Journal of Chromatography, 632 (1993) 91-96
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2603

High-performance liquid chromatography with ion pairing and electrochemical detection for the determination of the stability of two forms of vitamin C

Kathryn M. De Antonis, Phyllis R. Brown and Zhen Yi

Department of Chemistry, University of Rhode Island, Kingston, RI 02881 (USA)

Paul D. Maugle

P.D.M. & Associates, Norwich, CT 06360 (USA)

ABSTRACT

An ion-pair method with sodium octanesulfonate and ethylenediaminetetraacetic acid was used for the analysis of extruded salmon and trout feeds for ethyl cellulose-coated L-ascorbic acid and one of its more stable analogues, dipotassium ascorbyl-2-sulfate dihydrate. For enhanced sensitivity electrochemical detection was used. The procedure used to extract the vitamin C from the feeds played a vital role in obtaining good recoveries. When the ascorbic acid was extruded manually, the recovery was poor. However, when a polytron homogenizer was employed, the vitamin C recoveries were greatly improved.

INTRODUCTION

Although most animals are able to synthesize their dietary requirements of vitamin C, certain types of fish cannot [1]. It is, therefore, imperative that these fish acquire vitamin C from their diet. Since unprotected vitamin C is unstable under ordinary conditions of light, temperature and moisture, protected forms of vitamin C must be developed and evaluated for use in vitamin-supplemented feeds.

Two protected forms of vitamin C, ethyl cellulose-coated L-ascorbic acid (Cl) and dipotassium ascorbyl-2-sulfate dihydrate (C2), were analyzed by HPLC to determine their stability in manufactured feed matrices. These feeds were extruded trout feed, extruded salmon feed and moist salmon feed.

In order to evaluate effectively Cl and C2 in feeds, good methods of analysis are required to identify and quantify these dosage forms. The important requirements for a reliable method are adequate sensitivity and recovery, and the ability of differentiate between the signal from the vitamin C and the matrix.

Vitamin C and its analogues in feeds and tissues have been analyzed by a variety of techniques. For example, Quadri *et al.* [2] used a spectrophotometric method to analyze C2 in pelleted fish feed, as did Soliman, *et al.* [3] who analyzed a variety of protected forms of vitamin C including C2. The stability of these preparations was observed during both feed manufacture and long-term storage. The analytical method used was adapted from the method of Roe [4]. De Antonis et al. [5] compared three HPLC methods for the analysis of Cl and C2 in shrimp tissues. Of these three methods, two used ion-pair chromatography and one used ion-exchange chromatography; all three used UV detection. Skelbaek *et* al. [6] analyzed two forms of vitamin C (not in-

Correspondence to: Dr. Phyllis R. Brown, Department of Chemistry, University of Rhode Island, Kingston, RI 02881, USA.

0021-9673/93/$06.00

cluding Cl or C2) in pelleted fish feed both during and after manufacture. The analysis was done using reversed-phase HPLC with tetrahexylammonium bromide as an ion-pairing agent and UV detection at 243 nm. Schuep *et al.* [7] examined the stability of C2 in pelleted fish feed using reversed-phase HPLC with UV detection at 254 nm. Wang *et al.* [8] used ion-pair chromatography with tetrabutylammonium phosphate and electrochemical detection for vitamin C analysis. In the present work, Cl and C2 were analyzed with sodium octanesulfonate (SOS) as the ion-pairing agent and electrochemical detection for enhanced sensitivity.

Little is known about the stability of C2 during extrusion manufacturing, which is more rigorous than pelleting, and storage. This project was designed to examine the stability of C 1 and C2 in feeds both during and after the manufacture process.

EXPERIMENTAL

Apparatus

Chromatographic analysis was performed using a Series 3B liquid chromatograph (Perkin-Elmer, Norwalk, CT, USA), a Model 625 autoinjector with a 20-μl loop (Micromeritics, Norcross, GA, USA) and an LC-4B amperometric detector with a silver/silver chloride reference electrode and a glassy carbon working electrode (Perkin-Elmer). Peak heights were recorded with an Omniscribe strip-chart recorder (Houston Instruments, Austin, TX, USA), and peak areas were electronically integrated with a Model 3600 data station, using CHROM2 software (Perkin-Elmer). A reversed-phase Nucleosil C_{18} column (I. Molnar, Berlin, Germany; 25 cm $\times$ 4.6 mm I.D., 5-μm particles) was used with a guard column packed with Sepralyte C_{18} (25 μm).

