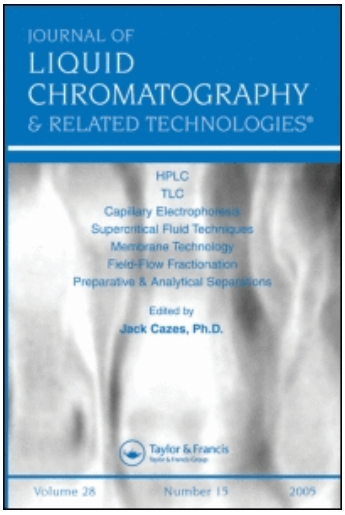

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Authentication of L-Ascorbyl-2-sulfate in Salmonid Gastric Tissue: HPLC/Electrospray Ionization Mass Spectroscopic Verification

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AUTHENTICATION OF L-ASCORBYL-2-SULFATE IN SALMONID GASTRIC TISSUE: HPLC/ELECTRO-SPRAY IONIZATION MASS SPECTROSCOPIC VERIFICATION

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ABSTRACT

New findings establish the intact transfer of the L-ascorbyl-2-sulfate ester across the stomach membrane in salmon. In addition, measurement of the ester has been confirmed in a commercial fish diet. The procedure used a non ion-pairing C_{18} reverse phase column for the separation of L-ascorbyl-2-sulfate (AS) from coho salmon gastric tissue. The AS fraction was eluted from the column with 0.1 M ammonium acetate pH 5.0 and rechromatographed to ascertain purity. When chromatographed under the same conditions, the collected AS fraction had the same retention time as the purified standard of L-ascorbyl-2-sulfate dipotassium salt. The purified AS fraction was then confirmed as to structure by Electrospray Ionization Mass Spectrometry . The AS fraction gave the same Electrospray Ionization Mass Spectrometry results as the standard AS when subjected to mass spectrometry-mass spectrometry (MSMS) analysis.

INTRODUCTION

A stable form of vitamin C has been sought for moist and dry feed by the aquaculture and animal industries. They needed a form of C that would maintain activity during high temperature processing and storage. Several products had been synthesized and were available to feed manufacturers. Previously, one of these products (AS) has been identified as an extractive in fish liver (1, 2 and 3).

After discovery of AS in 1971 (4) and the synthesis of AS (5), there was keen interest in utilizing this product as a source of vitamin C in animal feed. However, before the product could be fully evaluated in the intact animal, there needed to be an unequivocal method for identification of the vitamin C form (AS) in biological tissue. Most HPLC methods of identification have been fraught with co-elution problems (6, 7). This co-elution problem had made it difficult to quantify the AS in tissue and feeds, leading to skepticism by feeders. It was important for investigators to validate specifically what was being measured and the exact amount.

The purpose of this work was to establish credibility in the identification of AS in fish food and in tissue so that studies of the product could move forward.

METHODS AND MATERIALS

Equipment:

The analytical column used was an Alltima (Alltech Associates) C_{18} reverse-phase column, 250 mm x 4.6 mm and a 5 µm particle size. A hand-packed Alltima guard column was used to protect the analytical column.

The HPLC equipment used was a Perkin-Elmer Corporation (P E) (Norwalk, CT) model 250 isocratic pump with an in line PE solvent filter, a PE model 290 UV/Vis detector, set at 254 mm, and a Bioanalytical Systems Inc. (BAS) electro-chemical detector, set at +0.72 volts. The data system was linked to a PE Nelson model 950 interface and an Epson III+ computer. The software used was the PE Nelson 2100.

The HPLC method and conditions were as previously described (7). Briefly, the column was run at room temperature (25°C) with a mobile phase of 0.10 M ammonium acetate pH 5.0 and a flow rate of 0.75 ml/min. The column pressure ranged from 2000 to 2200 psi., depending upon the column age, condition, and nature of pre-column filters.

Tissue was homogenized with a Brinkmann Polytron homogenizer and centrifuged with an Eppendorf Minifuge (Brinkmann Instrument Co., Westbury, N.Y.).

The AS and AA standards were obtained from Pfizer, Inc., Groton, CT and Hoffmann La Roche Co., Nutley, N.J. respectively.

Mass Spectroscopy:

Samples were analyzed on a triple-quadrupole Sciex API III instrument (PE/SCIEX Thornhill, Ontario, Canada). Samples were infused into the electrospray source via a 50 micron i.d. fused silica transfer line using a Harvard Apparatus pump at 1-3 uL/min. Positive ion mass spectroscopy (MS) and mass spectroscopy-mass spectroscopy (MS/MS) were run with an orifice voltage of 70 to 80 volts. Negative ion electrospray MS and MS/MS used an

orifice voltage from -70 to -90 volts. The interface temperature was maintained at 52° C. For tandem mass spectrometry (MS/MS), precursor ions were selected with the first of three quadrupoles (Q1) for collision-induced dissociation with argon in the second quadrupole (Q2). The third quadrupole (Q3) was scanned with a mass step of 0.20 daltons (Da) and 1ms/step. In order to select a mass from Q1 for MS/MS studies, parent ion transmission was maximized by reducing the resolution of Q1 to transmit a 2 to 3 m/z window about the selected parent ion, and Q3 resolution was adjusted to approximately 50% valley between peaks 3 Da apart. Spectra were collected and analyzed using proprietary software from Sciex Corporation.

The mass spectrometer was calibrated to eight different poly (propyleneglycol) masses.

Solvent and Tissue Preparation:

The solvent system for the mobile phase (0.10 M ammonium acetate pH 5.0) was made as follows: 0.1 M reagent grade acetic acid was titrated to pH 5.0 with isothermally distilled ammonium hydroxide.

Tissue extracts were made as follows: The 5% trichloroacetic acid (TCA) extract was made by homogenizing 1 vol. of tissue to 4 vols. of glass distilled water for 30 sec. While homogenizing, 5 vols. of 10% TCA were added, and homogenizing was continued for another 60 sec. This extract was centrifuged and supernatant solution was decanted and filtered through a 0.45 mm syringe filter. The filtered solution was used for the HPLC determination.

HPLC Peak Collection:

The AS retention time was 3.23 minutes when using the ammonium acetate (0.1 M and pH 5.0) mobile phase (AS shown chromatographed with AA in Figure 1). The AS peak was collected at the outlet from the ultraviolet (UV) detector with a time frame of 3.0 to 3.5 minutes (vol. = 375 µl). This peak was rechromatographed to establish purity and quantity.

