LIQUID CHROMATOGRAPHIC DETERMINATION OF A COMBINED FORM OF L-ASCORBIC ACID (L-ASCORBATE 2-SULFATE) IN FISH FEEDS AND FISH TISSUES BY SOLVOLYTIC RELEASE OF L-ASCORBIC ACID

by

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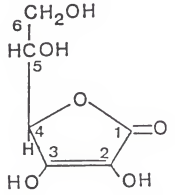
TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

L-Ascorbic acid (AsA), or vitamin C, plays a critical role in all living organisms. Vitamin C protects cells from the toxic by-products of aerobic metabolism (Halliwell, 1982), assists in the hydroxylation of collagen, lysine, and proline (Barnes and Kodicek, 1972), and invigorates immune cells (Li and Lovell, 1985; Bendich, 1987).



L-Ascorbic Acid

Although most vertebrate animals synthesize L-ascorbic acid, several species lack the gene to produce L-gulonolactone oxidase, the enzyme required to convert L-gulonolactone to L-ascorbic acid (Chatterjee et al., 1975). Primates, guinea pigs, fish , bats, insects and some birds depend upon a dietary source of vitamin C for normal growth.

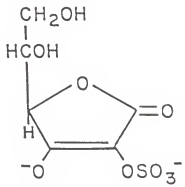
Vitamin C is known to be a required component of diets for intensively cultured salmonids, catfish, eel, shrimp and most carp (Kitamura et al., 1965; Wilson, 1973; Lim and Lovell, 1978). As in mammalian systems, L-ascorbic acid in fish appears to be involved in collagen synthesis. Fish reared on diets deficient in vitamin C develop signs traceable to impaired collagen biosynthesis. These include malformed vertebrae (lordosis or scoliosis), deformed support cartilage, delayed wound repair and reduced growth (Arai et al., 1972; Halver et al., 1972).

Vitamin C is frequently unstable in formulated feeds, principally due to its reaction with air. In the presence of cupric or ferric ions, and with sufficient water activity , L-ascorbate reacts with molecular oxygen to give dehydroascorbic acid, which rapidly decomposes to a variety of compounds with loss of vitamin C activity (Liao and Seib, 1988). Hilton and co-workers (1977) found a 70% loss of supplemental L-ascorbic acid during pelleted feed preparation, and virtually complete destruction after seven weeks of storage at 25^{o}C. Wang et al. (1988) followed the loss of AsA in several feeds stored at 25^{o}C and 40^{o}C in polyethylene bags. AsA showed the following half-lives in the feeds: 62 days and 42 days in a starter trout feed at 25^{o}C and 40^{o}C, respectively; and 8 days and

6 days in a grower trout feed at 25°C and 40°C, respectively.

L-Ascorbate 2-Sulfate (AsAS)

To increase stability, alternate forms of AsA have been investigated. One such form, L-ascorbate 2-sulfate (AsAS, or vitamin C_2), was reported to be much more stable in processed and stored foods and feeds than L-ascorbic acid (Quadri et al., 1975; Soliman et al., 1987).



L-Ascorbate 2-Sulfate

L-Ascorbate 2-sulfate is readily available in pure crystalline form. The compound can be synthesized in high yield (80%) by sulfation of L-ascorbate at pH 9.5-10.5 using trimethyl amine-sulfur trioxide (Seib et al., 1974). Chlorosulphonic acid can also be used for regioselective 2-sulfation of L-ascorbate (Hayashi et al., 1975).

Natural occurrence of L-ascorbate 2-sulfate

L-Ascorbate 2-sulfate was first isolated and purified from the undeveloped cysts of brine shrimp by Mead and Finamore (1969). Since then, it has been found in the urine of humans, monkeys, rats and guinea pigs, as well as in the tissues of the rat and trout (Mumma and Variang, 1972; Hornig et al., 1973). L-Ascorbate 2-sulfate in fish is thought to be synthesized by ascorbic acid-sulfotransferase acting on AsA (Farooqui, 1980; Benitez and Halver, 1982). Mead and Finamore (1969) postulated AsAS may have a dual metabolic role as a storage form of both ascorbic acid and sulfate.

Vitamin potency of L-ascorbate 2-sulfate

The vitamin C potency of L-ascorbate 2-sulfate appears to be species-dependent; it prevents scurvy in fish (Halver et al., 1972), but not in the guinea pig (Campeau et al., 1973; Kuenzig et al., 1974). In most animals, AsAS given by parenteral administration is rapidly excreted, which indicates little or no transport into tissue (Omaye et al., 1982). In contrast, salmonids fed ^{35}S-labeled AsAS showed an ability to absorb ascorbate 2-sulfate into their tissues (Halver et al., 1975). Moreover, a sulfohydrolase was isolated from rainbow trout liver and shown to convert AsAS to AsA and sulfate. That enzyme has been proposed as the modulator of cellular levels of AsA in fish, with its activity

controlled by feedback inhibition by AsA (Benitez and Halver, 1982).

Liquid chromatography (LC) under pressure has been used to demonstrate the presence of both AsA and AsAS in whole body homogenates of rainbow trout (RBT) (Halver et al., 1983; Tucker and Halver, 1986). Tucker and Halver (1984) studied the body pool of both AsA and AsAS in RBT. They reported that AsAS is a major storage form of vitamin C in RBT.

Brine shrimp, a traditional feed for aquarium fish as well as prawn culture, provides vitamin C, at least partially, in the form of AsAS. The AsAS present in cysts is apparently converted to AsA for use during embryonic development and larval emergence (Golub and Finamore, 1972). Several species of fish larvae have been reared on dry flaked diets using AsAS as the sole source of vitamin C (Tucker et al., 1980).