Chromatographic method

The column was a Nucleosil C_{18} (I. Molnar; 25.0 cm $\times$ 4.6 mm I.D.). The mobile phase was 500 ml of an aqueous solution containing 3.2812 g of sodium acetate, 0.0168 g of ethylenediaminetetraacetic acid (EDTA), 0.1082 g of SOS and 26.3 ml of methanol. The pH was adjusted to 4.0 with glacial acetic acid. The mobile phase was filtered through a 0.45-μm filter prior to use. The flow-rate was 1.0 ml/min, and column was at room temperature. The electrochemical detector potential was varied from 0.70 to 0.95 V. The optimal potential value for Cl was 0.75 V and the optimal potential for C2 was 0.95 v.

Chemicals

Two commercially available forms of vitamin C were used as references, ethyl cellulose-coated t-ascorbic acid (Cl) (97% pure, 3% ethyl cellulose) (Hoffman LaRoche, Nutley, NJ, USA) and dipotassium ascorbyl-2-sulfate dihydrate (C2) (97% pure, 48% ascorbic acid equivalence) (Pfizer, Specialty Chemicals Division, Groton, CT, USA).

The following chemicals, which were of reagent-grade quality, were used without further purification. EDTA and SOS were purchased from Sigma (St. Louis, MO, USA), glacial acetic acid and methanol from Fisher Scientific (Fairlawn, NJ, USA) sodium acetate from Matheson, Coleman, and Bell (Norwood, OH, USA) and dithiothreitol (DTT) from Eastman Kodak (Rochester, NY, USA).

Doubly distilled and deionized water was prepared on site and filtered through a 0.45-μm filter (Millipore, Bedford, MA, USA) prior to use in the mobile phase.

Dry extruded feeds

Three one-ton batches of dry extruded trout feed and three one-ton batches of dry extruded salmon feed were manufactured by Archer Daniel Midland (Tuscalusa, AL. USA). The levels of Cl and C2 were different in the three batches of each feed. Three target treatment levels were made: (1) 250 mg/kg C2, (2) 350 mg/kg C2 and (3) 2200 mg/kg C 1. These are further described as T1, T2 and T3 in the trout feed and S1, S2 and S3 in the salmon feed.

Twenty-five-pound samples of the six types of supplemented feeds (three treatment levels of trout feed and three treatment levels of salmon feed) were taken at three points in the manufacturing process. The first sample was taken immediately after supplementation, the second was taken at the die plate and the head of the extruder and the third was taken at the bagging operation after the feed was dried and cooled. In addition, baseline samples were taken prior to the addition of the vitamin C. The samples were frozen with dry ice and transported to the University of Rhode Island for storage and analysis.

Moist feeds

Duplicate batches of a soft moist salmon (33% moisture) containing the same ingredients as the extruded salmon feed were made at the University of Rhode Island's aquaculture facility using a Hobart (Toledo, OH, USA) laboratory-scale soft pelleting machine. The first batch was supplemented at a level of 1000 mg/kg Cl, and the second batch was supplemented at a level of 350 mg/kg C2.

After cold pelleting, two samples of each feed were taken. One was immediately frozen (−70°C) using a bath of methanol and dry ice, and the other was stored at room temperature for 3 h prior to freezing. Samples of each feed were analyzed for Cl and C2 levels. In addition, the feeds were screened for pro-oxidants prior to their long-term stability evaluation.

In a separate study, C2 stability was examined in a commercial soft moist feed and its respective premanufacture mash provided by Conners Brothers (Blacks Harbor, New Brunswick, NJ, USA). The target potency of this batch was 250 mg/kg C2. The feed samples were received in a thawed state 3 days after manufacture and were stored at room temperature. Samples of the premanufacture mash and the finished feed were analyzed for C2 content five times in an 8-day period.