Tissue Collection:

Each coho salmon smolt (weighing about 400 grams)was force-fed AS in the following manner: the smolt was anesthetized with Tricaine Methane Sulfonate (MS 222) until it could be handled without struggling; then a polished-end glass tube (3.5 mm ID) was inserted through the mouth into the stomach. A #13 gelatin capsule was filled with 75 mg of AS mixed with crystalline serum albumin (3+1 parts respectively). This small capsule was forced through the glass tube with a 6 in. (Puritan) applicator stick. The glass tube was removed and the fish was returned to the tank. In order to make sure the capsule was not regurgitated, the fish had to be observed carefully for about 3 minutes .

After 24 hours, the fish was sacrificed and the stomach removed. The stomach cavity was opened and washed clear of any solids or absorbed substances, after which the portion from

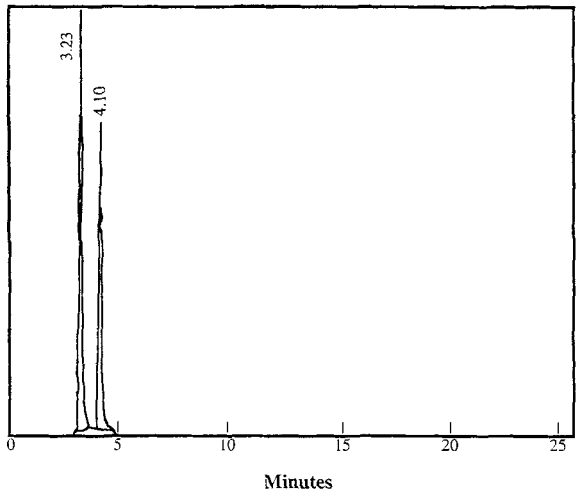


Figure 1 HPLC standards, L-ascorbyl-2-sulfate (3.23 min.) and ascorbic acid (4.10 min.) from a C18 Altima column using a mobile phase of 0.1 M ammonium acetate pH 5.0. Retention time in minutes

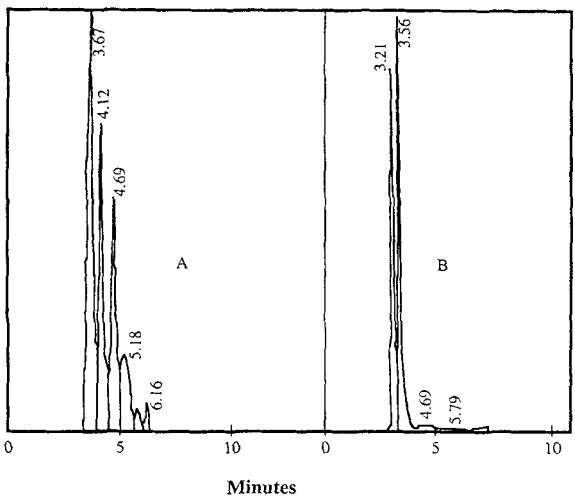


Figure 2 A A typical HPLC spectrum of a 5 % TCA gastric tissue extract from a control fish, fed a normal commercial fish diet containing ascorbic acid (AA).

Figure 2 B A typical HPLC spectrum of a 5 % TCA gastric tissue extract from a fish force-fed L-ascorbyl-2-sulfate (AS). Note the absence of ascorbic acid in the force-fed fish gastric tissue extract. The 3.56 min. peak represents an unknown that is enhanced in the presence of AS.

the esophagus to the large intestine was excised. The excised tissue was washed 10 times with 50 volumes of glass distilled water. The final rinse was saved and checked for any residual AS. The residual AS found in the rinse water represented 0.35 % of that found in the tissue. The gastric tissue was extracted with TCA as described above and injected into the HPLC.

RESULTS AND DISCUSSION

HPLC Data:

Figure 1 shows a typical HPLC computer profile of AA and AS standards using the mobile phase and other conditions listed above. These standard profiles are shown for comparison purposes with Figures 2 A and 2 B below. In Figure 1, the AS had a retention time (RT) of 3.23 minutes and AA had a RT of 4.10 minutes at medium to high concentrations. These retention times did not vary under the prescribed conditions stated above except when the concentrations were high or very low. For example, when the concentration approached the detection limits of 2 ng and 1 ng for AS and AA, respectively, the RT's were 3.17 and 4.10 minutes (7). At high concentrations they shifted to 3.29 and 4.16, respectively. Therefore, at the standard concentrations of 500 and 200 ng of AS and AA they are as seen in Figure 1. It was found that when the retention times were earlier, the column was not equilibrated.

Figure 2 A illustrates a typical HPLC profile of a 5% TCA extract of coho stomach tissue from a control fish. The tissue is from a fish that has been fed a commercial fish diet containing AA as the vitamin C source. The AA peak is clearly discernible at the retention time of 4.12 minutes. Attention is brought to the fact that there is no indication of a peak at 3.2 minutes, the retention time for AS in this system. Figure 2 B is a typical profile of a similar gastric tissue extract from an experimental fish force-fed AS as described above. The tissue extract was taken at 24 hours post feeding time. Minimization of the other peaks in this profile is due to the high concentration of AS found in the gut tissue extract. The AS peak represents 2.043 µg whereas the AA seen in Figure 2 A represents one tenth of that amount, 0.255 µg.

Note the two large and sharp peaks in Figure 2 B at RT's 3.21 (AS) and 3.56 minutes. The latter peak, which varies from 3.54 to 3.67 minutes is found in both experimental and control fish extracts. It is possibly a tissue matrix peak or carrier in nature since this peak is enhanced whenever AS is fed to the fish. Figures 3 A and 3 B are computer enlargements of Figures 2 A and 2 B. In the TCA extractives of the control fish seen in the 3 A enlargement there are no indications that would affirm the presence of AS. Figure 3 B enlargement defines AS presence in the TCA gastric extract . By way of contrast, Figure 3 C illustrates the change in the control fish profile of 2 A when 200 ng of AS standard is added to the 5% TCA gut extract.