Assay of L-ascorbate 2-sulfate (AsAS) and combinations of AsAS and L-ascorbic acid (AsA).

Several methods have been reported to quantitate AsA and AsAS in food, feed or animal tissue. Baker et al. (1973) first reported that AsAS could be quantitated by modifying the classical colorimetric procedure (Roe-Kuether, 1943) using 2,4-dinitrophenylhydrazine (DNPH). Those workers found that heating AsA or AsAS with DNPH in

9 N sulfuric acid for either 15 min at 95°C or 3 h at 60°C gave equivalent conversion to a colored osazone. On the other hand, warming AsA or AsAS in the oxidation/coupling reaction to 37°C for 1 h gave the osazone from AsA only, but not from AsAS.

Terada et al. (1978) reported another modification of the Roe-Kuether (1943) method. They used 2,6-dinitrophenolindophenol (Tillman's reagent) and potassium bromate to oxidize AsA and AsAS, respectively. The oxidized product was then heated with DNPH in 9 N sulfuric acid for 3 h at 60°C, and the osazone produced was determined as in the original method by Roe and Kuether (1943). In this method, potassium bromate oxidized both AsA and AsAS, but Tillman's reagent oxidized AsA only at 37, 60, and 90°C for 5 h.

Tucker and Halver (1981) used the analytical procedure of Baker et al. (1973) to assay fish tissue for total ascorbic acid equivalents (AsA and AsAS) and free AsA. AsAS was hydrolyzed by heating the oxidation/coupling reaction mixture to 100°C for 20 min, instead of the 95°C for 15 min used by Roe and Kuether (1943). Upon release of AsA from AsAS in the acidic coupling reaction mixture, AsA was oxidized by cupric ion, and the oxidation product converted to the osazone upon reaction with DNPH.

Liquid chromatography (LC) under pressure was described first to separate AsA and AsAS by Bigler and

Kelly (1975). They used an ion-exchange column with UV detection and reported a sensitivity of 150 ng of AsAS. Reverse-phase LC under pressure with UV detection has been proposed to assay fish tissue for AsA and AsAS (Felton and Halver, 1987). Chromatograms of extracts of dried rainbow trout with 5% trichloroacetic acid showed baseline resolution of AsA, but not AsAS. No data was presented on AsAS levels in tissue, nor was the sensitivity of the method reported.

LC under pressure with electrochemical (EC) detection (Tsao et al., 1984) was shown to be approximately two-orders of magnitude more sensitive than UV detection (Bigler and Kelly, 1975). Tsao et al. achieved baseline resolution of AsAS in human urine using a reverse-phase column eluted with a 9.5/90.5 (v/v) mixture of ethanol and 30 mM acetate buffer containing 1.5 mM octylamine. The oxidation potential in the EC detection used was +0.91 V, which is somewhat higher than that (+0.88 V) reported by Pachla and Kissinger (1979). As pointed out by Tsao et al. (1984), UV-detection of AsAS in LC is far less sensitive than EC-detection and the chromatograms of biological extracts using UV-detection often show interfering components.

In summary, a general, sensitive, and accurate method is still needed to assay AsAS in biological tissue and aquaculture feeds. The published methods based on osazone

formation from DNPH suffer from possible interference by sugars at the high temperatures used in the hydrolysis/oxidation/coupling reaction (Roe, 1961; Zloch et al., 1971). Furthermore, as found in this work, heating the hydrolysis/oxidation/coupling reaction to 95-100°C caused cloudiness in the colored solution, which resulted in error in the assay. The LC assay methods using UV-detection for AsAS in biological tissues lack sensitivity and specificity. LC with EC-detection appears to be sensitive and specific when applied to urine. However, as found in this work, this method could not be applied to the determination of AsAS in other biological samples because of the high applied potential. In our laboratory many components in the extracts were oxidized under the high applied potential (+0.91 V), and those components interfered with the determination of AsAS.

New approach to assay for L-ascorbate 2-sulfate

Another approach to assay for AsAS is based on its ease of acid-catalyzed solvolysis to AsA (Seib et al., 1974). The AsA released can then be quantitated by LC with EC-detection, which is a sensitive and accurate method (Kissinger and Pachla, 1987; Hung et al., 1987).

The hydrolysis of sulfate esters has been studied by several workers. Burstein and Lieberman (1958) studied the solvent effect on hydrolysis of a steroid hydrogen

sulfate. Solvolysis was reported to proceed by first-order kinetics in a variety of organic solvents of low polarity. Seib et al. (1974) investigated the hydrolysis stability of ascorbate 2-sulfate under certain conditions and found a 1500-fold rate acceleration as the solvent composition was changed from water to 99% methanol.

In this investigation, it is proposed that, first, a sample be assayed for free AsA. Second, the sample be subjected to acid-catalyzed solvolysis, and the mixture assayed for total AsA (free and released). If it is assumed that AsAS is the major bound form of L-ascorbate in fish tissue, the difference between the two assays gives the level of AsAS.

Objectives

The objectives of this investigation were two-fold:

(1) to develop a sensitive assay method for determination of L-ascorbate 2-sulfate using LC under pressure with isocratic elution, and

(2) to apply the method to determine the level of AsAS and AsA in selected fish tissues.