Standard preparation

Standard solutions for Cl and C2 in the concentration range 0.0001–0.01 mg/ml were analyzed. The solution were prepared in water.

Sample preparation

The vitamin C was extracted from the fish feeds using two techniques, a manual technique and a more rigorous technique which employed a polytron homogenizer.

In the manual extraction procedure, 0.5 g of the feed sample was ground to a fine powder with a mortar and pestle. The sample was then dissolved in 5 ml of a solution containing 6% metaphosphoric acid (mPA) (to precipitate the proteins [9]) and 0.2% DDT (for protection of sulfhydryl groups [10]) and vortexed for 2 min. The mixture was centrifuged at 3000 g for 3 min, and the supernatant was filtered through a 0.45-μm filter (Millipore). The filtrate was then diluted to 100 ml.

When the polytron was used, the feed sample was ground and dissolved as previously described. The resulting solution was homogenized in three aliquots using the polytron. The supernatants from each aliquot were then pooled, diluted to 100 ml and filtered through 0.45-μm filters as previously described.

RESULTS AND DISCUSSION

Screening for pro-oxidants

There was no evidence of the presence of pro-oxidants which can accelerate the degradation of the ascorbic acid. The levels of moisture, lipid, protein, ash, fiber, calcium, potassium and phosphorus were determined. The levels were found to be very close to those measured in a typical commercial trout or salmon feed, indicating that these are appropriate batches of feed for this study.

Extraction procedure

The levels of Cl and C2 determined for the extruded feeds using the manual extraction procedure are significantly lower than the levels found using the Polytron. The ascorbic acid levels were determined using each of the extraction techniques for the samples taken after storage for 30 days at 25 and 40°C (Table I). The ascorbic acid way extracted from the feed more thoroughly using the polytron than the manual technique. In all cases there was an increase in ascorbic acid level when the polytron was employed.

Chromatographic method

Aqueous standard solutions containing Cl and C2 were analyzed. The retention times of Cl and C2 were approximately 3.5 and 4.5 min, respectively. The method exhibited good linearity for Cl and C2 in the concentration range 0.0001-0.01 mg/ml. The correlation coefficients for both dosage forms were 1.000 over two orders of magnitude.

The two forms of ascorbic acid were adequately separated from the feed matrix. All of the possible interfering peaks from the matrix were eluted after the ascorbic acid (see Figs. 1 and 2 for chromatograms of feeds containing Cl and C2).

Ascorbic acid stability

During manufacture, less than 5% of the C2 and greater than 98% of Cl was lost. These data in-

TABLE I

COMPARISON OF MANUAL AND POLYTRON SAMPLE PREPARATION

Sample[a]	Dosage form	Target level (mg/g)	Level after 30 days (mg/g)			
			25°C		40°C	
			Polytron	Manual	Polytron	Manual
Trout:						
T1	c 2	250	278	129	279	149
T2	C2	350	351	219	359	279
Salmon:						
S1	c 2	250	278	139	254	179
S2	C2	350	395	314	369	299

[a] T3 and S3 were not monitored since the Cl levels were so low after manufacturing. T1 = trout feed with 250 mg/kg C2; T2 = trout feed with 350 mg/kg C2; T3 = trout feed with 2200 mg/kg Cl; S1 = salmon feed with 250 mg/kg C2; S2 = salmon feed with 350 mg/kg C2; S3 = salmon feed with 2200 mg/kg C 1.

dicate that C2 survived the rigorous extrusion manufacture process whereas the Cl did not (Table II).

The amount of C2 remaining in the extruded trout and salmon feeds was examined on a monthly basis for 6 months after manufacture. Cl was not monitored since there was an insignificant amount remaining after manufacture. After 6 months of storage, the extruded trout and salmon feeds retained 81–85% of the potency after manufacture which corresponds to 77-8 1% of the target potency. Due to inhomogeneity in the samples, the levels appear to increase with time at some points

In the soft moist feed 87% of the C2 survived cold pelleting, 85% remained after 3 h at room temperature, and 81% remained after 24 h at room temperature. Only 16% of C 1 survived the cold pelleting. After 3 h at room temperature only 4.8% of Cl remained, and after 24 h at room temperature no Cl was detected.