Figure 4 A, an enlargement, was made in order to show the smaller peaks occurring after the peaks of interest (similar to a 5% TCA gut extract of a fish force-fed AS as seen in Figure 2

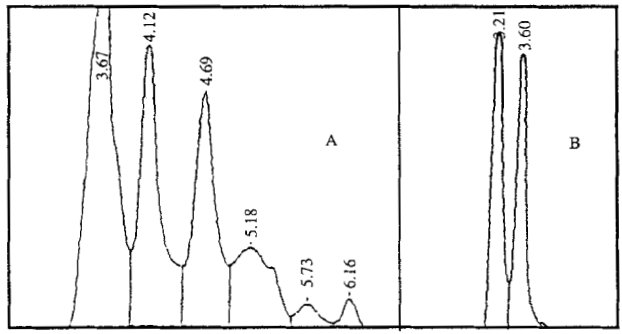


Figure 3 (A) and (B) are computer enlargements of figure 2 A and 2 B, more clearly detailing the peaks of interest.

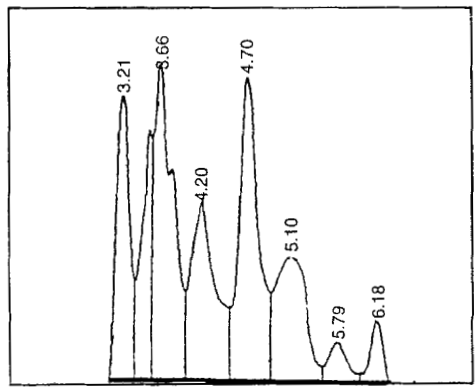


Figure 3 (C) This is a profile of the 5 % TCA extract from gut tissue of the coho salmon on a commercial diet containing AA as the vitamin C source to which is added 200 nano grams of AS. (Also a computer enlargement)

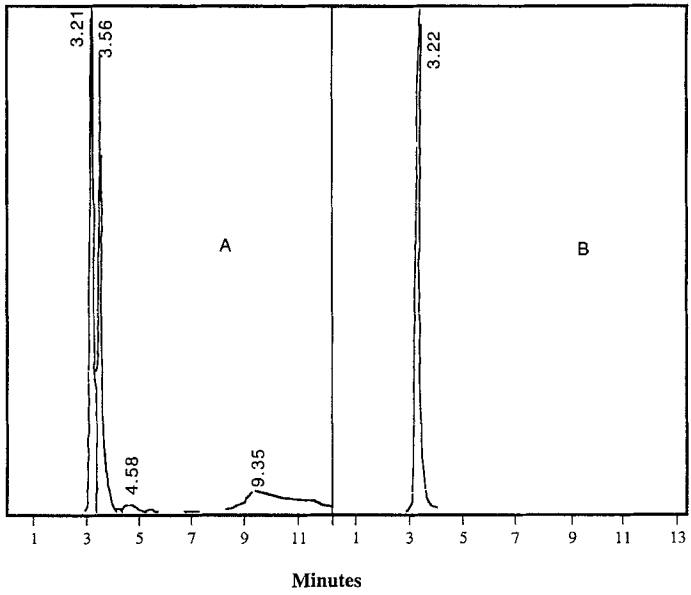


Figure 4 (A) A similar HPLC spectrum of a 5% TCA gastric tissue extract from a fish force-fed AS. 4 (B) is the spectrum of the eluted first peak from the extract at RT of 3.21 shown in 4 (A).

B). Note that there is no AA peak in this stomach extract, since there is no peak appearing between 3.89 and 4.59 where AA should be retained. Figure 4 B is the HPLC profile of the eluted AS peak seen at RT of 3.21 in Figure 4 A. The eluted UV detector peak from the 5% TCA gut extract was collected from 3.0 to 3.5 minutes (as described above). Note its relative purity . The eluted peak amount represents 0. 214 μg . The collected fraction is homogeneous as determined by the HPLC profile.

Previously it has been hypothesized that AS may be desulfated in fish gut. These new findings indicate that AS may be transported intact across the gastric membrane rather than being desulfated to AA by stomach acid and/or enzymes.

Figure 5 is the electro-chemical (EC) profile of the 5 % TCA gut extract from the force-fed fish seen in the 4 A UV profile . The spectrum is obtained from the tandem electrochemical detector connected to the outlet from the UV detector. There is a lag time of 0.3 minutes between the UV and EC detector spectrum before the spectrum of the latter appears. At RT 3.95 minutes, the EC peak is the oxidation of the UV peak (with a RT of 3.6) that is seen in Figures 3 A, B, and C. This peak is oxidizable at a glassy carbon electrode with a potential of +0.72 volts; and it is also enhanced when AS is present, posing an interesting transport possibility.

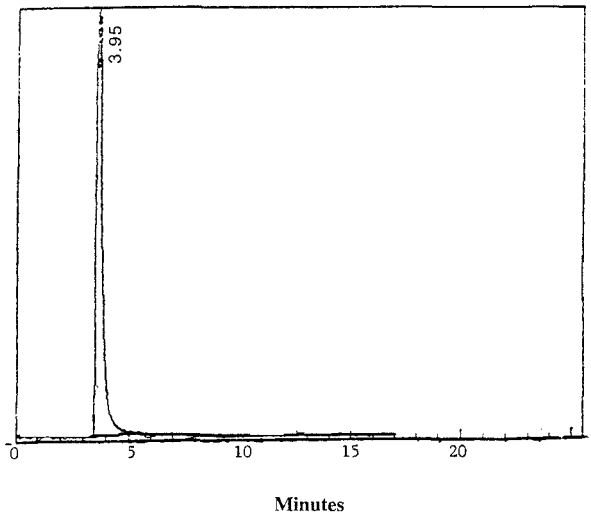


Figure 5 This electro-chemical detector spectrum at RT of 3.95 minutes, is the oxidation of the 3.5 minute UV detector peak of the 5% TCA gut extract from the force-fed fish seen in Figure 4 A.

Figure 6 A is a UV spectrum of a 5% TCA extract from a commercial diet supplemented with AS (50 mg/100g). Note that the AS retention time (3.29 min.) is characteristic of a high concentration, as previously described. The RT difference is in hundreds of a second. The dietary AS peak amount represents 1.32 µg. The AA peak is very small since it represents a residual AA of 20 µg/g from storage diet. The AS peak calculates to a dietary concentration of 52.50 mg/100 g as determined by standard addition, representing a recovery of 105 %.

Figure 6 B is the spectrum of the eluted AS peak from the diet extract shown in Figure 6 A. The separation of the AS peak from a diet extract is very difficult because there is a contaminating peak (appearing to the left of the major peak).

