Journal of Chromatography, 207 (1981) 449–453
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,547

Note

Resolution of ascorbic, dehydroascorbic and diketogulonic acids by paired-ion reversed-phase chromatography

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(First received September 16th, revised manuscript received November 25th, 1980)

Investigation of the interrelationships of ascorbic acid and its oxidation and hydrolytic derivatives in food products requires rapid qualitative and quantitative analysis. Because interest in oxidative changes in food products is increasing, it is of particular importance to determine simultaneously ascorbic acid, dehydroascorbic acid, and 2,3-diketogulonic acid. Overlapping specificities of colorimetric and titrimetric chemical assays for the individual components make such assays impractical for rapid measurements in processed food products. Marchesini *et al.*[1] proposed an enzymatic method for the determination of the various ascorbic acid derivatives. The method was free of most interferences, but does not seem to lend itself to repeated rapid determinations on a large number of samples.

The preferred method for determination of ascorbic acid is the reduction of 2,6-dichlorophenolindophenol. Dehydroascorbic acid must be determined by difference after reduction to ascorbic acid and subsequent reduction of 2,6-dichlorophenolindophenol. The 2,6-dichlorophenolindophenol method does not measure the biologically unavailable 2,3-diketogulonic acid. Ascorbic acid can be determined by its ultraviolet absorbance at 245 nm, but Hegenauer and Saltman[2] report that measurement at 260 nm avoids some interferences. An alternative procedure for the determination of dehydroascorbic acid is the reaction of the hydrolytic product, 2,3-diketogulonic acid, with 2,4-dinitrophenylhydrazine. Dehydroascorbic acid can be differentiated from ascorbic acid by its more rapid reaction with 2,4-dinitrophenylhydrazine. Dehydroascorbic acid is differentiated from 2,3-diketogulonic acid by reducing the dehydroascorbic acid to ascorbic acid. Hegenauer and Saltman[2] have used a combination of these methods to monitor an ion-exchange separation of ascorbic, dehydroascorbic, and diketogulonic acids.

The development of high-performance liquid chromatography (HPLC) has substantially improved the separation of vitamins and organic acids. The separation of ascorbic acid in vitamin mixtures by ion-exchange chromatography has been reported by Williams *et al.*[3] and reversed-phase partition chromatography by Wills *et al.*[4]. Wagner *et al.*[5] have measured ascorbic acid on urine samples by reversed-phase partition chromatography. Sood *et al.*[6] and Wills *et al.*[4] reported the use of paired-ion chromatography to effect separation of water-soluble vitamins from vitamin concentrates. The purpose of our work was to extend the use of paired-ion chromatography to the simultaneous assay of ascorbic, dehydroascorbic and diketogulonic acids.

0021-9673/81/0000–0000/$02.50

EXPERIMENTAL*

Analyses were performed with a Waters Associates liquid chromatograph (Milford, MA, U.S.A.), equipped with a U6K injector, a 6000A solvent delivery system, an M450 variable-wavelength detector set at 210 nm and an LDC dual-wavelength monitor (Laboratory Data Control, Miami FL, U.S.A.) set at 254 nm. Separations were achieved on two Waters Assoc. μBondapak C_{18} columns (30 cm $\times$ 3.9 mm I.D.).

The mobile phase was prepared from distilled water which was further purified by passing it through a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). Disodium phosphate was added at various concentrations from 0.004 to 0.04 *M*. The mobile phase was completed by addition of the counter-ion reagents, which were either PIC-A (Waters Assoc.) or 0.7 ml of tri-*n*-butyl amine/l, which were added prior to filtration through a 0.22 μm Millipore filter. Final pH adjustment was made by addition of 4 *M* H_3PO_4, as required. The mobile phase was pumped through the columns at 0.7 ml/min for all experiments. Fruits, juices and vegetables were prepared according to procedure of the American Association of Official Analyticai Chemists[7]. The only further purification necessary was to pass the extracts through a C_{18}-SepPak (Waters Assoc.). Ascorbic acid was obtained from Eastman Chemicals, Rochester, NY, U.S.A., and the dehydroascorbic acid and 2,3-diketogulonic acid were prepared as described by Hegenauer and Saltman[2].

