione con 2,6-diclorofenolindofenolo, il monitoraggio degli acidi organici a 214 nm permette di quantificare in modo esatto la vitamina C in esame. Nel caso dei succhi surgelati, le lunghezze d'onda di monito-

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obtained using the reference method, indicating that the HPLC method is not suitable for the determination of vitamin C in this juice.

raggio selezionate portano all'ottenimento di livelli di acido ascorbico significativamente differenti da quelli ottenuti con il metodo di riferimento, indicando che il metodo per HPLC non è adatto alla determinazione della vitamina C in questo tipo di succo.

INTRODUCTION

Vitamin C (L-ascorbic acid) is one of the most important organic acids in fruits and vegetables, in relation to their nutritional value. Its content is not only used as a nutritional index, but it is also used to evaluate processing effects, since vitamin C is highly unstable. Several analytical methods have been developed in the last decades to determine the vitamin C content in fruits and juices. 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 (DE ASSIS *et al.*, 2001) 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 (PARK *et al.*, 1983).

Determination of ascorbic acid by means of electrochemical and enzymatic methods are also well represented in the literature (AKYILMAZ and DINÇKAYA, 1999; IJERI *et al.*, 2001). However, the majority of the methods are based on high-performance liquid chromatography (HPLC) (MARINI and BALESTRIERI, 1994; ARENA *et al.*, 2001), that allows the simultaneous determination of ascorbic and dehydroascorbic acids (NISPEROS-CARRIEDO *et al.*, 1992) or the sep-

aration of ascorbic acid and isoascorbic acid (BUI-NGUYÉN, 1980). Attention has also been focused on methods for the simultaneous determination of organic acids and vitamin C in juices (LEE, 1993; CÁMARA *et al.*, 1994), since organic acids play an important role in the organoleptic properties of juices. These last methods are also based on HPLC, using a buffer solution as mobile phase in order to avoid ionization of the organic acids due to variations in pH.

Organic acids are usually monitored at wavelengths in the range of 206-220 nm (BLANCO GOMIS, 2000), although specific methods for ascorbic acid usually call for higher wavelengths, usually in the range of 245-260 nm (NISPEROS-CARRIEDO *et al.*, 1992; LEE and COATES, 1997).

The main objective of this study was to evaluate the suitability of an HPLC method for the analysis of organic acids to determine accurately the content of vitamin C in orange juices. Several wavelengths of detection were selected and the 2,6-dichlorophenolindophenol titration method was used as the reference method for the determination of vitamin C.

MATERIALS AND METHODS

Samples

Thirty-three samples of orange juice were selected: 14 ultrafrozen orange juice (UFOJ) samples and 19 orange juices

from concentrate (OJFC). UFOJ were obtained from Valencia late oranges during the 1999 season. Samples were provided by the CCH Zumos Vitafresh industry, (Almonte, Spain). After squeezing the oranges, the juice was cooled and immediately frozen by means of an industrial freezing tunnel. Hence, the juice had not undergone high temperature or low pressure treatment. The samples were kept in a freezer chamber between -18° and -21°C until analyses. Defrosting was carried out by means of a Samsung M1713 microwave oven at 800 watts for 11 min. OJFC samples were purchased from local markets as representative examples of commercially available orange juices from concentrates. Before analysis they were kept in a cool, dry place. OJFC were prepared by reconstituting either frozen concentrate orange juice (orange juices concentrated in a high vacuum evaporator and frozen) or concentrated orange juice.

Each sample was analysed in triplicate both by HPLC and by the titration method.

Methods

2,6-dichlorophenolindophenol titration

The titration method is based on the reduction of the sodium salt of the blue dye by ascorbic acid, resulting in the formation of a colourless derivative and dehydroascorbic acid. The endpoint of titration is indicated by the persistence of the pink colour of the solution (AOAC, 1999). The solution of 2,6-dichlorophenolindophenol was prepared by dissolving 50 mg of the sodium salt of the dye (Panreac, Montcada i Reixac, Spain) and 42 mg of sodium bicarbonate (Panreac, Montcada i Reixac, Spain) in 200 mL water, removing the insoluble material with Albet 243 filter paper. This solution was standardised daily against a standard solution of ascorbic acid and stored in a refrigerator.