Mass Spectroscopy Data:

Figure 7 illustrates a typical MS spectrum of standard L-ascorbyl-2-sulfate, Astos standard. The solubilized form of L-ascorbic acid-2-sulfate has a calculated mass of 255 Da. Note that the major base peak of the standard has a m/z of 255.2 Da. This mass peak was used for the MS/MS analysis. Figure 8 is the MS spectrum of the eluted peak from the HPLC run of the coho gut extract . It is basically identical to the Astos standard seen in Figure 7. The change in Argon density may have caused the difference in the mass peak intensity at 227 Da and 241

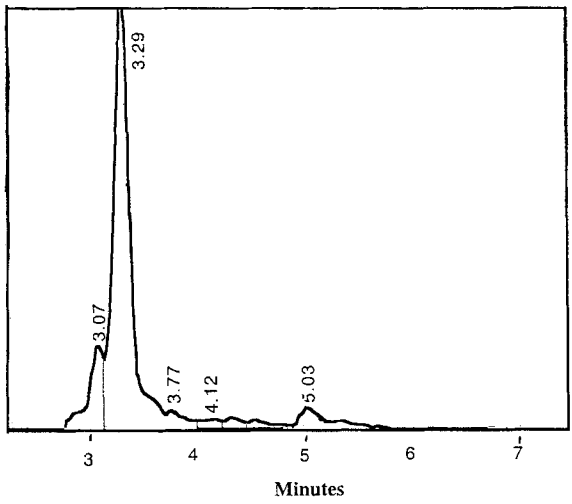


Figure 6 (A) An HPLC spectrum of the 5% TCA extract from a commercial diet containing internal AS standard (at RT of 3.29 min.) and a small quantity of AA at RT 4.12 minutes. Expanded spectrum.

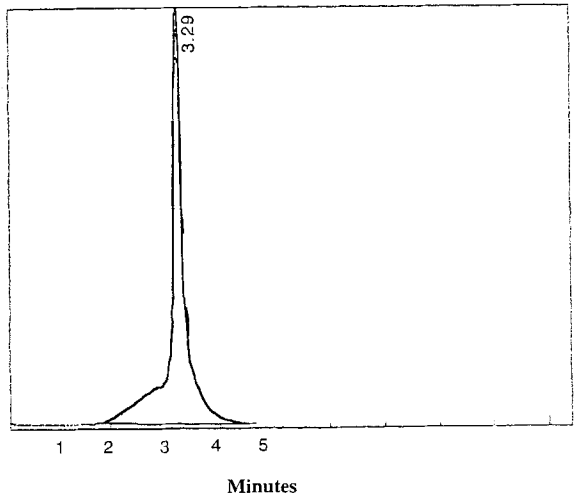


Figure 6 (B) is the spectrum of the eluted AS peak at RT of 3.29 min. from the commercial diet extract shown in Figure 6 (A) above. Expanded spectrum.

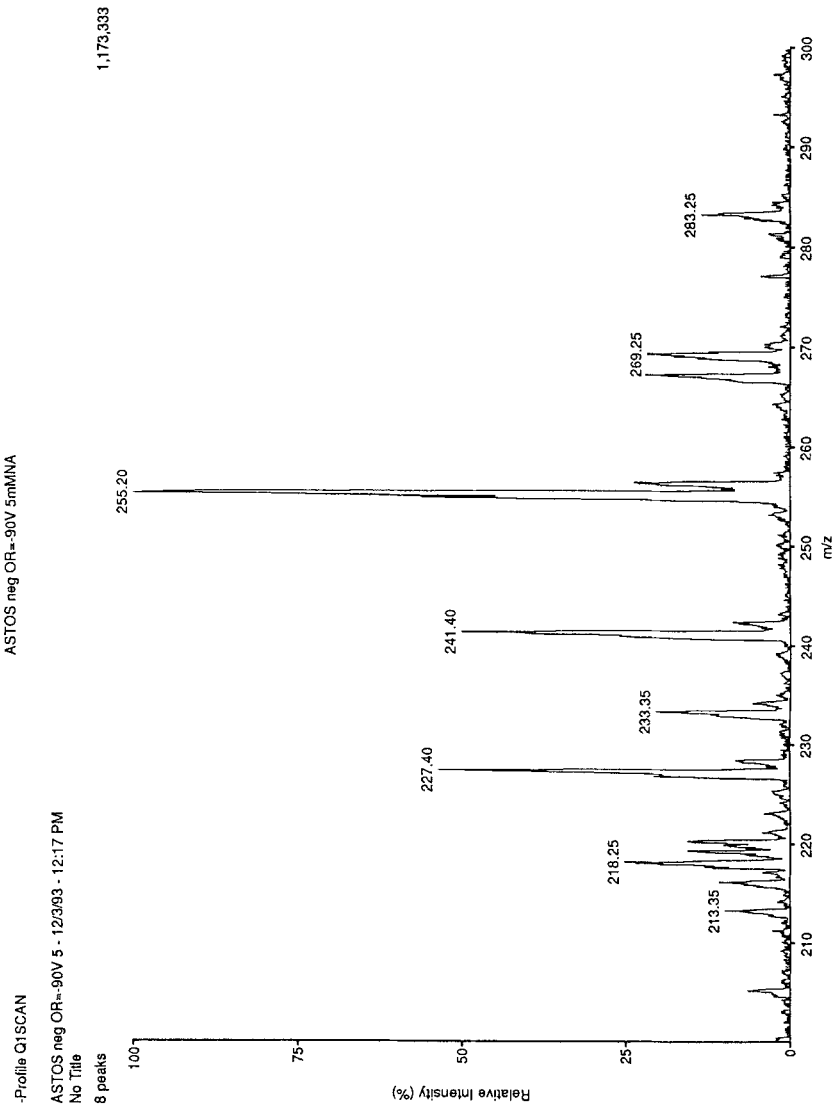


Figure 7 An Electro-Spray ionization mass spectrum of L-ascorbyl-2-sulfate (Astos) standard. X axis = m/z and Y axis = relative intensity