RESULTS AND DISCUSSION

Preliminary experiments showed that phosphoric acid with some additional sodium phosphate present was superior to sulfuric acid, hydrochloric acid and trifluoroacetic acid for buffering the mobile phase. Initial experiments were carried out with PIC-A, as specified by the manufacturer, although a high ultraviolet background at lower wavelengths made this material less desirable than the tri-*n*-butyl amine. The first variable to be studied in detail was the effect of pH on the separation of ascorbic, dehydroascorbic, and diketogulonic acids. Table I shows the results of varying the pH on the separation of the three compounds. It can be seen that at pH 5.3 resolution was excellent. Further lowering of the pH actually had little effect on the resolution, although slight differences in retention of dehydroascorbic and diketogulonic acid were observed. The next variable to be evaluated was the effect of ionic strength of the phosphate buffer. Results of these comparisons are shown in Table II. From the data it appears that low concentrations of phosphate increase the retention of all three derivatives. At very high concentrations (0.02–0.04 *M*) the diketogulonic and dehydroascorbic acid retentions are again reduced. It is apparent from this experiment that 0.008 *M* phosphate retains the ascorbic acid on the column longer and affords acceptable separation of all three compounds. Results of a typical separation of the three components are shown in Fig. 1. In preliminary experiments a flow-rate of 0.7 ml/min was found to be optimal. At greater flow-rates, the retention

* Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

TABLE I

EFFECT OF pH RETENTION OF ASCORBIC, DEHYDROASCORBIC AND DIKETOGULONIC ACIDS

Void volume (V_0) = 4 min. Retention times are averages of four determinations.

pH	*Retention times (min)*		
	Ascorbic	*Diketogulonic*	*Dehydroascorbic*
7.1	4 ± 0.04	4 ± 0.05	4 ± 0.04
6.5	4.8 ± 0.1	—	4.8 ± 0.0
5.3	6.0 ± 0.1	8.0 ± 0.3	6.0 ± 0.1
7.5	6.0 ± 0.1	8.2 ± 0.2	6.2 ± 0.1
4.0	6.0 ± 0.1	8.2 ± 0.2	6.2 ± 0.1
3.4	6.0 ± 0.1	7.0 ± 0.2	6.5 ± 0.2

TABLE II

EFFECT OF PHOSPHATE CONCENTRATION ON RETENTION TIMES OF ASCORBIC ACID OXIDATION PRODUCTS

Solvent: 0.7 ml/min, pH 3.4. Retention times are averages of four determinations.

Phosphate (M)	V_0	*Retention times (min)*		
		Ascorbic	*Dehydroascorbic*	*2,3-Diketogulonic*
0	4	4.8 ± 0.1	5.2 ± 0.2	6.0 ± 0.1
0.004	4.0	6.0 ± 0.1	6.5 ± 0.2	7.0 ± 0.2
0.008	4.0	6.8 ± 0.1	8.4 ± 0.2	9.2 ± 0.1
0.012	4.0	6.4 ± 0.1	7.8 ± 0.3	8.6 ± 0.1
0.016	4.0	6.2 ± 0.1	7.2 ± 0.2	8.2 ± 0.1
0.020	4.0	5.6 ± 0.1	6.4 ± 0.2	7.9 ± 0.1
0.040	4.0	5.2 ± 0.1	5.8 ± 0.2	6.6 ± 0.2

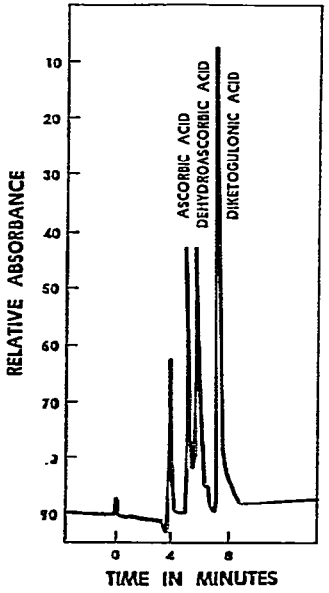

Fig. 1. Typical separation of ascorbic acid, dehydroascorbic acid and 2,3-diketogulonic acid, carried out on Waters μBondapak C_{18} columns (30 cm × 3.9 mm I.D.) at pH 5.3 with 0.008 *M* phosphate buffer and 0.7 ml/l tri-*n*-butyl amine in the mobile phase.

times were extremely short, and ascorbic acid and dehydroascorbic acid were not well separated. Because of the limited retention of these compounds in this system, the only acceptable separations we obtained were with two μBondapak C_{18} columns. A third μBondapak C_{18} column may prove useful for samples containing poorly retained interfering compounds.

