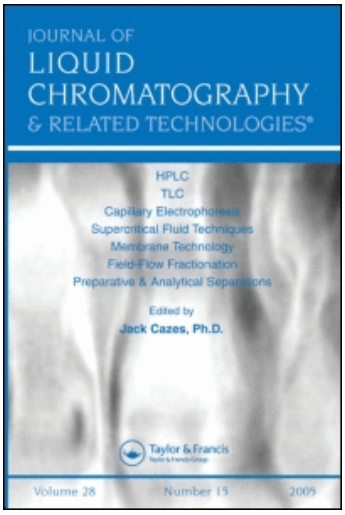

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A QUANTITATIVE ESTIMATE OF ASCORBIC AND ISOASCORBIC ACID BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: APPLICATION TO CITRIC JUICES

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ABSTRACT

The present report deals with the optimization and verification of a simple HPLC isocratic method useful for determining ascorbic and isoascorbic acids in standard solutions and fruit juices. Sample preparation is minimal and does not require derivatization. The method uses an octadecyl reversed phase, a mobile phase which contains cetyltrimethyl ammonium bromide 0.05M as the ionic pair and potassium dihydrogen phosphate as a buffer, at pH=4.5, and 4-hydro-xyacetanilide as internal standard.

The proposed method makes it possible to quantify ascorbic and isoascorbic acid in less than 13 minutes, with precision (C.V. 3.5%) and accuracy (recovery of 98%).

INTRODUCTION

It is a fairly common practice to fortify certain foods with vitamin C. Besides increasing the vitamin value, this process gives foods antioxidant

activity. Ascorbic and isoascorbic acids both have antioxidant activity but the vitamin value of the latter is 20 time lower (1). On the other hand, the use of isoascorbic acid as an antioxidant is not permitted in EEC countries.

It is clear, therefore, that an analytical method that can be used to measure the ascorbic and isoascorbic acid contents of food samples and to detect possible adulterations is of interest. Methods of vitamin C analysis based on its oxidation–reduction potential do not allow us to differentiate between ascorbic and isoascorbic acids, because both have the same potential redox. A review of the bibliography shows that the methods proposed for differentiating between the two are HPLC techniques based on the use of a weak anion exchange column (amino bonded) (1,2,3) or on paired ion reversed phase chromatography (4,5,6,7). Detection is carried out using an electrochemical detector or UV.

The aim of our work is to optimize an HPLC method in the ion pairing mode that is useful and adequate for determining ascorbic and isoascorbic acid in food samples, using an octadecyl reversed phase column. The quality of the method is verified by determining the analytical parameters of the method applied to standard solutions and to fruit juice samples.

MATERIALS

Reagents

- Extractant: Metaphosphoric acid from *Aldrich*
- Solvents: Methanol from *Romil*
- Standards: L–ascorbic and isoascorbic acids from *Aldrich*
- 4– hydroxyacetanilide– internal standard from *Merck*
- Distilled–deionized water *Millipore– MilliQ* was used to prepare all solutions Solutions were filtered through a *Millipore* HPLV 0047 0.45 μmfilter.

High Performance Liquid Chromatography

The chromatograph (Shimadzu–Japan) was equipped with an LC–6A pump an SPD–6A detector and an SCL–4A integrator and SCL–6A control system.

The detector was set at a wavelength of 254 nm.

A Spherisorb ODS C_{18} column (l= 250mm, ⊕ = 4mm and particle size 5 μm) was used for the analysis.

Sample preparation

1 mL fruit juice (enriched with 400μg ascorbic acid / 1 mL of juice) was diluted by adding metaphosphoric acid 2.5% to complete the volume to 50 mL. The dilution depends on the estimated concentration of ascorbic acid, which has to be in the range of 5 to 25 μg/mL. The amount of 4' hydroxyacetanilide needed to reach a concentration of 3 μg/mL was also added. The solution was then filtered and was ready for use in the chromatograph.

RESULTS

The development of an HPLC method implies the selection and optimization of chromatographic conditions, column and mobile phases, and the most adequate internal standard.

Given the fact that the column chosen for our study, Spherisorb C_{18}, is apolar, a reagent that would act as an ionic pair was needed in the mobile phase to obtain good resolution of both isomers, and retention times that would allow quantification of ascorbic and isoascorbic acids and separation from the other components of the sample.