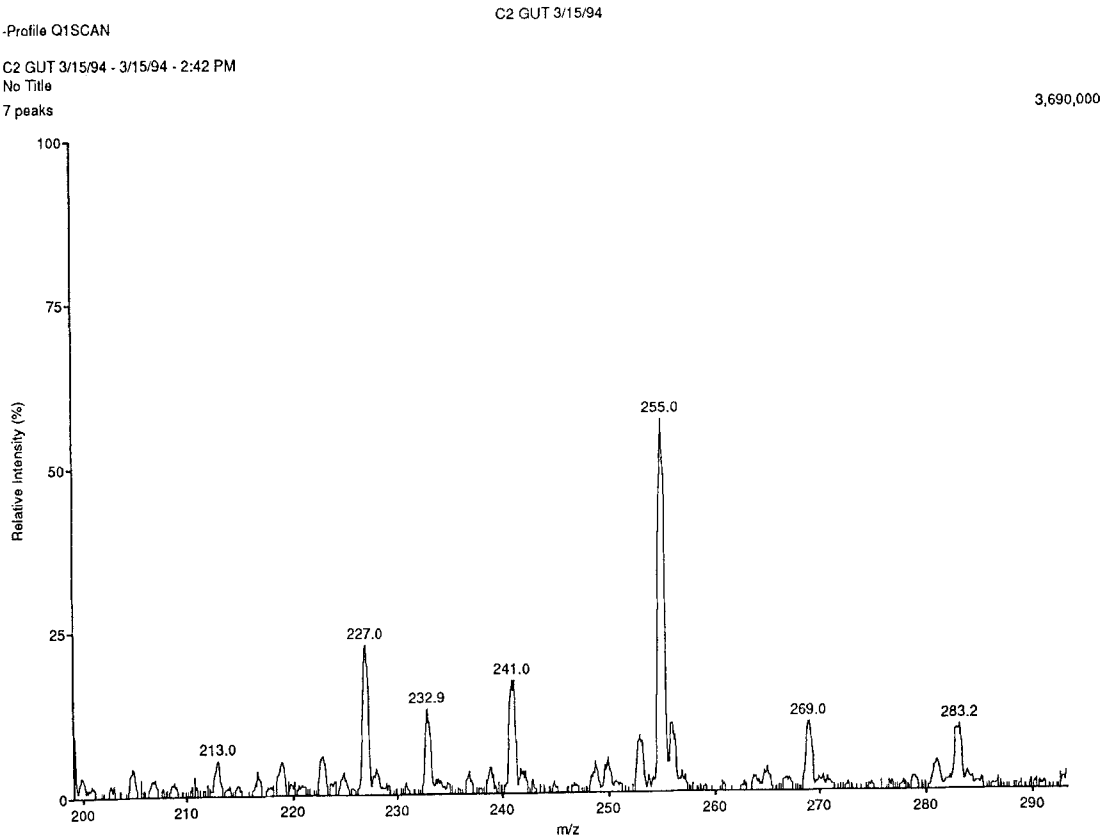


Figure 8 An Electro-Spray ionization mass spectrum of AS eluted from 5% TCA gut extract HPLC run shown in Figure 4 (B) above. The difference in mass peak intensity at 227 is related to argon density difference.

Da. Argon densities effect the intensities of the daughter ions. For example in higher argon density the smaller daughter ions predominate; whereas with less argon density, the converse is true.

When running a mass spectrometer MS/MS program , the instrument focuses exclusively upon the base peak of interest. All other mass peaks are filtered out. Most peaks in a mass spectrum appear at integral mass numbers. However multiplied charged ions can appear at fractional masses, i.e. 59.1 vs 59, as seen in Figure 10. These fractional numbers can also be caused by differences in carbon C_{12} to C_{13} ratios. Figure 9 depicts the ion fragments produced when the MS / MS program is performed on the base peak 255 of standard AS.

It should be emphasized that in MS/MS, only unimolecular reactions are important in electron ionization. In the electron interaction with the parent molecule, the first result is the formation of the molecular ions by injection of another electron. Then upon further degradation, part of the molecular ions will fragment into ions such as seen in the spectrum. Table 1 lists the likely ion fragments from the MS/MS data of the base peak of 255.2 (shown in Figure 7 for standard Astos). The table shows formula weights of the fragments and their most probable structures. Note, these structures exist only in unstable gaseous forms. Unequivocal proof for establishing the identity of an unknown would be for its MS/MS fragments to compare with those of the standard compound in Figure 9. Figure 10 includes figure 9 and shows this comparison. It offers convincing evidence that the Astos standard is the same as the eluted peak from the AS force-fed coho. Any minor differences in mass fragments between the two can be due to argon gas density differences and the differences discussed above.

Figure 11 is an MS spectrum of the 3.25 min. RT peak eluted from an HPLC run of a 5% TCA dietary extract. The diet was supplemented with AS. Note the similarity of the eluted dietary extract MS to that of standard AS shown in Figure 7. The fragment peaks of interest are 227, 233, 241, 255, 269 and 283 as seen in the Astos run. Figure 11, the MS spectra of the eluted dietary AS peak, illustrates and confirms the elution of AS from the diet matrix. A co-eluted impurity from the diet may have caused the unknown fragments that are not the same as AS standard. However, this mass spectrometer fragmentation offers strong evidence that the 3.25 min. HPLC peak is AS.

Figure 12 is the MS spectrum of the eluted HPLC peak (with a RT of 3.5 min. seen in Figure 2 B). This eluate was from the HPLC run of the 5% TCA gut extraction from the AS force-fed-fish. Note the fragment peaks are identical to the Astos spectra (227, 233, 241, 255, 269, and 283). The other different fragment peaks with masses of 213, 222.8, 239, 249.9, and 281 may be associated with the " carrier" substance that has a retention time of 3.5 to 3.6 min. This peak is found as well in the spectra of the control fish TCA extract (not force-fed AS or containing AS) seen in Figure 2 A.

The finding of this RT peak with its AS component is consistent with the fact that the 3.56 minute retention time peak is enhanced when fish are fed AS—a finding that may offer indirect evidence of an AS carrier found in fish gastric tissue. It is possible that this apparently -bound AS fraction may account, in part, for the low reported uptakes of AS in fish (8). The