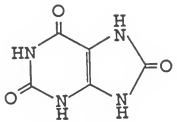
Results and Discussions

Determination of free L-ascorbic acid (AsA) in feed, fish meal or fresh fish tissue; possible interference by uric acid (UA)

Determination of AsA using liquid chromatography under pressure with electrochemical detection (LC-EC) is now a routine method (Pachla and Kissinger, 1987). Sample preparation for AsA assay by LC-EC is readily done by extraction with 6% metaphosphoric acid containing DTT (Augustin et al., 1981; Lookhart et al., 1982), followed by dilution with 0.05 M cold perchloric acid. Chromatograms typical of those for the assay of free AsA in fish feed, fish meal and fresh fish tissue are shown in Fig. 1. Extracts from a catfish feed spiked with 25 ppm AsA, freeze-dried whole-body rainbow trout (RBT) and fresh rainbow trout liver all gave chromatograms (Fig. 1) showing the AsA peak eluting at R_T 6.0 min with base-line resolution. The peak eluting at R_T 7.1 min was assigned to uric acid (UA) in the extracts of the samples.

Uric acid is a catabolic product of purine nucleotides that is excreted by insects, birds, fish and mammals. Uric acid is an electroactive substance, and its response to electrochemical detection at +0.72 V is almost identical to that of an equivalent amount of AsA. Uric

acid may interfere with AsA determination of aquaculture samples assayed by LC-EC. Chromatograms for AsA assay of fish tissue using two different mobile phases are shown in Fig. 2. The chromatogram in Fig. 2A shows a major peak eluting at R_T 6.5 min, which was shown by spiking experiments to be due to co-elution of AsA and UA. When the same extract of fish tissue was assayed using a different mobile phase, the chromatogram (Fig. 2B) showed two peaks eluting with R_T 6.0 min and R_T 7.1 min that were identified by spiking with standard AsA and UA.



Uric Acid

When a fish feed, fish meal or fresh fish tissue was spiked with 25-150 ppm of AsA and assayed by LC-EC, a linear response was observed (Fig. 3). Compared to AsA dissolved in 6% aqueous metaphosphoric acid, the recovery of AsA from spiked samples varied between 94-108% (Table 1). For that reason, we constructed a separate standard curve for each sample.

Upon assay for AsA in catfish feed, freeze-dried whole body RBT, and wet RBT liver, the coefficients of variation were 0.9, 0.4, and 0.4%, respectively. The

sensitivity of the assay method for AsA was 0.4 ng in the 20 µL of extract, which was equivalent to 10 ppm AsA in a sample. An example chromatogram using the most sensitive detection mode is shown in Fig. 1A for a fish feed that contained 10 ppm AsA.

The assay method for AsA in fish feed, fish meal and fish tissue is outlined in Fig. 4; the total time period for assay was approximately 30 min.

Solvolysis of L-ascorbate 2-sulfate (AsAS) in a model system

Seib et al. (1974) reported the rapid conversion of AsAS to AsA in an acidic methanol media, but no quantitative data were reported. In the present investigation, the release of AsA from AsAS in dry and moist methanol containing acetyl chloride (AcCl) was followed with reaction time using LC-EC. Fig. 5 shows that solvolysis of AsAS in dry, acidic methanol at 25°C gave quantitative release of AsA after 20 min.

As expected, Fig. 6 shows that the rate of solvolysis was directly dependent on the concentration of acid, while Fig. 7 shows the rate was inversely related to the water present in methanol. At the highest concentration of acid (3% by volume of added acetyl chloride), the time required for 98% release of AsA from AsAS increased from 5 to 20 min as water concentration

changed from 0 to 2% by volume. At the lowest level of added acetyl chloride (1%), the 98% solvolysis time increased from 20 to 60 min (Fig. 7) as the water level increased from 0 to 2%.

In addition to the acceleration effect of acid, it has been long known (Burstein and Lieberman, 1958) that the rate of solvolysis of a sulfate ester is strongly influenced by the ionizing power of the solvent. The less polar the medium, the faster the rate of cleavage of the sulfate ester bond. Thus, an increase in the concentration of water in a solution retards solvolysis of a sulfate ester greatly, which was demonstrated for AsAS by Seib et al. (1974).

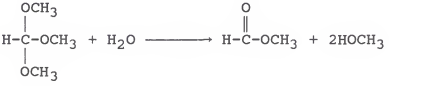
The retarding effect of 0 to 2% water in methanol on the time needed to solvolyze AsAS could be countered by increasing the level of acid in the medium. The curves in Fig. 7 show that by adding 2-3% (by volume) acetyl chloride to the solvolysis medium, 98% solvolysis of AsAS was achieved in 30 min or less when the medium contained as much as 2% water.

When the solvolysis reaction mixture contained relatively large concentrations of water (2-8% in methanol), trimethyl orthoformate (TOF) could be used to remove the water so that > 95% recovery of AsA was observed in 30 min at 25^{o}C (Fig. 8). The amount of TOF added to a solvolysis reaction had to be optimized (Table

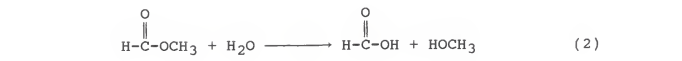
2). When the molar ratio of TOF/H_2O in a solvolysis mixture was less than 0.5 (water level was > 2%), recovery of AsA was low due to the slow solvolysis of AsAS, whereas when the ratio of TOF/H_2O was grater than 1.6, the recovery of AsA was low due to formylation of AsA. The extra peak seen in the chromatogram in Fig. 9 was assumed to be a formyl ester of AsA.

