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HPLC Assay of Ascorbic Acid in Fresh and Processed Fruit and Vegetables

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ABSTRACT

A method is reported to estimate ascorbic acid (AA) content in fresh and preserved fruit and vegetables. Following its extraction by HPO_3, AA is separated by HPLC on a strong anionic pellicular (Partisil 10 SAX) column, isocratically eluting with 0·1 M *sodium acetate buffer solution at pH 4·25, and detecting at 250 nm by a variable wavelength UV detector. The amount of AA is estimated by adding benzoic acid as an internal standard and processing data by an attached electronic printer plotter integrator.*

The method has shown a high selectivity and speed together with a very good reliability in the range 0·04% to 1% AA.

INTRODUCTION

Because of the significance of ascorbic acid (AA) as a quality index for fresh and processed food, as a vitaminic factor and as an antioxidant, a quick and routine method of its estimation is still of great interest.

HPLC analytical methods are now replacing the traditional titrimetric and colorimetric methods based on the reducing properties of the ascorbic acid (Roe & Kuether, 1943; Robinson & Stotz, 1945; Freed, 1966; Pelletier & Brassard, 1977); however, these HPLC methods cause problems of interference due to other reducing substances or end point

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determination, especially with coloured samples. This new instrumental technique is now becoming a good alternative to other modern analytical methods such as GC (Schlack, 1974), polarography (Jadhar *et al.*, 1975), enzymatic techniques (Kelly & Latzeko, 1981) and microfluorimetry (AOAC, 1975).

Different procedures of ascorbic acid assay have been developed over the last few years using HPLC, namely on both weak (Mai-Huong, 1980; Dennison *et al.*, 1981; Geigert *et al.*, 1981) and strong (Pachla & Kissinger, 1976; Marsili *et al.*, 1981; Busling *et al.*, 1982) ion-exchange columns and on reversed phase even ion-paired columns (Sood *et al.*, 1976; Carnevale, 1980; Rückemann, 1980; Augustin *et al.*, 1981; Finley & Duang, 1981; Busling *et al.*, 1982; Moledina & Flink, 1982). From the review of the methods published from 1976–1981 made by Hasselmann & Diop (1983), a great increase in HPLC applications for AA analysis is apparent.

The published HPLC techniques were surveyed, with the aim of carrying out a suitable method to quickly monitor the ascorbic acid content of fruit and vegetables during processing and storage.

According to our previous observations, ascorbic acid was easily separated within a very short time on a column packed with an NH_2-bonded phase acting as a weak anion-exchanger, eluting either with phosphate or acetate buffer solutions (Giangiacomo *et al.*, 1983). However, such stationary phases showed a slight solubility in the aqueous mobile phases, resulting in a reduced efficiency and stability. Therefore, we took into consideration the use of a porous silica-based strong exchanger that was more stable and withstood buffer salt changes over a wide range of pH and high temperatures without suffering a loss of column efficiency.

EXPERIMENTAL

Apparatus

A Jasco Twincle liquid chromatograph was used, equipped with a variable loop injection valve fitted upon a stainless steel column (250 × 4·6 mm ID) prepacked with Partisil 10 SAX (Whatman) and coupled to a Uvidec 100 II Jasco variable wavelength detector.

A Shimadzu Data Processor Chromatopac C-R 1 A, was employed.

Materials

A 6% aqueous solution of metaphosphoric acid (Merck) was used for diluting samples and standards. Standard solutions of 0·1% and 0·01% L(+)-ascorbic acid (AA) (Merck) in 6% metaphosphoric acid, equivalent to 1 μg/μl and 0·1 μg/μl of vitamin C, respectively, were made up. A 0·01% aqueous standard solution of benzoic acid (Baker) (IS) equivalent to 0·1 μg/μl, was used. A 0·1 M sodium acetate buffer solution, pH 4·25, was prepared according to Long. All other reagents were of analytical grade.

Before HPLC analysis, the standard solutions were filtered on Millex GV 0·22 μm (Millipore); the buffer solution was filtered on a Millipore membrane filter HA 0·45 μm.

Methods

Sample preparation

Fruit juices and syrups: samples were diluted with 6% metaphosphoric acid so as to give a concentration of AA above 0·05%.

