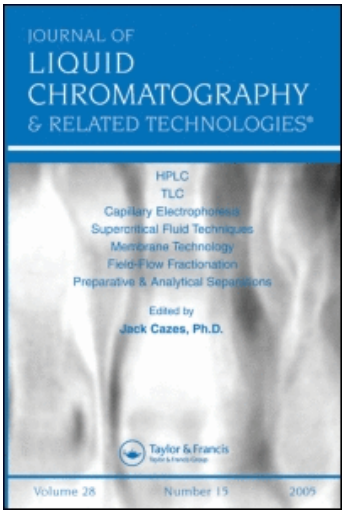

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SIMULTANEOUS HPLC ANALYSIS OF L-ASCORBIC ACID, L-ASCORBYL-2-SULFATE AND L-ASCORBYL-2-POLYPHOSPHATE

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ABSTRACT

L-Ascorbic acid is a difficult vitamin to quantitate by HPLC due to its low retention and poor resolution using the previously available column technologies. This is particularly evident in aquatic feed and meals, where various interferences deter the vitamin's accurate determination and quantitation. Furthermore, these matrices often contain other 'stabilized' forms of vitamin C, necessitating several determinations utilizing a variety of chromatographic methods.

An improved HPLC method is introduced, here, for the simultaneous separation of L-ascorbic acid, L-Ascorbyl-2-Sulfate and L-Ascorbyl-2-polyphosphate. This method utilizes two novel inert HPLC columns, Inertsil C_4 connected to an Inertsil C_{18}. The mixed phases, chemistry and packing of these columns have allowed improved separation of the earlier components (which include ascorbic acid) as well as that of the latter eluting ones (L-Ascorbyl-2-Sulfate and L-Ascorbyl-2-polyphosphate).

Various ion-pairing reagent concentrations, buffer ionic strength and pH were investigated to achieve the optimum mobile phase conditions necessary for a simultaneous and universal separation.

The final method carries out the analysis using one HPLC chromatographic system with ultra-violet detection, one mobile phase containing n-octylamine as an ion-pairing agent and the same column set for the simultaneous analysis of all the Vitamers. Percentage relative standard deviations for ascorbic acid were less than 0.5 % for the peak areas and retention times, minimum detection limits of less than 250 ppb with UV detection and recoveries in the 97 % range.

The method was then utilized to quantitatively asses bulk storage losses of commercial Ascorbyl-2-sulfate and Ascorbyl-2-polyphosphate at the typical tropical storage conditions of 37°C and 47% relative humidity.

INTRODUCTION

Vitamin C or *Ascorbic acid*, (C1), a white crystalline solid, is a water soluble micronutrient essential for normal growth and health. Various fish species and shrimp, guinea pigs and primates (non-human and human) cannot synthesize adequate ascorbic acid *in vivo*.[1] Lack of sufficient vitamin C in aquatic diets results symptoms such as poor growth, lordosis, loss of scales, reduced egg hatchability, impaired hydroxylation of collagen, internal fin hemorrhages and mortality.[2,3] Consequently, a dietary source of vitamin C is needed to meet their requirements. Ascorbic acid has been commonly added to the feed to supplement the diet. However, ascorbic acid is unstable in the feed and water as it is easily destroyed by oxidation.[4] The decomposition is accelerated in the presence of salts, moisture, heat and high pH. Substantial losses also result during feed manufacturing, storage, pelleting and extrusion.[5] Consequently, over the years, overages of ascorbic acid have been added to the diet to offset losses and permit vitamin C fortification at nutritional levels.

Stabilized forms of vitamin C have been introduced to permit fortification of feeds at cost effective levels. A variety of coating agents and ascorbic acid derivatives have been developed. These include silicone, oil and cellulose coatings as well as stearate, palmitate, sulfate and phosphate esters. The most effective derivatives to-date, recently being the focus of attention in the field of