In order to obtain > 95% solvolysis of AsAS at a given moisture level in methanol, an optimum level of TOF must be chosen to substitute for methanol in the solvolysis reaction mixture. Table 3 summarizes the level of TOF used to substitute for methanol when the solvolysis mixture (methanol, TOF, and water) contained 2-8% water (by volume).

The consumption of water by TOF in the solvolysis reaction mixture decreases the polarity of the medium, and the rate of solvolysis of AsAS is increased. There are two reactions by which TOF removes water from a solvolysis mixture. The reaction of the orthoester with the first molecule of water (reaction 1), which occurs very rapidly, yields methanol plus methyl formate (Ahmad et al., 1979). The methyl formate then reacts with second molecule of water to give formic acid and methanol (reaction 2).



(1)



(2)

L-Ascorbate 2-sulfate determined in a fish feed, fish meal or fresh fish tissue by simultaneous extraction and solvolysis to AsA

AsAS in a sample of fish feed, fish meal or fresh fish tissue was determined by the increase in AsA content after cleavage of the sulfate group using acid-catalyzed solvolysis.

In developing the assay method, we set the maximum assay time at one hour and the recovery of AsA from AsAS greater than 95%. Those results were achieved simply by adjusting the levels of acid and TOF which controlled the rate of solvolysis of AsAS in the methanolic extraction/solvolysis medium.

Dithiothreitol (DTT) was included in the extraction/solvolysis medium to preserve AsA released from AsAS. Furthermore, a temperature of 25°C was chosen for extraction/solvolysis to minimize potential dehydration of AsA in the acidic medium.

Fish and feed samples to be assayed for AsAS contain varying amounts of ionizing components, thus the amount of acetyl chloride to be added to the methanol used for

extraction/solvolysis of a sample varied and was determined in a separate experiment. The sample was mixed in water, and the amount of acetyl chloride required to generate pH 0.5-0.9 was recorded. That amount was then used in the extraction/solvolysis medium.

The moisture content of a sample also had to be determined prior to the extraction/solvolysis step in the assay procedure for AsAS. The level of TOF added to the extraction/solvolysis medium was dictated by the moisture content of the sample. Based on the model solvolysis studies on AsAS, the following ratios of TOF/methanol were required in the extraction/solvolysis medium for a 2 g sample with varying moisture content:

Moisture in sample, wet basis, %	TOF/methanol, mL/mL
5 - 20	1 / 19
21 - 50	3 / 17
51 - 90	7 / 13

If the moisture content of a 2g sample was 20, 50, and 90%, theoretically 1, 3, and 7 mL of TOF would be sufficient to react with the 0.4, 1.0, and 1.8 g of water in the samples, respectively, provided both reactions (1) and (2) proceeded to completion.

One potential problem with the solvolysis assay method for AsAS is the accessibility of AsAS inside animal cells to the acid-methanol. It would be expected that during extraction of animal cells, methanol would readily disrupt the lipid bilayer of the cell membranes. We examined this point by subjecting solvolysis reaction mixtures of liver and freezed-dried fish meal, respectively, either to grinding in the Tekmar homogenizer alone, or to grinding in the Tekmar followed by exhaustive grinding using an Elvehjen apparatus. No increase in recovery of total ascorbic acid was found in the extraction/solvolysis mixtures after exhaustive grinding in the Elvehjen apparatus (Table 4).

The assay procedure for total AsA equivalents (AsA + AsAS) in fish feed and fish tissues is outlined in Fig. 10. Chromatograms typical of those for free AsA and total AsA equivalents (AsA + AsAS) by the solvolysis assay are shown in Fig. 11. Once again, the AsA peak was base-line resolved. The peak in Fig. 11A represented free AsA in the whole-body rainbow trout, while the peak in Fig. 11B was the sum of free AsA plus AsAS. The level of AsAS in the sample was readily determined by difference.

To determine the recovery of AsAS by the assay procedure in Fig. 10, several samples were spiked with AsAS equivalent to 25-100 ppm AsA. A 94-101% recovery of AsAS in the form of AsA was obtained when compared to

adding AsA directly to the samples immediately prior to assay (Table 5). When AsA (25-100 ppm) was added to the samples, recoveries were 95-100% (Table 5) compared to standard solutions of AsA in 6% aqueous metaphosphoric acid containing 0.2% DTT.

Fig. 12 shows the standard curves constructed for assaying total ascorbic acid equivalents (AsA + AsAS) in freeze-dried fish meal by the solvolysis method outlined in Fig. 10. No significant difference was found between the recovery of AsA and AsAS subjected to the extraction/solvolysis treatment. The same results were obtained for a fish feed or fresh fish tissue, even though the curves are not presented here. These results confirm that total ascorbic acid equivalents (AsA + AsAS) can be determined by the solvolysis method, and the level of AsAS can be obtained by subtracting free AsA from total ascorbic acid equivalents.

The solvolysis assay method for AsAS gave excellent reproducibility. The coefficients of variation of three extracts from freeze-dried, whole-body RBT, catfish feed, and wet RBT liver were 7.8, 0.8, and 1.1%, respectively.

L-Ascorbate 2-sulfate in fish tissue determined by two additional assay methods from the literature

Direct determination of AsAS with LC-EC was done for several fish tissues. Samples were prepared by extraction

with 6% aqueous trichloroacetic acid at 25°C (Tucker and Halver, 1984). The extracts were centrifuged, diluted with 0.05 M perchloric acid, and analyzed for AsAS by LC-EC. Compared to LC-EC assay of AsA, when using LC-EC to determine AsAS, the applied oxidation potential was increased from +0.72 V to +o.91 V (Tsao et al., 1984), and the methanol in the mobile phase was increased from 5 to 10%. Chromatograms typical of those for direct assay of AsAS by LC-EC are shown for fresh fish tissues in Fig. 13.