The standard solution of ascorbic was prepared daily by dissolving 25 mg of high-purity L-ascorbic acid (Panreac, Montcada i Reixac, Spain) in 25 mL of metaphosphoric acid (Panreac, Montcada i Reixac, Spain) aqueous solution (3% w/v).

HPLC

The HPLC apparatus consisted of a Hewlett-Packard 1100 system (Hewlett-Packard, Palo Alto, CA, U.S.A.), equipped with a quaternary pump, a diode-array detector and a column temperature control module. A 20 μL loop was used for injection. The column was a C-18 ODS Hypersil (2504 mm, 5 μm packing) with a guard-column of the same material.

The chromatographic procedure used was based on the isocratic method reported by LEE (1993), although slight modifications were made. Two solvents: a 20 mM aqueous solution of KH_2PO_4 (pH = 2.8) and methanol were used. Elution was carried out according to the following program: 0 min 100% KH_2PO_4 ; 5 min 96% KH_2PO_4 + 4% MeOH; 15 min 100% KH_2PO_4 .

The column was kept at 25°C and the flow rate was 0.7 mL/min. The eluate was monitored at 200, 210, 214 and 230 nm. The chromatograms were recorded using ChemStation software (Hewlett Packard; Palo Alto, CA, U.S.A). The column was washed with water at the end of each day.

Identification of ascorbic acid in the samples was made by comparison of the spectra and retention times with those obtained for the standard solutions. Peak purity was established by comparing the spectra which were obtained at the up-slope (at peak half-height), apex, and downslope (at peak half-height) against a baseline reference. The spectra were normalized and overlaid for comparison. The limits of detection (LOD) of ascorbic acid at the different wavelengths selected were calculated according to the formula $\text{LOD} = \alpha + 2s_{y/x}$ (MILLER and MILL-

ER, 1993), where $s_{y/x}$ is the standard deviation of the calibration curve and a is the intercept.

Preparation of samples and standard solutions for HPLC

Samples were prepared as follows: 9 mL of juice and 1 mL of metaphosphoric acid solution (3% w/v) were mixed. The mixture was centrifuged at 5,000 rpm for 5 min and filtered through PVDF Millipore filters (13 mm, 0.45 μ m).

The standard solutions of ascorbic acid (Panreac, Montcada i Reixac, Spain) were made from a 1 g/100 mL stock solution in 3% (w/v) metaphosphoric acid. Calibration curves at three concentration levels (100, 400 and 800 mg/L) were obtained for the different wavelengths monitored.

Statistical analysis

For statistical analyses Statistica v. 5.5 software (STATSOFT, 1999) was used. Significant differences were evaluated by means of analysis of variance (ANOVA) for repeated measurements ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Table 1 shows the regression equations for the calibration curves for ascorbic acid, r values and LOD for the HPLC method at the different wavelengths tested.

Tables 2 and 3 show the concentration of vitamin C obtained by HPLC and the 2,6-dichlorophenolindophenol titration method, for OJFC and UFOJ, respectively. Determination at 200 nm led to the highest standard deviations for both juices, indicating that quantification at this wavelength was less accurate. The results obtained by the reference method showed that the vitamin C content in UFOJ was quite homogeneous. The average concentration was 360.85 ppm, and the minimum and maximum contents were 319.55 and 382.69 ppm, respectively. In OJFC, the range of vitamin C varied widely, since some of the samples were enriched juices. Most of the samples ($n = 11$) ranged from 400 to 800 ppm. The minimum and maximum contents were 125.80 and 1,191.84 ppm, respectively.