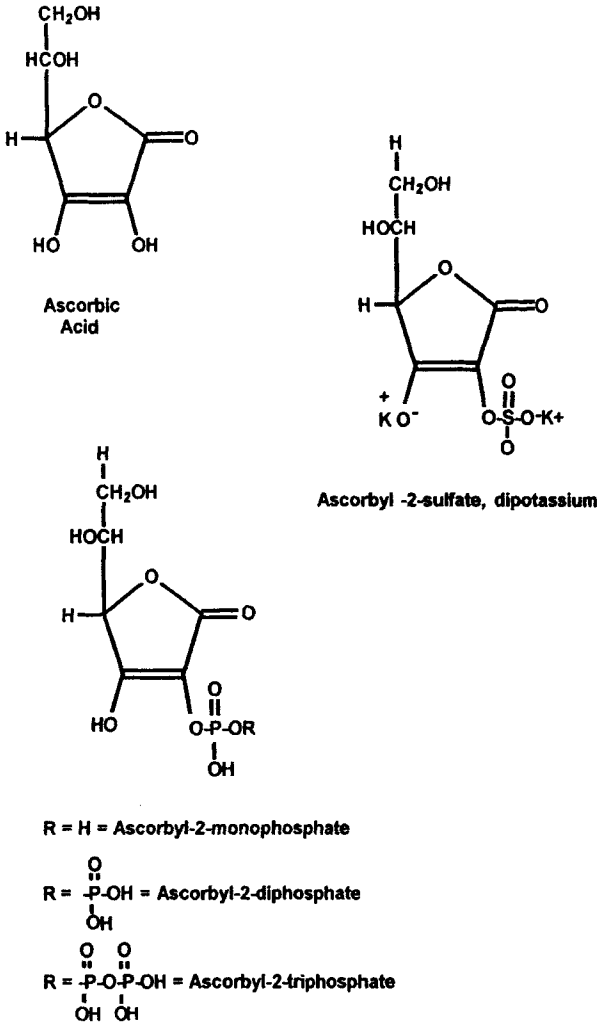

Figure 1. Chemical structure of vitamins.

scientific fisheries, are the 2-sulfate (C2) and 2-polyphosphate (C3) esters of L-ascorbic acid (Figure 1). The chemical and, therefore, the physical properties of these esters are quite different from ascorbic acid. For example, because of it's proven stability[6] and natural occurrence,[2,7] interest in L-ascorbyl-2-sulfate as a source of Vitamin C to enrich foods has grown.

The stability is the result of the protected OH-group at the C2 position of ascorbic acid by the sulfate ester group. When ingested, an enzyme known as ascorbate-2-sulfohydrolase converts the ascorbyl-2-sulfate back to ascorbic acid.[8]

New information,[9] showing that Ascorbyl-2-sulfate is absorbed directly into the lumen for further conversion into ascorbic acid, has further illustrated the mechanism of the vitamer's utilization. In order to assess the stability of the various commercially available vitamins, their potency, equivalency to ascorbic acid and ingestion, an accurate determination and quantitation is necessary. The various aquatic feeds and body part matrices has made this endeavor challenging. Various analytical methods have been available for the determination of ascorbic acid in particular. These have included titrimetric methods, derivatization reactions, enzymatic methods and chromatographic methods.[10-12] The most specific, quantitative, sensitive and rapid have been those methods utilizing High Performance Liquid Chromatography (HPLC) using reversed phase (C_{18} or ion paired C_{18}) and bonded phase (such as amine) columns.[13-15] Ultraviolet (UV) and electrochemical (EC) have been the two most commonly employed detectors.[5,16,17] Washko et al.[12] gave a good review of the earlier existing methods.

Since then, several attempts are worth mentioning. Felton and Halver[13] analyzed, simultaneously, for the three vitamers using C_{18}, n-octylamine ion-pair chromatography. Peak efficiencies were poor and, consequently, total peak resolution was not achieved. Wang et al. introduced enzyme shifting, which allowed the hydrolysis of all the ascorbyl-polyphosphate to ascorbic acid. The latter was analyzed using C_{18} with the ion pairing agent tetrabutylammonium phosphate. A column heater and electrochemical detection were necessary. Two years later, Wang and Seib[17] investigated acid catalyzed methanolysis to determine ascorbyl-2-sulfate alone as ascorbic acid. Hoffman et al.[19] compared three different chromatographic techniques for reliability, sensitivity and recovery for the separation of C1, C2 and C3.