In a separate study of C2 stability of the soft

TABLE II

ASCORBIC ACID LEVELS OBTAINED BY THE POLYTRON EXTRACTION TECHNIQUE

Sample	Dosage form	Target level (mg/kg)	Level after supplementation (mg/kg)	Level at head of extruder (mg/kg)	Level after drying (mg/kg)	Percentage after manufacturing
Trout:						
T1	c 2	250	290	N.M.[a]	286	98.6
T2	C2	350	379	N.M.	363	95.8
T3	Cl	2200	2240	N.M.	10	0.04
Salmon:						
S1	c 2	250	292	N.M.	283	96.9
s2	c 2	350	399	N.M.	395	98.6
s3	Cl	2200	2199	N.M.	40	1.83

[a] N.M. = Not measured.

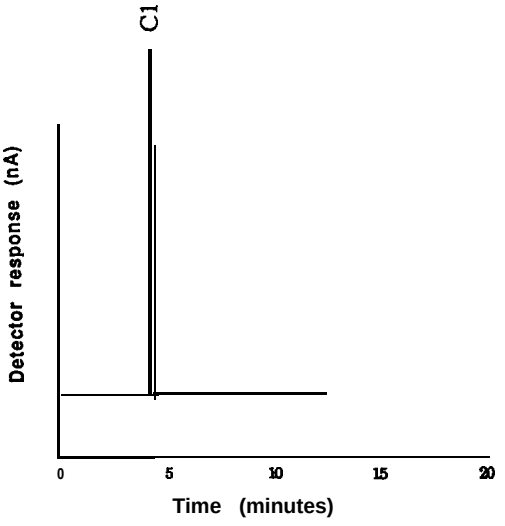

Fig. 1. Chromatogram obtained using a Nucleosil C_{18} column (I. Molnar; 25.0 × 4.6 mm I.D.). The mobile phase was 500 ml of an aqueous solution containing 3.2812 g of sodium acetate 0.0168 g of EDTA and 0.1082 g of SOS and 26.3 ml of methanol. The pH was adjusted to 4.0 with glacial acetic acid. The flow-rate was 1.0 ml/min, and the column was at room temperature. The electrochemical detector was set at 0.75 V, 200 nA full scale. The sample was a trout feed containing Cl.

moist feed, samples of a commercial soft moist feed and its premanufacture mash were provided by Conners Brothers. When this feed was analyzed over an 8-day period, 78% of the C2 remained in the premanufacture mash after 2 weeks, and 66% remained in the feed after 2 weeks (Table III). The increase in C2 content from the premanufacture mash to the finished product is due to the loss of water in the feed.

TABLE III

C2 LEVEL OBTAINED IN MOIST FEED AND PREMANUFACTURE MASH FROM CONNERS BROTHERS

Day	Premanufacture mash (mg/kg)	Feed[a] (mg/kg)
0	223	300
2	211	270
4	198	205
6	185	201
8	176	199

[a] Target amount is 250 mg/kg.