Fruit and vegetables: 10–30 g portions of fruit or vegetable, cut into small pieces, were obtained from a representative sample, so as to provide, at least, an estimated AA content of 0·05%. The AA was extracted by homogenising the material in a 100-ml centrifuge tube in the presence of 25 ml of 6% metaphosphoric acid; the suspension was centrifuged at 6000 rpm and the extract was transferred into a 100-ml volumetric flask, after filtration through Whatman N.4 filter paper.

The procedure was followed through once more and the second extract was combined with the first; the volume was brought to the mark with 6% $(HPO_3)_n$ solution. Before injecting, the extract was filtered on a Millipore membrane filter HA 0·22 μm. It is advisable to filter the extract through a disposable column packed with a C_{18} reverse phase, such as a Waters Sep-Pak C_{18} or a Baker disposable extraction column, previously conditioned by methanol, to clean up the solution, when necessary.

These solutions can be stored at +4 °C for up to 8 h, before HPLC analysis.

HPLC analysis

The separation was achieved at room temperature (~20 °C) according to

the following parameters:

(a) Stationary phase: Partisil 10 SAX;
(b) mobile phase: 0·1 M sodium acetate, pH 4·25;
(c) flow: 1·3 ml/min;
(d) detection: 250 nm at 0·08 AUFS range;
(e) sample: 5 to 10 μl;
(f) internal standard: 10 μl 0·01 % benzoic acid;
(g) retention time (during the middle life time of the column): ascorbic acid about 3·7 min, benzoic acid about 5·1 min.

Calculations

To quantify the AA content, method No. 43 of the Internal Standard as run by the Chromatopac Data Processor and described in its instruction manual was adopted.

According to this procedure it is not necessary to draw a calibration curve because a substance (e.g. AA) can be directly estimated using an analytical factor previously determined by a calibration run with standard solutions of the substance in comparison with an internal standard (IS, e.g. benzoic acid) according to the formula:

$$F = \frac{C - C_{IS}}{C_{IS}\left(\frac{A}{A_{IS}} - 1\right)}$$

where: F = analytical factor; C = concentration of the substance (AA); C_{IS} = concentration of IS (benzoic acid); A = peak area of AA; and A_{IS} = peak area of IS.

It is then stored and used to automatically calculate the concentration of AA in unknown samples according to the specific program included in the instrument.

The analytical factor F was obtained by injecting amounts varying from 1 to 10 μl of the AA standard solutions together with 10 μl of 0·01 % benzoic acid solution as an internal standard.

Routine maintenance

At the end of every day, the system was flushed with 10 column volumes of distilled water to avoid the possible overnight solid deposits in the equipment. The system was also periodically flushed with methanol for safe maintenance.

RESULTS AND DISCUSSION

After choosing the acetate buffer solution, in order to fix the parameters of the separation, standard solutions of AA and IS were chromatographed according to different values of ion strength and pH of the mobile phase to get the highest resolution versus the least time of elution, bearing in mind the maximum achievable sensitivity.

Benzoic acid was chosen as an internal standard, because it was sharply separated from ascorbic acid and at a very close retention time. It was previously ascertained that there were no other peaks at the same retention time in the extract. In the case of the presence of both natural and added benzoic acid in the vegetables or other interfering peaks, it

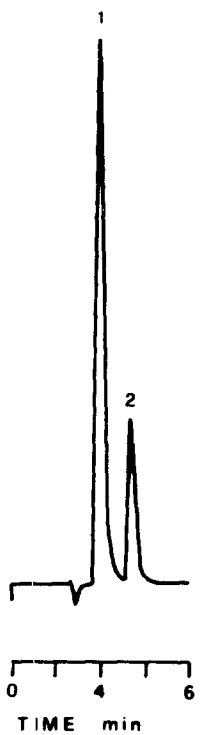

Fig. 1. HPLC separation of a standard solution containing 0·01% of ascorbic and benzoic acids. 1, Ascorbic acid; 2, benzoic acid.