Several extraction techniques (meta-phosphoric versus trichloroacetic acid), mobile phases (n-octylamine versus tetrabutylamine) and columns (reversed phase and ion exchange) were compared. Felton et al.[20] later attempted to eliminate the use of ion-pairing agents through the use of an Altima C_{18} column to separate ascorbic acid, ascorbyl-2-monophosphate and ascorbyl-2-sulfate. With only a difference of approximately two minutes between the vitamer peaks, the elution order was also reversed with C3 eluting after C2 and followed by C1.

Some of these methods seem to work only when the matrix is simple. For more complicated samples, such as fish feed, the methods suffered from poor resolution and peak efficiencies. This necessitated laborious, time consuming sample preparation, which, in turn, resulted in poor quantitation and reproducibility.

This work, an extension to the above attempts, uses the most optimal conditions, with major improvements in peak resolution and efficiency, leading to better quantitation and reproducibility. This is a consequence of the use of two different, novel columns. Inertsil C_4 and Inertsil C_{18}, in sequence. In addition, this method allows the simultaneous determination of all the vitamin C forms using the same column and HPLC conditions. This 'universal' method requires minimal sample preparation and overall analysis time.

MATERIALS

Apparatus

Two consecutive columns, one Inertsil C_4, followed by an Inertsil C_{18} (Metchem Technologies, Inc., Torrance, CA), each 15cm long, 4.6cm in internal diameter and 5μm particle diameter, were used in sequence for the separation. An LDC pump, autosampler, and spectrafocus detector (Thermo Separation Products, San Jose, CA) were employed. Centrifugation was performed using a Beckman J2-21 centrifuge (Beckman Instruments, Inc., Somerset, NJ).

Chemicals

The mobile phase is a solution made up of 0.1M anhydrous sodium acetate, the buffer (Fisher Scientific Co., Pittsburgh, PA), 174 uL/L n-octylamine, the ion pairing agent, (Sigma Chemical Co., St. Louis, MO), and 200 mg/L disodium (EDTA, chelates divalent metals which otherwise accelerates oxidation of the vitamins) (Fisher Scientific Co., Pittsburgh, PA). This solution is brought to pH 5 with glacial acetic acid (Fisher Scientific Co., Pittsburgh, PA). Acid phosphatase from potato (Boehringer Mannheim Corporation, Indianapolis, IN) is used for the enzyme shifting procedure, while dipotassium-ascorbyl-2-sulfate dihydrate, L-ascorbic acid (Sigma Chemical Co., St. Louis, MO), and L-ascorbyl-2-monophosphate magnesium salt (Wako Chemicals Inc., Richmond, VA) are used as the external standards. Meta-

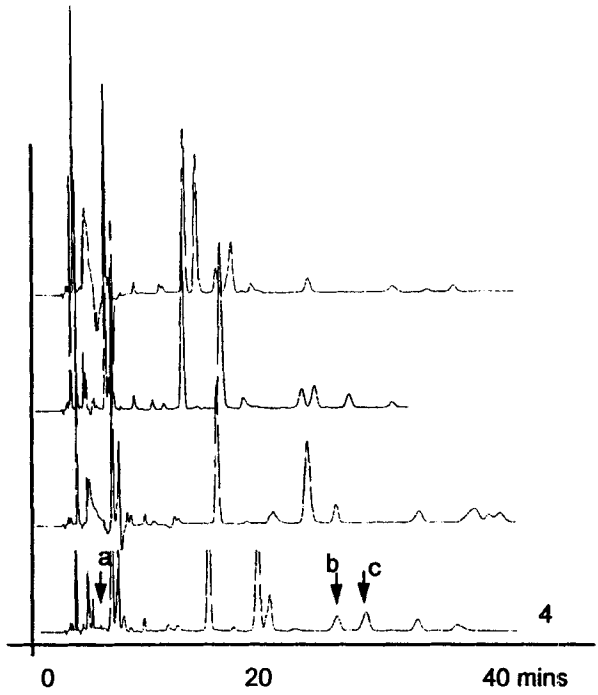

Figure 2. Effect of pH and ionic strength changes in the mobile phase. 1) pH 5.0, 0.15 M NaOAc; 2) pH 4.7, 0.1 M NaOAc; 3) pH 5.3, 0.1 M NaOAc; 4) pH 5.0, 0.1 M NaOAc (optimum separation). a = position of ascorbic acid, b = ascorbyl -2-sulfate, c = ascorbyl-2-monophosphate.

phosphoric acid (used to denature the proteins and prevent hydrolysis of the Lactone ring) and 1,4-dithiothreitol (DTT, Cleland's reagent, used to retard oxidation), (both from Aldrich Chemical Co., Milwaukee, WI) are essential components in the extraction and solvation solutions.