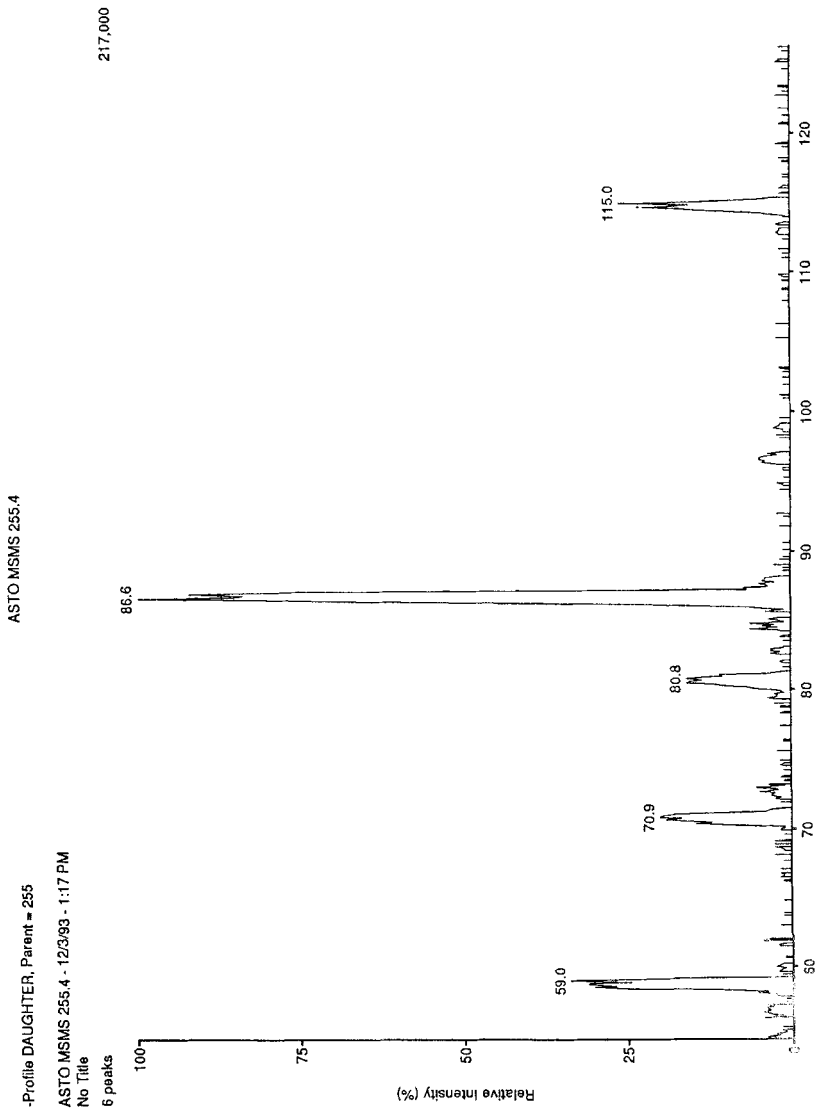


Figure 9 An Electro-Spray ionization mass spectrum of a MS/MS fragmentation of L-ascorbyl-2-sulfate standard.

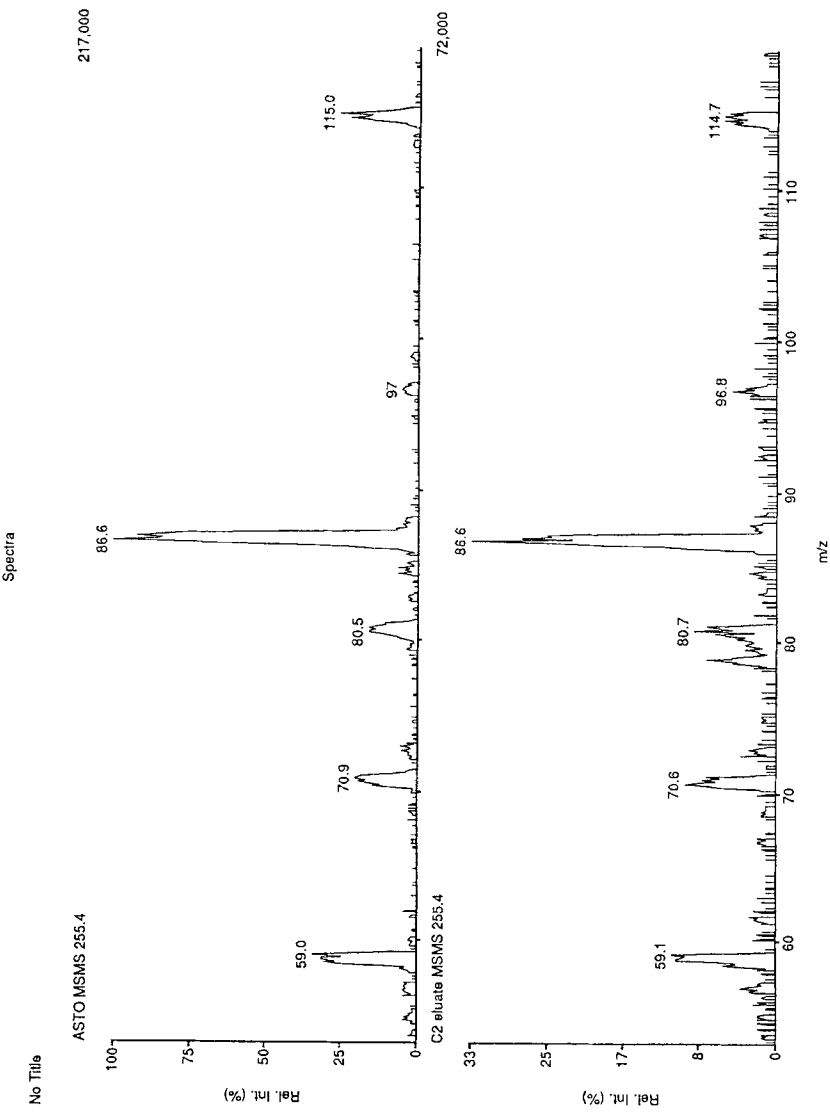
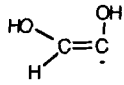
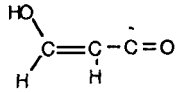
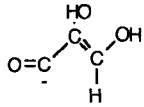
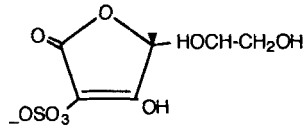


Figure 10 An MS/MS spectral comparison of standard AS (top).and the eluted peak at RT of 3.22 from the 5% TCA gastric extract of fish force-fed AS.

TABLE 1
Summary of fragments resulting from the MS/MS fragmentation of Astos (AS) standard. Order of fragments are from top to bottom with calculated atomic masses and proposed structures. Fragments are referenced to the parent compound L-ascorbic acid-2-sulfate shown at bottom of table.