To test the separation of ascorbic acid and its oxidation products in the analysis of food products, several orange juice products and one orange drink were analyzed for ascorbic acid, dehydroascorbic acid and 2,3-diketogulonic acid. The same extracts were checked for ascorbic acid according to the American Association of Official Agricultural Chemists[7]. Dehydroascorbic acid was determined by difference after reduction by the same method. The results in Table III show an excellent correlation between the ascorbic acid values determined by HPLC and by titration. There was considerable variation in the levels of dehydroascorbic acid between samples. After extraction, the HPLC method requires 15 min per sample, giving not only data for ascorbic acid, but also for dehydroascorbic and 2,3-diketogulonic acids. To obtain data for dehydroascorbic acid by titration procedure, a reduction and second titration is required. It does not give data for the 2,3-diketogulonic acid, but such information may be a valuable index of "overprocessed" or otherwise damaged products.

TABLE III

COMPARISON OF HPLC AND TITRATION METHOD FOR ASCORBIC ACID DETERMINATION IN ORANGE JUICE

Concentrations in mg/100 ml, as served (averages of triplicate determination).

Product	*Ascorbic acid*		*Dehydroascorbic*		*2,3-Diketogulonic*
	Titrimetric	*HPLC**	*Titrimetric*	*HPLC***	*HPLC***
Fresh orange juice	31.8 ± 0.4	32.7 ± 0.3	2.6 ± 0.4	2.5 ± 0.3	1.8 ± 0.3
Frozen orange juice conc. No. 1	32.8 ± 0.3	31.6 ± 0.4	5.8 ± 0.5	6.5 ± 0.4	3.1 ± 0.4
Frozen orange juice conc. No. 2	46.0 ± 0.3	45.3 ± 0.3	4.2 ± 0.4	4.9 ± 0.3	4.4 ± 0.3
Frozen orange juice conc. No. 3	41.8 ± 0.4	43.7 ± 0.3	10.1 ± 0.4	12.0 ± 0.3	5.1 ± 0.3
Artificial orange drink	37.8 ± 0.3	39.7 ± 0.4	>0.1 ± 0.1	0	
$\bar{x}$	37.98	38.6			
Average uniformity	1.02				
Correlation coefficient	0.970				
Standard deviation	6.0	6.2			

* Measured at 254 nm.
** Measured at 210 nm.

For additional comparisons, several other plant extracts were evaluated in Table IV. Of particular interest are the extracts of spinach, which were held at room temperature for extended periods of time. Table IV shows that there is considerable oxidation to dehydroascorbic acid and hydrolysis to 2,3-diketogulonic acid during that time. Most of the ascorbic acid in the initial extracts can be accounted for by the sum of the three compounds, thus adding support to the credibility of the method.

Recently Bui-Nguyên[8] has reported the separation of ascorbic acid and isoascorbic acid by HPLC. In this work it was reported that the separation on C_{18}

TABLE IV

COMPARISON OF HPLC AND TITRATION METHOD FOR ASCORBIC ACID DETERMINATION IN PLANT EXTRACTS

Concentrations in mg/100 ml or mg/100 g fresh material (average of duplicate determination).

Sample	*Ascorbic acid*		*Dehydroascorbic*	*2,3-Diketogulonic*
	Titrimetric	*HPLC*	*HPLC*	*HPLC*
Tomato juice	14.4 ± 0.3	14.3 ± 0.3	8.1 ± 0.3	6.1 ± 0.3
Green pepper	126 ± 0.6	120.0 ± 0.7	20 ± 0.7	Trace
Spinach (fresh extract)	47.4 ± 0.5	49.7 ± 0.3	11.2 ± 0.4	2.0 ± 0.5
Spinach (extract after 1 h)	30.1 ± 0.4	32.3 ± 0.5	20.1 ± 0.5	8.7 ± 0.5
Spinach (extract after 2 h)	25.2 ± 0.6	24.8 ± 0.6	23.0 ± 0.6	10.1 ± 0.8

columns with and without ion pairing was not good. In the current procedure good separation was obtained above pH 3.5, but isoascorbic acid was a shoulder on the ascorbic acid peak at lower pH values.

At higher pH values the separation seemed to be acceptable. The isoascorbic acid appears between ascorbic acid and dehydroascorbic acid on the chromatogram.

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