METHODS

Extraction Conditions of Aquatic Feeds

Upon receipt of the feed sample, the contents are stored in the freezer (-80°C) prior to analysis time. From each feed, two batches, 5 grams each, are

well ground and mixed using a mortar and pestle. Two 1.0 g samples are weighed (after reaching ambient temperature) into centrifuge tubes (record the exact weight), from each of the five-gram batches. This replication in sampling tests both the efficiency of vitamin mixing in the feed as well as the analytical extraction procedure.

The bags are then well sealed and stored at -80°C for any future re-analysis. To each 1.0 g add 20 mL of extraction solution. The extraction solution is prepared by mixing 1% metaphosphoric acid and 0.2% DTT to give a pH of approximately 2.16.

Doubly distilled, deionized water and high purity grade reagents are used throughout. Use of amber glass ware is recommended to minimize light exposure.

Each sample is placed in a sealed container and vortexed for 5 min or shaken for 5-10 minutes. A Tissumizer can be used, for approximately 30 seconds, if the sample is bulky. Note that excessive use of the Tissumizer may speed up the vitamin decomposition through the introduction of oxygen. Sonication is not recommended as it may heat up the sample. The samples are then centrifuged for 10 min at 13,000 rpm and -5°C.

The supernatant is consecutively filtered, first through 0.45μm, then 0.2μm acid resistant (e.g. PTFE) filters. Solid phase extraction (SPE) should be avoided if possible. The filtrate is then immediately chromatographed. Each sample is injected at least twice. Resultant areas are compared to those of the external standards.

Enzyme Shifting of Ascorbyl-2-Polyphosphate

When (and only if) ascorbyl monophosphate is detected, to 1 mL of filtrate add another 1.0 mL of acid phosphatase enzyme solution to initiate enzyme shifting. This method is adapted from Wang and Seib[17] and Maugle[21] with modifications. The solution is shaken and left in the dark for at least one hour before chromatography.

The enzyme solution consists of 4 mg/mL acid phosphatase enzyme dissolved in 0.1M sodium acetate (anhydrous) / 0.2% DTT solution (pH 5.0), and is prepared fresh. The final, higher pH on mixing the enzyme solution with that of the filtrate (pH ~3.5), was found to be more optimal for the enzyme.

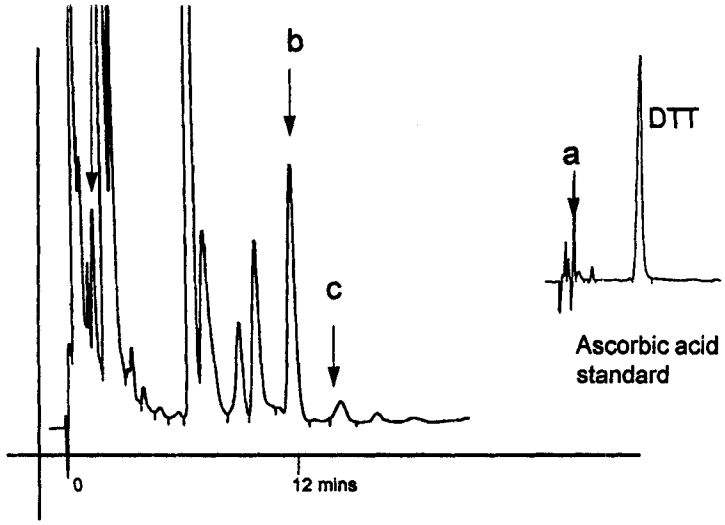

Figure 3. Separation of an aquatic feed using a Nova Pak C18 column, octylamine, EDTA, pH 5, 0.1M NaOAc. a= Ascorbic acid, b= Ascorbyl -2-sulfate, c= Ascorbyl-2-monophosphate.