Mass Number	Formula	Structure
59.0.	$C_2H_3O_2$-	
70.9	$C_3H_3O_2$-	
80.8	SO_3-	
86.6	$C3H3O3$-	
115.0	Unknown	
255	$C_6H_7O_9S^-$	

final proof for this eluted "carrier's" identity requires the more rigorous chemical identification that is being pursued.

During the study three separate coho 5 % TCA gut extracts were made. Two MS spectra are shown in Figure 13 and a third in Figure 8. They compare favorably with the standard AS seen in Figure 7. These three extracts were made in the manner described in the Methods section but were made at different time periods. The first one was made on 12/3/93, the second one on 2/1/94 and the third one on 3/15/94. As a result, the argon gas densities and sensitivities may vary slightly but the characteristic mass fragments are the same as Astos standard AS, 227, 233, 241, 255, 269, and 283.

In conclusion, the basic purpose of the mass spectrometer is to convert a sample into measurable products that are indicative of the original molecule (9). The work reported here is convincing evidence that the original molecule AS and the eluted tissue components do fragment

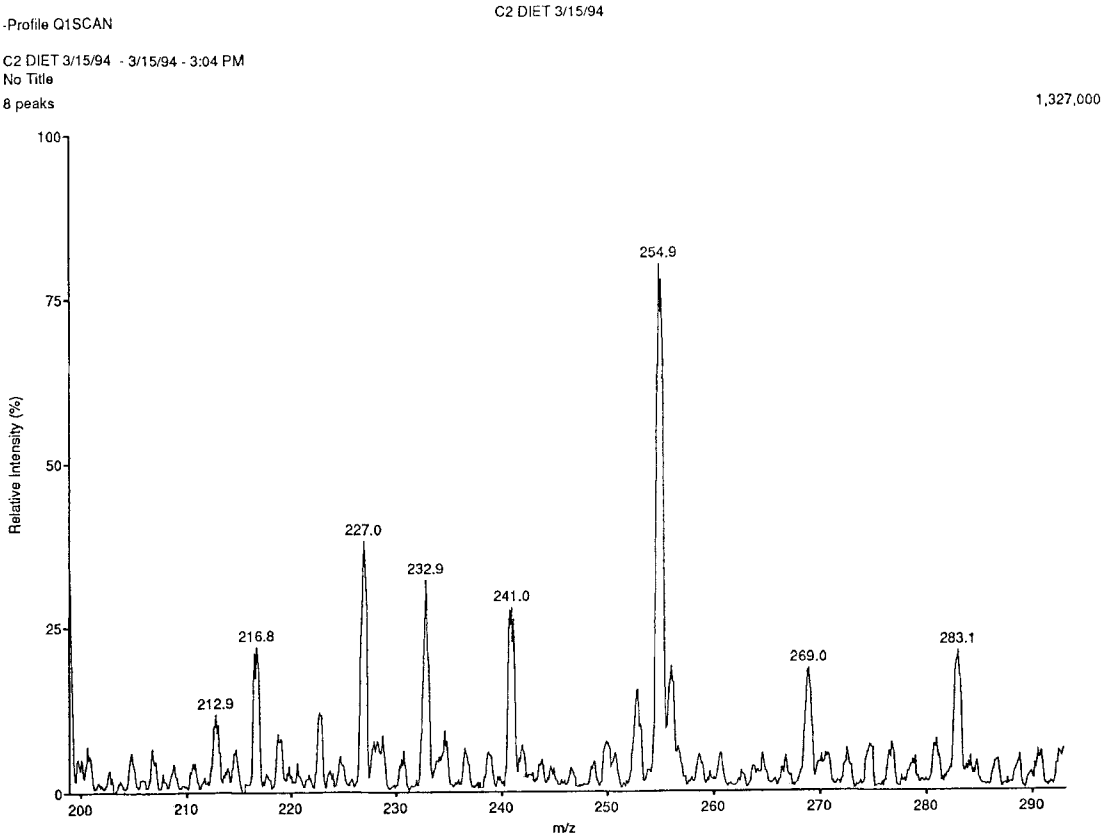


Figure 11 An Electro-Spray ionization mass spectrum of the AS peak eluted from a HPLC run of a 5% TCA extract from the commercial diet extract containing AS (Eluate seen in Figure 6 B).

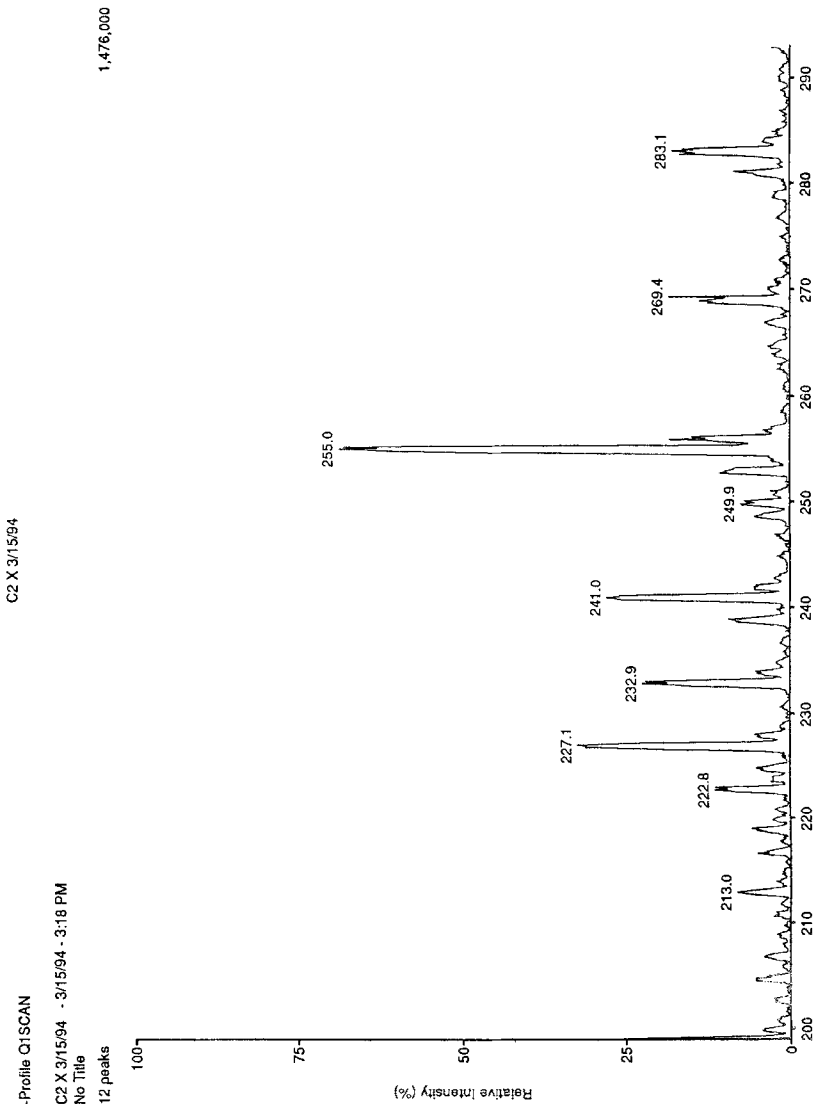


Figure 12 The An Electro-Spray ionization mass spectrum of the eluted HPLC peak (with the retention time of 3.5 min.) shown in figure 2 B.