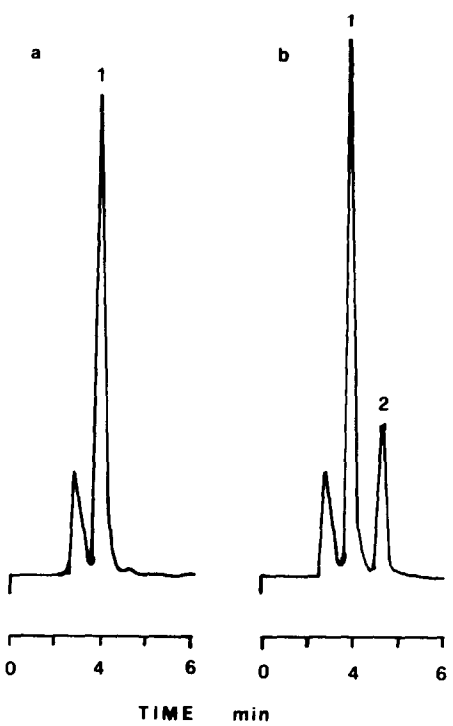

Fig. 2. Ascorbic acid analysis of zucchini squash: (a) without internal standard; (b) with internal standard. 1, Ascorbic acid; 2, benzoic acid.

TABLE 1
Analytical Factor of Increasing Concentrations of AA (IS Benzoic Acid 0·01 %)[a]

Ascorbic acid (%)	*Analytical factor*
0·005	0·338 6
0·01	0·261 1
0·02	0·251 4
0·03	0·247 3
0·04	0·220 9
0·05	0·223 3
0·06	0·219 7
0·07	0·227 6
0·08	0·225 1
0·09	0·220 4
0·1	0·222 7
0·2	0·218 9
0·3	0·215 5
0·4	0·225 7
0·5	0·212 7
0·6	0·212 6
0·7	0·221 4
0·8	0·224 6
0·9	0·225 0
1·0	0·215 5

[a] Each value is the average of five injections.
$F = 0{\cdot}220\,0 \pm 0{\cdot}004\,0$ (99·8 %).
Relative standard deviation = 0·61.

should be substituted by another internal standard; otherwise, a calibration curve of ascorbic acid should be prepared. Therefore, the optimal conditions resulted as quoted in the experimental part. Figure 1 shows the chromatogram of a 0·01 % AA and a 0·01 % IS (benzoic acid) standard solution.

The analytical factor F was checked in the concentration range, 0·005 % to 1 % of AA (Table 1).

From 0·04 % to 1 % AA the value of F was nearly constant with a mean value of 0·2200, a standard deviation $S = 0{\cdot}001\,34$ and a relative standard deviation $C = 0{\cdot}61$. Therefore the method can be considered reliable in such range.

TABLE 2
Precision Test for the Method: Analysis of Zucchini Squash

Analysis	*mg/100 g (averages)*
1	28·43
2	27·58
3	28·41
4	28·21
5	26·98

$\bar{x} = 27{\cdot}57 \pm 0{\cdot}84$ (99 %).
Relative standard deviation = 0·63.

Precision was tested on five samples of the same stock of zucchini squash: Fig. 2 shows the separation of AA in the extract. It may be seen that there were no other peaks at the retention time of IS. The data referred to in Table 2 show the good precision of the procedure: $\bar{x} = 27{\cdot}57 \pm 0{\cdot}84$ (for $P < 0{\cdot}01$ %).

The recovery tests were carried out on 'Golden Delicious' apples. To four duplicated samples of apples increasing amounts of AA standard solution were added according to Table 3. These samples and the apple without any addition, used as a blank, were then submitted to the same complete analysis procedure. The data were corrected for the amount of AA naturally present in the fruit.

The recovery was good and comparable to that quoted in other HPLC

TABLE 3
Recovery of Ascorbic Acid Added to Apples

Ascorbic acid added (mg/100 g)	*Recovery[a] (mg/100 g)*	*Recovery (%)*
5	4·6	92·0
10	9·5	95·0
50	47·2	94·4
100	93·2	93·2

[a] Minus the natural content of the fruit (0·2 mg/100 g).
Average = 94·2 ± 1·68 (95 %).