Chromatographic Conditions

The column combination used in this work is used to improve the separation, especially for the early eluting peaks. Two Inertsil columns, a C_4 and a C_{18}, were connected in sequence, and equilibrated with the mobile phase (which contains the ion pairing agent n-octyl amine), along with the pumping system, overnight, before use. Once impregnated with the ion pairing agents these columns should be dedicated for this mobile phase only. In this way, reproducibility during the time of the analysis, and from day to day, is highly improved.

The ionic strength of the sodium acetate, and the pH of the mobile phase solution, were further manipulated to investigate enhanced resolution of the early eluting components, while maintaining acceptable analysis time.

For better reproducibility of the analysis, an isocratic mode was chosen at a flow rate of 1mL/min. Detection was carried out at 255 nm using a UV detector.

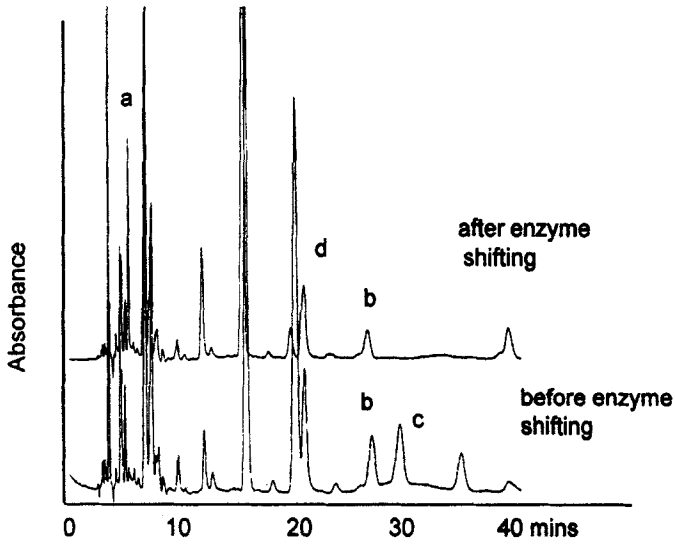

Figure 4. Separation of an aquatic feed using an Inertsil C4 and C18, octylamine, EDTA, pH 5, 0.1M NaOAc. a= Ascorbic acid, b= Ascorbyl -2-sulfate, c= Ascorbyl-2-monophosphate and d= DTT.

To effect a more rugged method, chromatographic conditions were chosen to allow for the more difficult feed matrices. Additionally, when sensitivity is required, this method can be easily adapted to glassy carbon electrochemical detection.

RESULTS AND DISCUSSION

The Chromatography

Increasing the pH of the mobile phase from 4.7 to 5.0 to 5.3, with manipulations of the amounts of glacial acetic acid, was found to increase the capacity factors of all the components, in turn increasing their retention times and their resolution (Figure 2). On the other hand, increasing the ionic strength via an increase in the sodium acetate concentration, resulted in a decrease in capacity factors and resolution (Figure2). Figure 3 shows a feed separation using a Waters Nova-pak C_{18} column, compared to that using the optimized conditions described by this method (Figure 4). Complete and clear