The ionic pair reagents assayed were: triethylamine (TEA), tributylamine (TBA) and cetyltrimethylammonium bromide (BCTMA), all of them at a concentration of 0.05M in aqueous solution. The assays were carried out at pH 4, 4.5 and 7, using phosphoric acid and potassium hydroxide to adjust the pH value. The usefulness of KH_2PO_4 as a buffer was also tested. The composition of the mobile phase was optimized by taking into account the pH value and the ionic strength. The possible advantages of using an organic solvent, methanol, which is less polar than water, were evaluated.

Conditions (reagent, concentration and pH values) and results (retention times and resolution factors) of the assays are summarized in Table 1.

TABLE 1

Optimization of Mobile Phase and Selection of Ionic Pair Reagent

R	KH_2PO	pH	MeOH	T_R	T_R	T	Rs
	M		%	AA	AI		
TEA	0	4	0	3.3	3.3	0.12	0.40
TEA	0	7	0	2.3	2.3	0	0
TEA	0.025	4	0	3.3	3.4	0.19	0.83
TEA	0.025	7	0	2.2	2.2	0	0
TBA	0	4	0	23.1	25.7	2.62	3.06
TBA	0	7	0	13.2	14.3	1.17	1.18
TBA	0.025	4	0	4.1	4.4	0.31	1.17
TBA	0.025	7	0	2.7	2.8	0.11	0.41
BCTMA	0	4	0	21.4	23.0	1.57	0.52
BCTMA	0	7	0	5.9	6.8	0.98	1.64
BCTMA	0.025	4	0	7.9	9.2	1.32	2.87
BCTMA	0.025	7	0	6.4	8.0	1.62	3.63
BCTMA	0.025	4.5	0	11.5	13.1	1.64	3.61
BCTMA	0.05	4.5	0	8.7	10.5	1.83	3.81
BCTMA	0.1	4.5	0	8.7	10.5	1.83	3.81
BCTMA	0.05	4.5	10	7.8	8.4	0.60	1.37

R: Ionic pair reagent; TEA: Triethylamine; TBA: Tributylamine;
BCTMA: Cetyltrimethylammonium bromide
(M): Molarity
T_RAA and T_RIA : Retention times of ascorbic and isoascorbic acid, respectively.
T: Difference between T_RIA and T_RAA; R_s: Ressolution factor of both acid peaks

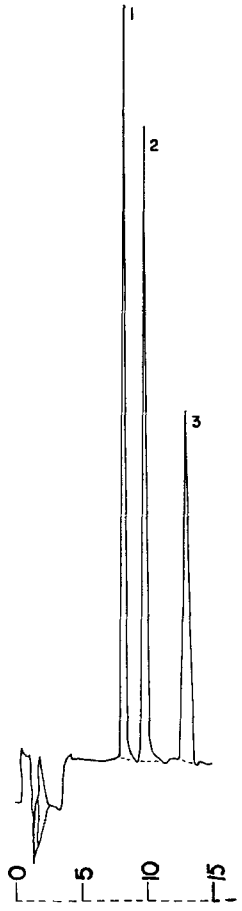

FIGURE 1.– Chromatogram of a standard solution of 5 μg/mL ascorbic acid, 5 μg/mL of isoascorbic acid and 3 μg/mL of 4' hydroxyacetanilide in HPO_3 2.5%.

1: Ascorbic acid; 2: Isoascorbic acid; 3: Internal standard

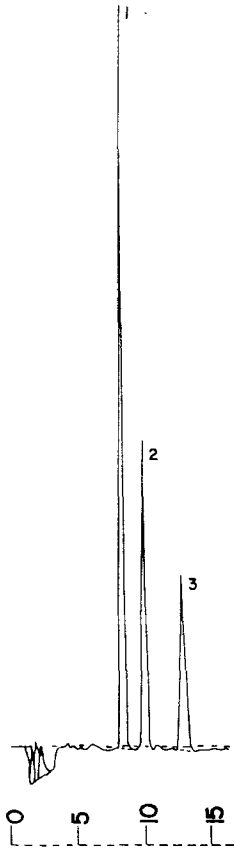

FIGURE 2.– Chromatogram of a diluted (1/50) juice sample to which 5 μg/mL of isoascorbic acid is added. Ascorbic acid content of the diluted sample: 8.95 μg/mL.