The maximum sensitivity of the LC-EC direct assay for AsAS was 50 ppm of AsAS or 26.5 ppm AsA equivalents (Fig. 13A). The low sensitivity of the direct assay method was due to the low response of AsAS to the EC detector, and to background interference in chromatograms because of oxidation of components in extracts at the high potential (+0.91 V) used. Most fish tissues assayed in this study by the solvolysis method contained less than 25 ppm AsA equivalents of AsAS. Thus, no AsAS peak was found in the LC-EC chromatograms of extracts of any fish tissue. A typical chromatogram for an extract of freeze-dried, whole-body RBT is shown in Fig. 13B.

In the direct assay method for AsAS by LC-EC, the mobile phase gave good resolution of the AsAS peak, but the AsA peak was poorly resolved (Fig. 13B, C). The level of AsA in a sample could only be estimated roughly (peak height) in the direct LC-EC method for AsAS.

AsA and AsAS in RBT liver, kidney, and skin were also determined by the modified DNPH method (Tucker and Halver, 1981). Samples were prepared by extraction with 6% aqueous trichloroacetic acid. Total ascorbic acid equivalents (AsA + AsAS) in extracts were determined by heating an extract in 9 N sulfuric acid at 100°C for 20 min followed by oxidation/coupling with the cupric/DNPH reagent. The color from osazone formation was measured spectrophotometrically and compared to a standard. The free AsA in the extract was determined in a separate experiment by warming the coupling reaction mixture to 37°C for 3 h. The calculation of AsAS in the extract of the sample was done by difference.

Tucker and Halver (1984) studied the distribution of vitamin C in RBT using a modified DNPH assay method (Tucker and Halver, 1981). The fish they studied were fed a diet containing 100 ppm of AsA. We also assayed RBT tissue by the modified DNPH method (Tucker and Halver, 1981). The data from the DNPH method in our laboratory are compared to the solvolysis data in Table 6. The two methods gave no difference for AsA levels in the tissues. But the amount of total AsA equivalents was higher for the DNPH method compared to the solvolysis method. We found that in the DNPH assay, the reaction mixture for total AsA equivalents showed cloudiness after heating to 100°C for 20 min (to hydrolyze AsAS, oxidize AsA, couple dehydro-AsA

with DNPH, and develop color). The cloudiness may have been caused by colloidal protein precipitation in the samples and explained the high absorbance and high levels of total AsA equivalents determined by the DNPH method. Other organic matter such as sugars in samples also introduces a positive error under the conditions used in the method (Roe and Kuether, 1943; Roe, 1961).

Determination of L-ascorbic acid and L-ascorbate 2-sulfate levels in selected fish tissues

Free AsA in RBT liver, kidney, skin and catfish fillet was determined by the assay method for AsA shown in Fig. 4. A separate determination of total ascorbic acid equivalents (AsA + AsAS) in the selected tissues was done by the solvolysis procedure shown in Fig. 10. AsAS in the sample was calculated by difference. The number of AsA equivalents can be converted to AsAS (disodium salt) by multiplying by a factor of 1.89. Typical chromatograms for freeze-dried, whole-body RBT and wet RBT liver are seen in Fig. 14, and the data are summarized in Table 7. It appears that AsA is the major form of ascorbate in those fish tissues, except in brine shrimp cysts, which contain very high level of AsAS (405 ppm AsA equivalents) but only traces of AsA. Small amounts of AsAS were found in RBT liver and kidney.

The level of AsA, but not AsAS, in the liver and in the kidney was directly proportional to dietary intake of ascorbate (Table 7). Liver from rainbow trout (RBT) was found to contain 40, 60, 106, and 123 ppm AsA on wet basis and 5, 14, 6, and 16 ppm AsA equivalents in form of AsAS when fish were fed diets containing 20, 40, 60, and 100 ppm AsA equivalents, respectively in the form of L-ascorbate 2-polyphosphate (AsPP). The level of AsA in the liver appeared to level off when the feed contained ≥ 100 ppm AsA equivalents.

Duplicate determinations of total AsA equivalents in extraction/solvolysis mixtures of the tissues agreed within 5 ppm. Thus, the solvolysis procedure for total AsA equivalents coupled with conventional LC-EC determination of AsA appears to be an excellent method to determine AsAS levels. The data in Table 7 indicate that the vitamin C nutritional status of fish or shellfish can be assessed by the solvolysis assay method for either free or total AsA in liver or kidney.

Conclusions

1, Solvolysis reaction conditions were devised wherein L-ascorbate 2-sulfate (AsAS) added to biological tissue (5-90% moisture) was converted quantitatively to L-ascorbic acid (AsA).

2, Fish tissue subjected to the solvolysis reaction conditions released AsA, which presumably was from AsAS.

3, AsA was the major form of vitamin C found in fish tissue, whereas AsAS was by far the form present in brine shrimp cysts.

4, AsA concentrations in fish liver or kidney were a good index of ascorbate levels in fish feed.

EXPERIMENTAL

General

Samples of fish meal, fresh fish tissue , and fish feed were stored in a freezer (-20°C) for less than one month before analysis. Dry feed samples were ground to pass a 40-mesh screen using an impact mill (Micromill, Cat. No. S-61691-02, Sargent-Welch Scientific Co., Skokie, IL). Fresh fish tissue or dry fish meal was ground together with extraction medium using either a Tekmar tissue homogenizer (Cat. No. S-61746-10D, Sargent-Welch Scientific Co., Skokie, IL), or a Elvehjen apparatus (Cat. No. 62400-744, VWR Scientific Co., Division of Univar, San Francisco, CA).