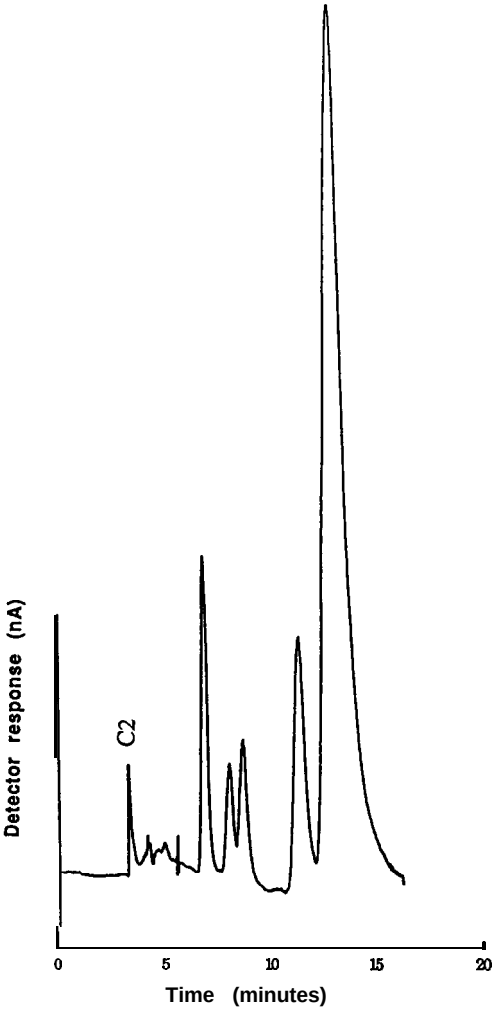

Fig. 2. Chromatogram obtained using a Nucleosil C_{18} column (I. Molnar; 25.0 cm × 4.6 mm I.D.). The mobile phase was 500 ml of an aqueous solution containing 3.2812 g of sodium acetate, 0.0168 g of EDTA and 0.1082 g of SOS, and 26.3 ml of methanol. The pH was adjusted to 4.0 with glacial acetic acid. The flow-rate was 1.0 ml/min, and the column was at room temperature. The electrochemical detector was set at 0.95 V, 20 nA full scale. The sample was a salmon feed containing C2.

CONCLUSION

The reversed phase HPLC system using ion pairing with SOS gives good linearity of Cl and C2 in the standard, and resolves the two forms of vitamin C from the feed matrix in the extract samples. The electrochemical detector gives adequate detection

for the ascorbic acid at the levels found in the feed matrices.

The extraction procedure greatly influenced the recovery of Cl and C2 from the dry extruded feeds. Data from samples extracted both with and without the homogenizer indicate that the homogenizer provides a more complete ascorbic acid extraction. In almost all cases, the homogenizer improved recovery of Cl and C2 from the feed, and the results are much more reproducible.

The data in this paper show that while almost none of the Cl activity remained after manufacture, greater than 95% of the C2 activity remained after manufacture, and les than 20% is lost during 6 months of storage at 40°C. In the most salmon feeds, 8 1% of C2 activity and none of the Cl activity was retained after 2 weeks of storage. Thus, C2 in commercial feeds is very stable during and after the manufacturing process of commercial feeds. Due to the instability of oils and vitamins other than vitamin C, commercial dry feeds are generally not stored for more than 90 days and soft moist feeds not longer than two weeks.

ACKNOWLEDGEMENTS

The authors wish to thank Archer Daniel Midland and Conners Brothers, Inc. for their assistance with this work. Partial support for this study was provided by Pfizer, Inc., Specialty Chemicals Division.

REFERENCES

1 B. W. Tucker and J. E. Halver, ***Fish Physiol. Biochem.*, 2 (1986) 151.**
2 **S.** F. Quadri, Y. T. Liang, P. A. Seib, C. W. Deyoe and R. C. Hoseney, *J.* Food *Sci.*, 40 (1975) 837.
3 A. K. Soliman, K. Jauncey and R. J. Roberts, *Aquaculture*, 60 (1987) 73.
4 J. H. Roe, ***The Vitamins,*** Vol. VII, Academic Press, New York, 1967.
5 K. M. De Antonis, P. R. Brown and P. D. Maugle, *J.* ***Liq. Chromatogr.,*** 14 (1992) 258 1.
6 T. Skelbaek, N. G. Anderson, M. Winning and S. Westergaard, ***Aquaculture, 84 (1990) 335.***
7 W. Schuep. J. Marmet and W. Studer, ***Aquaculture, 79*** (1989) ***249.***
8 ***X. Y.*** Wang, M. L. Liao, T. H. Hwa and P. A. Seib, *J.* ***Assoc.*** *Off.* ***Anal.*** Chem., 71 (1988) 1158.
9 J. A. Albrecht and H. W. Schaefer. ***J. Liq. Chromatogr..*** 13 (1990) 2633.
10 ***The*** *Merck Index*, Merck & Co., Rahway. NJ. 1 1th ed., 1989.