1: Ascorbic acid; 2: Isoascorbic acid; 3: Internal standard

The best resolution of the mixtures of ascorbic and isoascorbic acid is achieved with BCTMA 0.05 M in KH_2PO_4 0.05M at pH 4.5 as can be seen in Table 1.

The results obtained in the assays make it possible to propose the following chromatographic conditions:

- Mobile phase: 5 mmol/L BCTMA and 50 mmol KH_2PO_4 in aqueous solution at pH= 4.
- A flow-rate of 1 mL/min.
- 4'-hydroxyacetanilide was chosen as the internal standard because it had a high absorbance at the selected wavelength, and in the chromatographic conditions proposed had a retention time of 12.8 min., which differs from that of the analyzed acids and does not significantly increase the analysis time.

Application of the proposed analytical conditions makes it possible to obtain the chromatograms shown in Figures 1 and 2. The first one corresponds to the standard solutions and the second to two 1:50 dilutions of fruit juice samples in metaphosphoric acid; 5 μg/mL isoascorbic acid was added to one of the dilutions.

Analytical Parameters

To evaluate the quality of the proposed method the following parameters were measured: lineality, sensitivity, precision and accuracy (recovery method), applying the procedure to standard solutions and fruit juices.

- Lineality : A lineal response is obtained in the following ranges:

Ascorbic acid

Standard 1 - 80 μg / mL $y = -0.57 + 0.30x$ $r = 0.999$

Juice 2 - 60 μg/mL $y = -0.27 + 0.29x$ $r = 0.999$

Isoascorbic acid

Standard 1 - 80 μg/mL $y = -0.53 + 0.29x$ $r = 0.999$

Juice 2 - 60 μg/mL $y = -0.24 + 0.28x$ $r = 0.999$

The lineality ranges include the contents of ascorbic and isoascorbic acid in diluted fruit juice samples.

- Precision (relative standard deviation): The precision of the method is calculated from the analysis of 6 homogenous samples of a standard solution and of a fruit juice added with isoascorbic acid. The instrumental precision was checked from 9 consecutive injections of the same fruit juice added with isoascorbic acid. The following results were obtained.

	Ascorbic acid	Isoascorbic acid
Mean conc. µg/mL	468	492
Precision instrumental n = 9	2.6%	2.2%
Precision of the method n = 6	3.5%	3.1%

Reproducibility is good with variation coefficients for the whole method of about three.

- Accuracy: The accuracy of the method was verified by means of recovery assays, to three aliquots of a diluted juice, adequate amounts of ascorbic and isoascorbic acid were added in order to obtain added concentrations of 5, 10 and 20 µg/mL, respectively. The assay was repeated twice. The following results were obtained.

Aliquot	Conc. AA µg/mL	Conc IA µg/mL	Recovery % AA	Recovery % IA
Diluted Fruit juice (FJ)	4.2	–	–	–
FJ + 5	8.9	4.9	95.0	98.2
FJ + 10	14.4	9.8	101.7	97.8
FJ + 20	24.1	20.1	99.5	100.7
Mean recovery			98.7	98.9

Recovery was calculated by applying: (Found – Present /Added) x 100

Detection and Quantitation Limits (LOD and LOQ): They were calculated applying the method proposed by KNOLL (8).

	Ascorbic standard	Acid juice	Isoascorbic Standard	Acid Juice
LOQ µg /mL	0.031	0.035	0.037	0.040
ng	0.62	0.70	0.73	0.076
LOQ µg/mL	0.089	0.092	0.106	0.107
ng	1.8	1.9	2.1	2.1

Detection and quantification limits are very low in relation to ascorbic acid contents of fruit juices.

CONCLUSION

The proposed method is a simple HPLC isocratic one. Its mobile phase does not include organic solvents and uses an octadecyl reversed phase column, which is often used in laboratories because of its multiple applications.

The separation between ascorbic and isoascorbic acids is good; it lasts less than 13 minutes and the analytical parameters are adequate for analysing fruit juice samples.

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