The moisture content of a feed sample was measured by weight loss after heating the ground sample 1 h at 130°C, while fish meal was dried 18 h at 100°C. Fresh fish tissue was first dried by air at 25°C and low relative humidity, then dried 18 h at 100°C.

All chemicals were reagent grade unless otherwise stated. Dithiothreitol (DTT), tetrabutylammonium phosphate (TBAP), disodium ethylenediamine tetraacetate (EDTA), acetic acid, perchloric acid and methanol were liquid-chromatographic grade. Anhydrous trimethyl orthoformate (TOF) was from Aldrich Chemical Co., Milwaukee, Wis..

Pure potassium L-ascorbate 2-sulfate (AsAS) was prepared starting from sodium L-ascorbate 2-sulfate (Nikkol, Tokyo, Japan). Sodium L-ascorbate 2-sulfate (15 g) was dissolved in water (100 mL) and passed through a column of strongly acidic cation exchange resin in the potassium form (500 mL). The column was washed with two volumes of water, and the effluents combined and concentrated to a small volume under vacuum below 60°C. Potassium L-ascorbate 2-sulfate crystallized from 50% aqueous methanol with m.p. 160-163°C. Elemental analysis. Found: C, 21.63; H, 2.05; S, 9.36. Calculated for $C_6H_6K_2O_9S$: C, 21.68; H, 1.86; S, 9.65.

Liquid chromatography under pressure was done using a Knauer pump (Model 64, Sommteck, Inc., Woodcliff Lake, NJ), electrochemical detector with a glassy carbon electrode and type TL-5A flow cell (Model LC-4B, Bioanalytical Systems, West Lafayette,IN), Rheodyne loop injector, (20 µL, Alltech Assoc, Inc., Deerfield, IL), and an integrating recorder (Model C-R3AChromatopac, Shimadzu Co.). The mobile phase used to elute L-ascorbic acid (AsA) was prepared by mixing one volume of methanol with 19 volumes of 0.08 M acetate buffer (pH 4.5) containing 0.1 mM EDTA and 1.75 mM TBAP. The eluting solvent was filtered through a 0.45 µm filter membrane (Nylon 66, Supelco, Inc., Bellefonte, PA), and degassed using a water aspirator. Flow rate was 0.8 mL/min. The electrochemical

detector was set at +0.72 V, and the column was maintained at 25°C with a column heater (FIAtron CH-30 Column Heater, Fiatron Laboratory Systems, Oconomowoc, WI). A diluted sample extract was filtered through a syringe filter (ACRO LC3A, Gelman Science, Ann Arbor, MI) prior to duplicate injections (each 20 µL) using a Hamilton microliter syringe (Hamilton Co., Reno, NV).

Determination of L-Ascorbic Acid (AsA)

AsA in dry feed and fresh fish tissue was determined by a modification of the procedure described by Kissinger and Pachla (1987). Ground feed (2 g) was extracted with 6% aqueous metaphosphoric acid (20 mL) containing DTT (0.2 g) for 10 min at 25°C using a magnetic stir plate. When assaying formulated feeds, a 10 g sample size was often used to improve precision. After centrifuging 15 sec at 8160 x g, an aliquot (1 mL) of the supernatant was brought to volume (50 mL) using cold 0.05 M perchloric acid. The diluted extract was filtered through a syringe filter, and the filtrate immediately injected into the chromatograph.

AsA in fresh fish tissue was assayed using the same procedure, except the fresh tissue (2 g, wet weight) was first ground together with dithiothreitol (0.2 g) in 20 mL of a mixture of methanol/6% aqueous metaphosphoric acid (7/3, V/V). The grinding was done for 30 sec using

the Tekmar or Elvehjen apparatus; the temperature was maintained at 25°C.

A standard curve for AsA assay in a fish feed and fresh fish tissue was constructed by adding known amounts of standard AsA to a given sample, extracting the sample and analyzing the diluted 0.05 M perchloric acid extracts by LC. Peak heights, which were corrected for native levels of AsA, were plotted vs the amounts of standard added to a sample. The amount of AsA in the extract of an unknown sample was determined by comparing the peak height to that on its standard curve.

Solvolysis of L-ascorbate 2-sulfate (AsAS): use of trimethyl orthoformate (TOF) and acetyl chloride in methanol to give quantitative release of AsA from solutions of AsAS containing varying levels of water

L-Ascorbate 2-sulfate stock solution was prepared by dissolving dipotassium L-ascorbate 2-sulfate (0.2g) in 5 mL of water, adding 45 mL of methanol, and adjusting to volume (100 mL) with 10% aqueous methanol. L-Ascorbate 2-sulfate standard solutions were prepared daily by diluting 25 mL of ascorbate 2-sulfate stock solution to 250 mL with methanol.

To methanol (12 mL) was added DTT (0.5 g), water (1.0 to 1.8 mL, 0.06 to 0.1 moles), acetyl chloride (0.35 to 0.6 mL), and an aliquot (1 mL) of L-ascorbate 2-sulfate

standard solution containing 0.6 micromoles AsAS. The mixture was stirred at 25°C and trimethyl orthoformate (TOF) (7 mL, 63 millimoles) was added. Aliquots (1 mL) of the mixture were withdrawn with time, brought to volume (50 mL) using cold 0.05 M perchloric acid, and the mixture immediately injected into the chromatograph.

