SEPARATION OF THREE COMMERCIAL FORMS OF VITAMIN C (ASCORBIC ACID, ASCORBIC-2-SULFATE AND ASCORBATE-2-POLYPHOSPHATE) BY HPLC*

SAMUEL P. FELTON** AND JOHN E. HALVER

School of Fisheries
University of Washington
Seattle, Washington 98195

Abstract. A modification of an existing separation technique by this laboratory is described for the separation and quantification of the three commercially available forms of ascorbic acid. The technique has the potential for identifying the various metabolic and degradation products resulting from vitamins C_2 and C_3 metabolism. A microwave technique is used for tissue heat denaturation and extraction.

A new form of vitamin C (*l*-ascorbyl-2-polyphosphate) was recently introduced to the animal feeds industry by Rangen, Inc., Buhl, Idaho (1). The form is purported to have unusual and enhanced stability over that of *l*-ascorbic acid (C_1) in feeds, in water and in elevated temperatures at neutral pH (2). The reported half-life of C_3 as compared with vitamin C_1 is days vs. minutes for C_1 in water—i.e., 20-1,300 times more stable against oxidation. The product, ascorbate-2-polyphosphate, contains ascorbate-2-triphosphate (C_3) as the major component and is primarily destined for animal feeds for the present, with prospects of human consumption after further testing has been conducted.

L-ascorbate-2-sulfate (C_2), a naturally stored form of vitamin C_1 found in fish tissue (3) is also commercially available. It too has enhanced stability over that of C_1 and can be distinguished from vitamin C_1 by HPLC (4). Now, since the advent of C_3, a modification of the above technique is described to distinguish between the three popular feed additive forms of vitamin C.

Materials and Methods. Chemicals necessary for the study were of reagent grade quality—acetic acid, sodium hydroxide, trichloroacetic acid (TCA), sodium acetate from Baker Co., ethylene dinitrilotetra acetic acid (disodium salt, EDTA) (NCB), n-octylamine (Sigma Co.), L-ascorbic acid (Hoffman-LaRoche Co.), dipotassium L-ascorbate-2-sulfate dihydrate (Dr. Paul Seib, Kansas State University), and L-ascorbyl-2-polyphosphate (Dr. Blake Grant, Rangen, Inc., Buhl, Idaho). Analytical high performance liquid chromatograph (HPLC) model SP-8000 (Spectra-Physics, Inc., San Jose, California), was equipped with a 254 nm UV-detector and two C_{18} columns in tandem, 30 cm x 4 mm i.d. each (Column Resolution, Inc., San Jose, California), for the separation. A Sharp industrial model microwave oven was used for the denaturing of the liver tissue protein . Tissue and samples were homogenized with a Brinkmann Polytron homogenizer. Gelman filters and acrodisc (0.45 μm) were used for liquid and sample extracts before HPLC injection.

Liver tissue samples were homogenized in cold distilled water in a ratio of one part tissue to nine parts water. The tissue homogenate was placed in a semi-closed polyethylene tube for microwave denaturation.

Tissue homogenate was microwaved for one minute, then cooled in ice and brought to original volume. The denatured homogenate was then rehomogenized in the Polytron at top speed for one minute, then centrifuged at 10,000 rpm in an RC5B DuPont-Sorval centrifuge. Supernatant solution was decanted, saved and the pellet rehomogenized in the same volume of cold water. The microwave treatment was repeated and the ensuing steps repeated. The two supernatant solutions were combined and filtered through Gelman acrodisc (0.45 μm) filters. The extract was now ready for HPLC analysis.

Vitamin standards were dissolved in cold buffer and made up fresh each day. The mobile phase buffer used for the HPLC separation was made from a 0.100-M sodium acetate solution containing 200 mg of EDTA and 0.17 ml of n-octylamine per liter.

HPLC conditions were as follows. Isocratic elution, flow rate of 1.5 ml/min, pressure was at 2,500 psi and the detector equipped with a 254-nm filter.

Results and Discussion. Figure 1 represents a typical profile of L-ascorbic acid, L-ascorbic-2-sulfate and L-ascorbyl-2-polyphosphate with increasing retention times, respectively. The C_1 peak

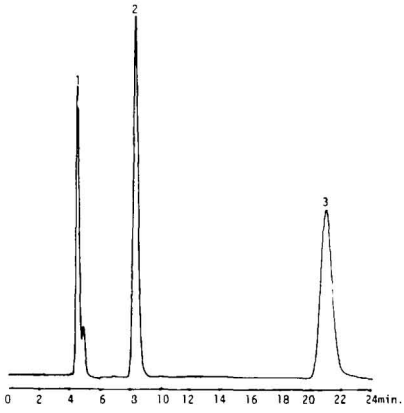

Fig. 1. A typical HPLC profile of vitamers C_1, C_2, and C_3 in 0.1M sodium acetate plotted in respective order.

represents 1 μg, the C_2 represents 2.0 μg, and the C_3 represents 7.3 μg. The C_3 peak occurs at about 12 minutes later than C_1 and about 9 minutes later than C_2. This long retention time for C_3 would be expected in view of triphosphate ester's characteristics on C_{18} type columns (5). It is anticipated that mono- and di-phosphate esters' retention times (rt) would fall in the rt areas between C_1 and C_3.

Figure 2 is a typical HPLC profile of a microwaved extraction of a fish liver sample to which has been added an aliquot of C_3. The C_3 peak represents 7.3 μg of purified C_3.

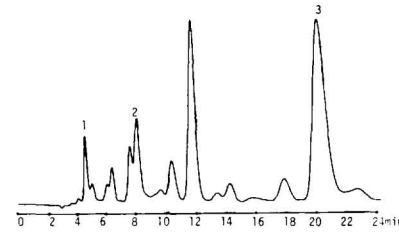

Fig. 2. A typical HPLC profile of a fish liver microwave extract in water with vitamin C3 added. Numbers 1, 2 and 3 refer to vitamers C_1, C_2 and C_3, respectively.

Cold water homogenates denatured by microwave technique was found to be very desirable whenever vitamin C_3 was present in the tissue or feed substance. Five percent TCA homogenization of tissue resulted in a 10% to 20% loss of vitamin C_3. This loss was probably due to residual phosphatase in the 5% TCA tissue extracts when C_3 was added to the tissue sample.

1. Grant BF, Seib PA, Orpron KC, Liao SL, Zeigler TR, O'Keefe T. Phosphorylated L-ascorbic acid: A stable form of vitamin C for aquaculture feeds. Proc 19th Annual Meeting of the World Aquaculture Society. Honolulu, Hawaii, Jan 4-8. 1988.
2. Technical Bulletin, Rangen Inc., L-ascorbyl-2-polyphosphate, PO Box 706, Buhl, Idaho 83316. 1988.
3. Halver JE, Smith RR, Tolbert BM, Baker EM. Utilization of ascorbic acid in fish . Ann NY Acad Sci **258**:81-102, 1975.
4. Felton SP, Halver JE. Vitamin C_1 and C_2 analysis using double column reverse phase high pressure liquid chromatography. Aquaculture and Fish Management **18**:387-390, 1987.
5. Brown PR, Hartwick RA, Krstulovic AM. Analysis of blood nucleotides, nucleosides, and bases by high pressure liquid chromatography. In: Hawk GL, Ed. Biological/Biomedical Applications of Liquid Chromatography. New York and Basel, Inc., Marcel Dekker, p. 295-331, 1979.

Received October 31, 1988. [P.S.E.B.M. 1989, Vol 190]
Accepted November 5, 1988.

0037-9727/89/1902-0217$2.00/0