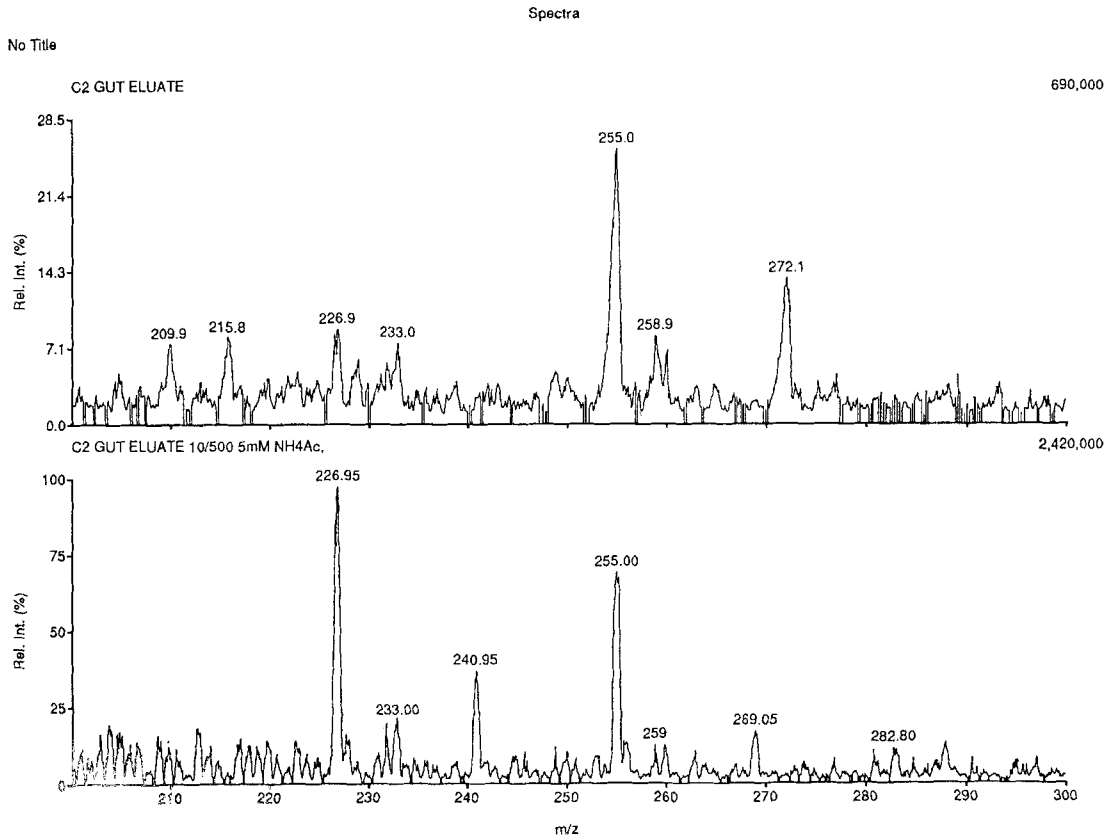


Figure 13 These Electro-Spray ionization mass spectra represent two additional eluted peaks from HPLC runs of 5% TCA gastric tissue extracts from fish force-fed AS. To be compared with Figure 8.

in an identical manner. The real value of this study is threefold: 1) By using the sensitive method of Electrospray Ionization Mass Spectrometry , to verify the existence of L-ascorbyl-2-sulfate in tissue, and to confirm earlier indications that relied upon a less precise HPLC identification; 2) to report to investigators and nutritionists that the previously documented HPLC methods are confirmed as viable analytical tools; and 3) to provide confidence to the aquaculture and animal industries that AS is confirmed in tissue as well as in diets.

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REFERENCES

1. Felton, S. P. and Halver, J. E, Vitamin C1 and C_2 analyses using double column reverse phase high pressure liquid chromatography, Aquaculture and Fish Management 18: 387- 390, 1987.

2. Felton, S P. and Halver, J. E. Separation of three commercial forms of vitamin C (ascorbic acid, ascorbic-2-sulfate and ascorbate-2-polyphosphate) by HPLC. Proceedings of Society for Experimental Biology and Medicine 190: 217, 1989.

3. Felton, S. P., Grace, R. and Halver, J. E., An improved method to measure L ascorbyl-2-sulfate (C_2).Federation of American Societies for Experimental Biology Journal 7: (3), A 74, 1993.

4. Johnson, C.L. Hammer, D.C. Halver, J. E., and Baker, E.M., Urinary metabolites of ascorbate in the rainbow trout .Federation Proceedings. 30:1822, 1971.

5. Tolbert, B., Isherwood, D.J., Atchley, R.W., and Baker E.M.Ascorbate acid sulfate: Synthesis and properties. Federation Proceedings 30: 1819, 1971.

6. Hoffman, K.M. Brown, P.R. and Maugle P.D., Comparison of three HPLC methods for the analysis of ascorbic acid in shrimp tissue. Journal of Liquid Chromatography 15 : (14), 2581-2610, 1992.

7. Felton, S.P. Grace, R. and Halver, J. E. A non-ion pairing HPLC method for measuring new forms of ascorbate and ascorbic acid. Journal of Liquid Chromatography 17 :(1), 123-131, 1994.

8. Dabrowski, K. Matusiewicz, M. and Blom, J. H. Review, Hydrolysis, absorption and bioavailability of ascorbic acid esters in fish. Aquaculture 124: 169-192, 1994.

9.. McLafferty, F.W. and Turecek, F. (eds.) Interpretation of mass spectra.4th Edition publ. University Science Books, Mill Valley, CA, 1993.

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