A standard curve for AsA was constructed by diluting a solution of AsA in 6% aqueous metaphosphoric acid with cold 0.05 M perchloric acid, and then injecting into the chromatograph. Peak heights were plotted against the concentration of AsA. Recovery of AsA released by solvolysis of AsAS was calculated by comparing peak heights to those on the standard curve.

Other solvolysis reactions were conducted as described above, except the reaction mixtures contained less water (either 0.4 to 1.0 mL or 0 to 0.4 mL), TOF (either, respectively, 3 mL or 0-1 mL), and methanol (either, respectively, 17 mL or 20-19 mL). All the reaction mixtures contained a total volume of 20 mL from a combination of TOF, methanol, DTT (0.5 g), and AsAS (0.6 micromoles). The acidity of a solvolysis mixture was varied using 0.35-0.6 mL of acetyl chloride.

Determination of total L-ascorbic acid equivalents (AsA + AsAS) in fish feed, fish meal and fresh fish tissue by

extraction/solvolysis followed by quantitation of total L-ascorbic acid (AsA)

Prior to extraction/solvolysis of AsAS in a sample of feed, fish meal or fresh fish tissue, it was necessary first to determine the amount of acetyl chloride to be used in the solvolysis reaction mixture. That was done by mixing the ground sample (2 g) in water (20 mL), and adding sufficient amounts of acetyl chloride to generate pH 0.5 to 0.9 (pH meter) in the aqueous mixture.

Ground feed or dry fish meal (2 g), containing 5 to 20% moisture content, was added to methanol (19 mL), TOF (1 mL), and DTT (0.5 g). The pre-determined amount of acetyl chloride (usually 0.8 mL) was added , and the mixture was stirred on a magnetic stir plate at 25°C for 30 min. After centrifugation at 8160 x g for 15 sec, an aliquot (1 mL) of the supernatant was brought to volume (50 mL) with cold 0.05 M perchloric acid, and the diluted mixture was filtered through a syringe filter. Immediately, the filtrate was injected into the chromatograph and assayed for AsA.

To assay fresh fish tissue with 51 - 90% moisture, the sample (2 g, wet basis) was ground 30 sec with methanol (13 mL) and DTT (0.5 g) using the Tekmar homogenizer. TOF (7 mL) was added followed by the predetermined amount (usually 0.6 mL) of acetyl chloride.

After solvolysis, the reaction mixture was diluted and analyzed for AsA as previously described.

Samples with a moisture content between 21 and 50% were assayed by the same method except the quantities of TOF and methanol were 3 mL and 17 mL, respectively.

When determining total ascorbic acid equivalents in fish feed, dry fish meal and fresh fish sample, a separate standard curve was constructed for each sample by adding the standard solution containing known amounts of AsAS. Depending on the moisture content of the sample, the appropriate extraction/solvolysis procedure was then followed as described above, and the standard curve (peak heights) constructed after correcting for any native levels of total ascorbic acid equivalents. When an unknown sample was assayed for total ascorbic acid equivalents, peak heights of AsA in the diluted extraction/solvolysis mixture were compared to those on the standard curve. AsAS (as AsA equivalent) in a sample was calculated by subtracting free AsA from total ascorbic acid equivalents.

Stability of AsA in the extraction/solvolysis step of the assay procedure

Dry fish meal and fish feed with 5-20% moisture (wet basis), soft-moist fish feed with 31% moisture, and fish meal or tissue with 70-90% moisture were spiked with AsA (25-100 ppm, wet basis) using a standard solution (200

ppm) of AsA in methanol. The samples were assayed for total ascorbic acid equivalents using the prescribed solvolysis treatment, which depended on moisture content. If a sample contained native ascorbic acid equivalents (both AsA and AsAS), recovery of spiked AsA was calculated by correcting for native levels of AsA and AsAS. The recovery of AsA added to a feed or fish meal sample was determined by comparison to a standard curve derived by adding AsA to a solvolysis reaction mixture with immediate work-up of the sample and injection into the chromatograph.

Direct determination of L-ascorbate 2-sulfate (AsAS) in fresh fish tissue using liquid chromatography under pressure with electrochemical detection

The wet tissue (1-2 g) was ground 30 sec with 6% aqueous trichloroacetic acid (20 mL) using the Tekmar homogenizer. After stirring an additional 10 min at 25°C, the mixture was centrifuged at 8160 x g for 5 min, and an aliquot (1 mL) of the supernatant was diluted with 0.05 M perchloric acid, filtered through a syringe filter, and injected into the chromatograph. When assaying directly for AsAS, the applied oxidative potential of the electrochemical detector was set at +0.91V, and the mobile phase was the same as that for AsA assay except

that 9 instead of 19 volumes of 0.08 M acetate buffer (pH 4.5) containing EDTA and TBAP were mixed with one volume of methanol.

Determination of free AsA and total ascorbic acid equivalents (AsA + AsAS) in fresh fish tissue using dinitrophenyl- hydrazine and spectrophotometry

Wet tissue (2 g) was ground with 6% trichloroacetic acid (20 mL) using a Tekmar homogenizer for 30 sec. The mixture was stirred an additional 10 min at 25°C on a magnetic stir plate, then centrifuged at 8160 x g for 5 min. The supernatant extract was used for analysis of AsA and total ascorbic acid equivalents according to the method described by Tucker and Halver (1981).