TABLE 4
Ascorbic Acid Analysis in Some Products

Products	*Ascorbic acid (mg/100 g of fresh product)*
Cauliflower:	
raw	103·2
blanched	42·8
Frozen green beans (stored at −20 °C for 12 months):	
raw	2·5
raw (frozen)	0·8
water blanched	2·6
120″ steam blanched	1·0
90″ steam blanched	2·0
Fresh pepper (Linea 81-375)	300·1
Fresh pepper (Lungo Rubens)	294·6
Fresh pepper (Linea 80-305)	238·2
Fresh pepper (Linea 81-245)	183·2
Pepper stored at +4 °C for three weeks:	
white	136·8
red	39·4
Overripe tomatoes stored at +4 °C for three weeks	12·4
Zucchini squash	27·6
Apricots var. San Castrese	10·4
Cherries var. Bargioni (overripe)	1·2
Cherries var. Vittoria	3·8
Apples 'Golden Delicious'	2·8

methods (Rückemann, 1980; Hofman *et al.*, 1981) and sometimes better for the reduced variability of the data (Dennison *et al.*, 1981).

The method can be applied to a wide range of concentrations of AA in fruit and vegetables as shown by Table 4. When it is necessary to estimate very low amounts of AA, as found for instance in overripe cherries and frozen green beans stored for 12 months (Fig. 3), as quoted in Table 4, the volume of solution should of course be increased. An incomplete resolution of AA can arise from other UV absorbing (at 250 nm) substances in such extracts. However, it was ascertained that the AA peak may be integrated and processed, although it was not completely resolved, without significant error. Therefore, the method may be considered

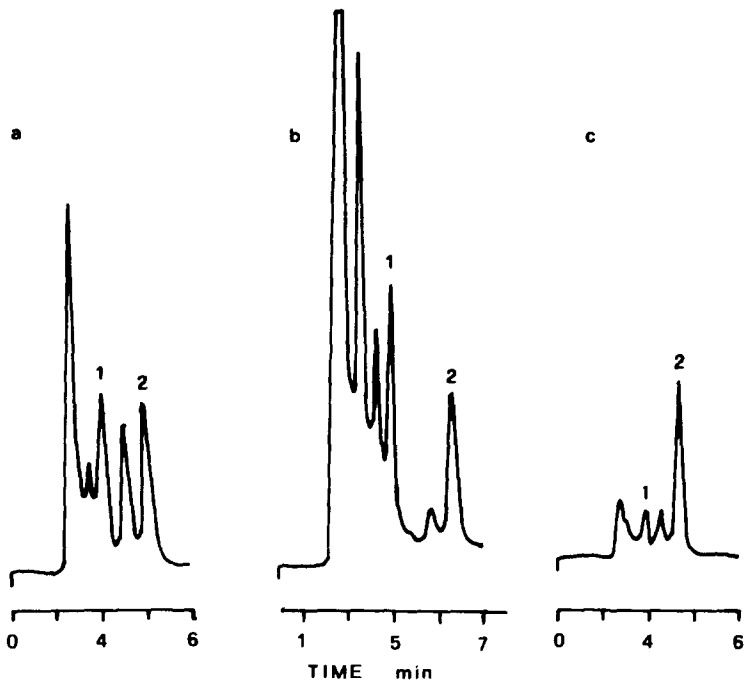

Fig. 3. Analysis of extracts from fruit and vegetables with a low content of ascorbic acid: (a) apricots; (b) overripe cherries; (c) frozen green beans stored at −20 °C for 12 months. 1, Ascorbic acid; 2, benzoic acid.

reliable even when an incomplete resolution of peaks from other compounds occurs.

The longer retention times shown in Fig. 3(b) should be attributed to the different life time of the column. This analysis was carried out using a freshly-conditioned new column, while the other two quoted examples were obtained after about thirty injections. Then the retention time was sufficiently stabilised for at least four months of routine work.

From the quantitative point of view, some shift of the retention time did not influence the accuracy of the analysis.

Table 4 indicates some effects of processing and storage on vitamin C content in fruit and vegetables. According to these data, the content of particularly AA-rich vegetables, such as peppers, appears to fall markedly after a few weeks of storage. Moreover, the effect of blanching is displayed by the losses of the AA in cauliflower; the content of vitamin C after 12

months of storage at −20 °C of frozen green beans is also very low but differentiated according to the pre-treatments.

Therefore the method reported here appears to be a useful and versatile procedure for research on the stability of processed fruit and vegetables, due to its high selectivity and speed. The total analytical time takes about one hour starting from the whole product. In routine operations at least 20 samples of fruit or vegetables can be analysed in one day, as shown by the practical application of the method to several hundred samples. For juices and syrup, needing only a sample dilution, the time of analysis is reduced to 15 min at maximum.

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