Analysis of variance (ANOVA) for repeated measurements ($\alpha = 0.05$) was applied, in order to test the same samples using different wavelengths. For OJFC (Table 2), an accurate quantification of vitamin C was obtained only at 214 nm, since the vitamin C levels were not significantly different from those of the reference method. According to the vitamin C levels determined with the reference method, the OJFC samples were divided into three groups: 0-400 ppm, 400-800 ppm and over 800 ppm (Table 4). For the first group, vitamin C levels were not significantly different from the reference method at any of the wave-

Table 1 - Regression equations and limits of detection (LOD) for ascorbic acid in OJFC and UFOJ using the HPLC method.

λ (nm)	Equations	r	LOD (ppm)
200	$c = -3.358 + 0.049A$	0.999	15.98
210	$c = -7.984 + 0.067A$	0.999	5.14
214	$c = 4.31 + 0.03A$	0.999	18.99
230	$c = -15.204 + 0.021A$	0.999	0.40

c refers to the ascorbic content (ppm) and *A* is the peak area of the chromatograms.

Table 2 - Vitamin C (ppm) in orange juices from concentrate (OJFC).

Samples	HPLC 200 nm	HPLC 210 nm	HPLC 214 nm	HPLC 230 nm	Reference
1	196.87±28.89	176.16±2.27	142.58±0.73	125.99±2.04	150.00
2	182.54±31.25	148.77±7.79	124.32±2.60	115.77±7.45	125.80
3	478.29±11.78	443.23±13.19	387.07±15.51	394.73±12.15	375.00
4	282.89±2.66	281.21±1.17	253.00±2.44	278.50±24.53	284.88
5	586.24±47.84	557.09±8.49	498.01±0.21	542.23±6.46	480.33
6	527.18±69.44	525.97±26.35	454.61±14.56	496.99±4.81	478.08
7	504.33±2.68	522.77±6.46	466.48±6.69	516.28±9.90	462.54
8	484.27±26.43	529.87±3.15	474.57±7.01	519.09±3.76	460.56
9	878.09±20.96	884.40±2.24	793.46±0.79	927.30±0.79	836.48
10	930.99±1.82	900.44±18.07	826.84±9.57	927.01±2.74	837.90
11	563.61±83.64	585.45±1.02	511.44±7.00	517.63±14.67	464.06
12	523.72±58.35	483.86±5.73	470.57±37.75	532.31±8.23	457.52
13	1,274.49±80.11	1291.74±32.43	1,148.75±45.84	1,291.85±31.93	1,149.10
14	1,229.05±18.78	1,223.28±5.89	1,126.80±8.29	1,282.13±16.30	1,191.84
15	688.23±49.10	660.89±6.97	601.98±2.50	664.21±16.19	598.08
16	739.66±5.60	734.10±12.96	627.16±12.49	679.67±3.08	631.80
17	452.79±35.90	465.77±13.81	398.40±2.04	440.71±0.36	423.64
18	531.92±25.21	485.79±27.08	436.72±2.00	460.49±16.17	421.20
19	616.96±7.04	643.19±1.46	575.77±1.70	636.46±0.38	550.08
Means	614.32±295.79 _a	607.58±301.20 _a	543.08±275.43 _b	597.33±321.29 _a	546.26±284.76 _b

Means with different subscripts within a row are significantly different at $p < 0.05$.

Table 3 - Vitamin C (ppm) in ultrafrozen orange juices (UFOJ).