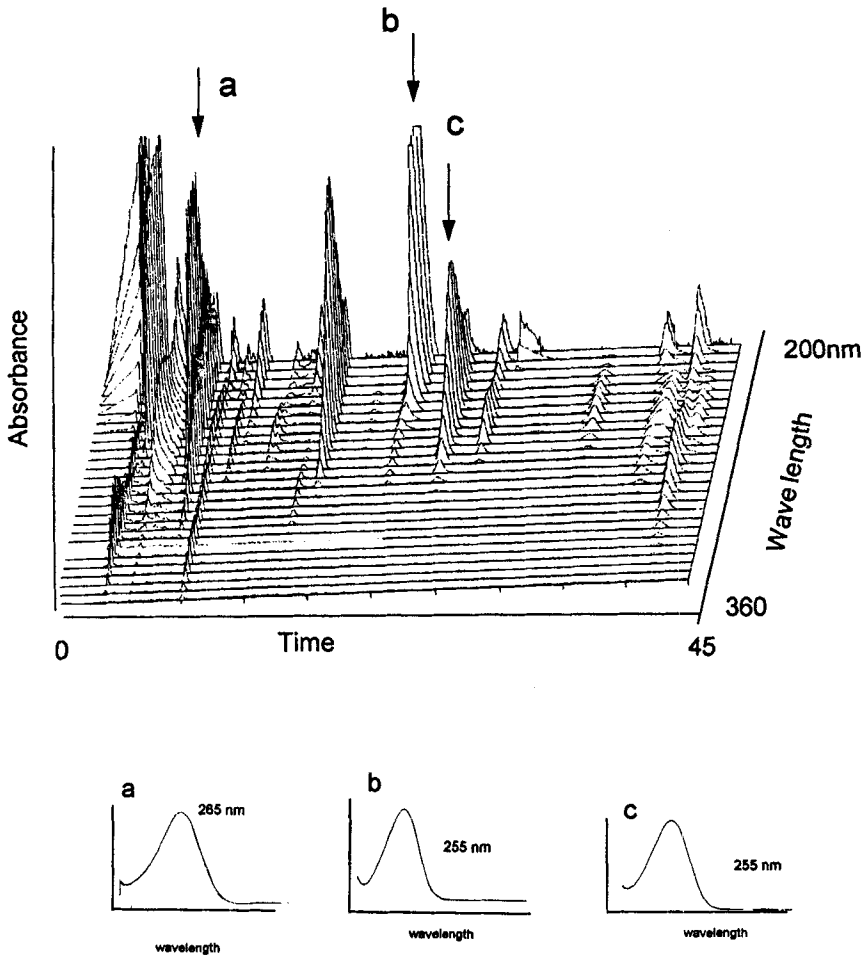

Figure 5. Three-dimensional plot for the aquatic feed sample. a = ascorbic acid, b = ascorbyl -2-sulfate, c = ascorbyl-2-monophosphate.

separation of the ascorbic acid peak is evident and repeatable, allowing a more accurate determination of the ascorbic acid content of the feed both before and after enzyme shifting of ascorbyl-2-polyphosphate. Ascorbyl-2-sulfate and ascorbyl-2-monophosphate are equally well resolved. The Inertsil C_4, being more polar, retains the more polar compounds, such as ascorbic acid, allowing better selectivity and, in turn, better resolution of the earlier eluting peaks. The

Inertsil C_{18} in conjunction, works to retain the more nonpolar compounds, in this case, the ester vitamins. Enzyme shifting, through the acid phosphatase hydrolysis of the phosphate moieties, quantitatively converted all the L-ascorbyl-2-polyphosphate to the equivalent amount of ascorbic acid. (Figure 4). This method eliminated the need for qualitative and quantitative determination of all the polyphosphate esters which are, with exception to the mono-ester unavailable as stable standards.

Retention times, peak heights and peak areas for L-ascorbyl-2-sulfate, using this method, were found to have percent relative standard deviations (%RSD) of 1.8, 0.64 and 1.2, respectively (n=10), while those of ascorbic acid were 0.48, 1.25 and 0.28, respectively (Table 1) . Recoveries in feeds were 97%.

The identities of the components were confirmed using external standards and the instrument's fast scanning capabilities. UV spectra of the three vitamins, in the optimum mobile phase, were recorded on-line and are shown in Figure 5. From these spectra, an optimum wavelength of 255nm was chosen for the analysis.

The analytical method described has been shown to be very useful for separation of ascorbic acid from other components in aquaculture feeds. It has also been used for routine analysis and for determination of properties of the various forms of stabilized C vitamers as indicated below.

Using the above chromatographic method, it was possible to quantitatively assess the stability and, hence, potency of two commercial forms of vitamin C, ascorbyl-2-sulfate and ascorbyl -2-polyphosphate. L-ascorbic acid is well known to be highly soluble; however, once dissolved in water, it is subject to rapid oxidative degradation.