Samples	HPLC 200 nm	HPLC 210 nm	HPLC 214 nm	HPLC 230 nm	Reference
1	612.61±1.73	453.14±71.33	394.67±7.90	322.48±6.30	337.81
2	693.83±9.56	497.84±66.77	444.97±5.53	341.71±7.39	319.55
3	696.36±32.04	499.16±4.69	357.52±3.37	355.08±7.01	319.55
4	735.08±39.07	618.22±33.16	474.60±16.30	381.79±12.85	378.24
5	732.12±1.64	609.75±6.68	477.69±1.95	381.15±18.68	378.24
6	749.24±38.61	548.55±95.61	477.33±14.87	414.38±1.70	365.63
7	693.98±44.64	566.17±40.94	469.23±17.37	407.28±5.48	360.75
8	691.72±18.36	559.83±9.19	462.71±10.98	408.09±4.80	367.50
9	729.42±38.46	592.68±47.15	479.60±12.80	416.65±2.01	362.60
10	701.07±151.09	464.66±56.59	448.42±61.17	395.58±15.45	382.69
11	642.58±103.94	495.69±43.64	445.86±29.68	394.14±10.13	366.51
12	801.67±144.54	502.87±49.02	468.82±60.73	396.48±8.73	360.08
13	643.59±213.20	492.12±97.87	455.48±82.41	410.73±19.59	373.18
14	673.09±92.01	500.78±121.05	458.74±20.16	386.99±5.43	379.50
Means	699.74±48.74 _a	528.68±53.32 _b	451.12±34.67 _c	386.61±28.49 _d	360.85±20.80 _e

Means with different subscripts within a row are significantly different at $p < 0.05$.

Table 4 - Average concentrations (ppm) of vitamin C in the different OJFC groups.

Group	HPLC 200 nm	HPLC 210 nm	HPLC 214 nm	HPLC 230 nm	Reference
0-400 ppm	285.15±136.17	262.34±133.42 _{a,c}	226.74±121.06 _b	228.75±133.35 _{b,c}	233.92±117.24 _{a,b}
400-800 ppm	565.36±87.10 _a	563.16±84.61 _a	501.43±71.73 _b	546.01±79.65 _a	493.44±69.27 _b
> 800 ppm _a	1,078.16±202.48 _a	1,074.97±212.73 _{a,c}	973.96±189.86 _b	1,107.07±207.79 _a	1,003.83±193.21 _{b,c}
Means with different subscripts within a row are significantly different at $p < 0.05$.					

lengths of detection. For the second group (400-800 ppm), vitamin C was accurately quantified only at 214 nm. For juices with vitamin C levels over 800 ppm, both 214 nm and 210 nm were suitable for accurately determining vitamin C.

For UFOJ, detection at 200 nm led to the highest standard deviations (Table 3) and the higher the wavelength of detection the lower vitamin C levels determined. The vitamin C content at 200 nm was nearly double that obtained at 230 nm. This is due to partial peak overlapping, since in UFOJ at the lower wavelength of detection, there was higher partial peak overlapping (Fig. 1). This was not true for OJFC; while the profile of organic acids and, therefore, the chromatograms of UFOJ were quite constant, those corresponding to the OJFC juices analyzed were quite different among themselves. The results of ANOVA for repeated measurements showed that vitamin C levels in UFOJ obtained at all the wavelengths tested were significantly different among themselves ($p < 0.05$) and in comparison with the reference method. However, the vitamin C content calculated at 214 and 230 nm correlated well with those obtained with the titration method ($r = 0.707$ and 0.690 , respectively) but poorly with those obtained at lower wavelengths ($r = 0.170$ and 0.352 , for 200 and 210 nm, respectively). This indicates that detection at 200 nm or even at 210 nm is not suitable, probably due to the interference of compounds which either could not be

detected or which do not occur in OJFC. With respect to the reference method, the vitamin C contents of UFOJ samples were all in the 0-400 ppm range. Whereas for OJFC in this group (Table 4) any of the wavelengths selected could be used to accurately determine vitamin C, but none of them were suitable for UFOJ.

In conclusion, the accuracy with which the vitamin C content can be determined using the HPLC method for organic acids depends on the wavelengths selected and on the type of sample. For OJFC with vitamin C contents in the range 400-800 ppm, the wavelength 214 nm should be selected. For lower vitamin C contents (lower than 400 ppm), any of the wavelengths tested in this study are suitable for an accurate quantification. However, the HPLC method studied overestimated the vitamin C content of UFOJ at all of the wavelengths tested, due to partial peak overlapping.

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