In order to retard oxidation during the time required for analysis, dithiothreitol (DTT) must be added or low ascorbic values will be obtained. Commercially available L-ascorbyl-2-polyphosphate (15% ascorbic acid equivalency) was found to be substantially insoluble in distilled water and seawater, floating to the top. Acidic solutions (1% metaphosphoric acid / 0.2% DTT with and additional 10% acetone) were required to effect complete solubility.

When exposed to conditions of high humidity (such as would be found in tropical feed mills, 47% relative humidity and 37°C), the stability of this ester was shown to progressively deteriorate reaching 18% loss of potency after 3

Table 1

Percent Relative Standard Deviations (n = 10)

Compound	% RSD Area	% RSD Height	% RSD Ret. Time
Ascorbyl-2-sulfate	1.2	0.64	1.8
Ascorbic acid	0.28	1.25	0.48

Table 2

Vitamer % Loss with Storage at 37°C, 47% Relative Humidity

Vitamin	Week 2	Week 3	Week 6
L-Ascorbyl-2-sulfate	1.7	0.1	4.1
L-Ascorbyl-2-poly-phosphate (15%)	4.7	18.4	34

weeks of storage (Table 2) and 34% loss in potency after 6 weeks bulk storage. L-Ascorbyl-2-sulfate dissolves readily and completely in both distilled water and sea water, but did not suffer a significant loss in potency when stored under the same conditions, losing only 4% in potency after six weeks storage (Table 2). This verifies the previous findings of Maugle[21] of the progressive loss in ascorbyl 2-polyphosphate potency after manufacture of an extruded salmon feed; this indicates that ascorbyl-2-sulfate provides a more cost-effective form of Vitamin C.

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REFERENCES

1. I. B. Chatterijee, Science, **82**, 1271 (1973).

2. J. Halver, R. Smith, B. Tolbert, E. Baker, Ann. N. Y. Acad. Sci., **258**, 81-102 (1975).

3. B. Tucker, J. E. Halver, Fish Physiol. Biochem., **2(1-4)**, 151-160 (1986).

4. U. Moser, A. Bendich, **Handbook of Vitamins,** Marcel Dekker, Inc., New York, 1991, p. 195.

5. K. De Antonis, P. Brown, Z. Yi, P. Maugle, J. Chromatogr., **632**, 91-96 (1993).

6. P. Seib, Y. Liang, C. Lee, C. Hoseney, C. Deyoe. J. C. S., Perkins I, 1220 (1974).

7. C. G. Mead, F. J. Finamore, Biochemistry, **8**, 2658-2655 (1969).

8. L. Benitez, J. E. Halver, Proc. Natl. Acad. Sci., **278**, 70-71 (1982).

9. S. P. Felton, R. Grace. J. Liq. Chrom., (1995) in press.

10. E. Baker, B. Tolbert, Anal. Biochem., **55**, 641-642 (1973).

11. B. Terada, B. Tolbert, M. Downing, E. M. Baker, Ann. N. Y. Acad. Sci., **258**, 48-69 (1975).

12. P. Washko, R. Welch, K. Dhariwal, Y. Wang, M. Levine, Anal. Biochem., **204**, 1-14 (1992).

13. S. Felton, R. Grace, J. E. Halver, Proc. Soc. Exptl. Biol. Med., **190**, 217 (1988).

14. W. Schuep, J. Marmet, W. Struder. Aquaculture, **79**, 249-258 (1989).

15. J. Albrecht, H. Schaffer, J. Liq. Chrom., **13(13)**, 2633-2641 (1990).

16. C. Tsao, M. Downing, R. Carlson, M. Knight, E. Baker, Ann. N. Y. Acad. Sci., **258**, 48-69 (1975).

17. X. Y. Wang, P. Seib, Aquaculture, **87**, 65-84 (1990).

18. X. Wang, M. Liao, T. Hung, P. Seib, J. Assoc. Off. Anal. Chem., **71(6)**, 1158-1161 (1988).

19. K. Hoffman, P. Brown, P. Maugle, J. Liq. Chrom., **15(14)**, 2581-2610 (1992).

20. S. Felton, R. Grace, J. E. Halver, J. Liq. Chrom., **17(1)**, 123-132 (1994).

21. P. Maugle, Bull. Aquaculture Assoc. Canada, **93**, 44-54 (1993).

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