Briefly, to assay for free AsA, an aliquot (2 mL) of the trichloroacetic acid extract and DTC reagent (0.6 mL) were mixed together in a 10 mL test tube. The DTC reagent was a mixture of 10:1:1 (v/v/v) 2% 2,4-dinitrophenylhydrazine in 9 N sulfuric acid / 1% aqueous thiourea / 0.5% aqueous cupric sulfate. The solution was mixed on a vortex mixer and placed in a water bath at 37°C for 3 h with gentle shaking. After that time, the reaction mixture was cooled immediately in an ice water bath, and 75% aqueous sulfuric acid (3 mL) was added. The reaction mixture was then held at 25°C for 30 min to

allow for color development. Absorbance was read at 515 nm against the blank (6% TCA) using a Varian spectrophotometer (Model DM-80, Varian Assoc., Inc., Walnut Creek, CA).

To assay total ascorbic acid equivalents (AsA + AsAS), the same procedure was followed, except the extract was heated with the acidic DTC reagent in a water bath at 100°C for exactly 20 min. AsAS was calculated by subtracting free AsA from total ascorbic acid equivalents.

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Table 1. Recovery of L-Ascorbic Acid (AsA) Added to a Fish Feed, Fish Meal, and Fresh Fish Tissue in AsA Assay.

Sample	Native AsA Level, ppm	Added ASA, ppm	Recovered AsA, ppm	Recovery of AsA, %
fish feed[a]	0	25	27	108
	0	75	77	102
	0	150	150	100
fish meal[b]	6	25	25	100
	6	75	74	98
	6	150	149	99
fresh fish tissue[c]	8	25	24	95
	8	75	71	95
	8	150	141	94

a, Catfish feed from Mississipi State University (Dr. E. H. Robinson), which contained no AsA.

b, Freeze-dried, whole-body rainbow trout from Rangen International Aquaculture Research Center (Dr. B. F. Grant).

c, Wet catfish fillet from supermarket.

Table 2. Release of AsA from L-Ascorbate 2-Sulfate (AsAS) in Methanol-Water Solutions Containing Acetyl Chloride, (AcCl) and Trimethyl Orthoformate (TOF).

Solvolysis Mixture, mL					Composition[a] of Solvolysis Mixture, %			Molar Ratio of TOF/H_2O	Release of AsA with Time, %				
MeOH	TOF	H_2O	AcCl	total	TOF	H_2O	AcCl		10′	20′	30′	40′	60′
20	0	0	0.6	20.6	0	0	3	-	99[b]	93	92	93	-
20	0	0.4	0.6	21.0	0	2	3	0	97	98	100	99	98
19	1	0.4	0.6	21.0	5	2	3	0.4	99	97	96	94	93
17	3	0.4	0.6	21.0	14	2	3	1.2	96	96	95	95	93
13	7	0.4	0.6	21.0	33	2	3	2.8	96	91	91	88	71
20	0	1.0	0.6	21.6	0	5	3	0	83	87	93	97	98
19	1	1.0	0.6	21.6	5	5	3	0.2	90	92	94	99	100
17	3	1.0	0.6	21.6	14	5	3	0.5	95	96	99	97	100
13	7	1.0	0.6	21.6	32	5	3	1.1	98	96	95	93	-
10	10	1.0	0.6	21.6	46	5	3	1.6	95	93	90	90	85
20	0	1.8	0.6	22.4	0	8	3	0	43	56	68	78	84
19	1	1.8	0.6	22.4	4	8	3	0.1	47	60	71	78	85
17	3	1.8	0.6	22.4	13	8	3	0.3	60	75	81	86	91
13	7	1.8	0.6	22.4	31	8	3	0.6	89	90	96	96	95
20	0	0	0.4	20.4	0	0	2	-	93	98	97	98	93
20	0	0.4	0.4	20.8	0	2	2	0	91	95	97	99	97
19	1	0.4	0.4	20.8	5	2	2	0.4	95	98	100	95	94
17	3	1.0	0.4	21.4	14	2	2	1.2	92	94	95	94	94
17	3	1.0	0.4	21.4	14	5	2	0.5	87	92	95	92	92
13	7	1.0	0.4	21.4	33	5	2	1.1	95	98	96	94	92
13	7	1.8	0.4	22.2	32	8	2	0.6	87	92	95	96	94

a, Composition of solvolysis mixture was calculated based on the total volume of the solvolysis mixture.

b, Obtained at 5 min of reaction time.

Table 3. Optimum Levels of Trimethyl Orthoformate for >95% Release of AsA in 30 min from AsAS in Acidic Methanol-Water Solutions Containing 0-8% by volume of Water.

Water in Solvolysis Mixture[a],		AcCl in Solvolysis Mixture,	TOF in Mixture,		Recovery of AsA,
mL	%	%	mL	%	%
0	0	3	0	0	92
0	0	2	0	0	97
0.4	2	3	1	5	96
0.4	2	2	1	5	100
0.4	2	3	3	14	95
0.4	2	2	3	14	95
1.0	5	3	3	14	99
1.0	5	2	3	14	95
1.0	5	3	7	32	95
1.0	5	2	7	32	96
1.8	8	3	7	31	96
1.8	8	2	7	31	95

a, Solvolysis reactions all contained 20 mL of a mixture of methanol, trimethyl orthoformate (TOF), and AsAS solution in methanol, along with variable amounts of water and acetyl chloride. The total volumes of the solvolysis reaction mixtures were 20.6 to 22.4 mL.

Table 4. Comparison of Tekmar and Elvehjen Homogenizers in the Extraction of AsA and in the Extraction/Solvolysis of AsAS. Data Presented in AsA Equivalents.

Sample	Replicate	AsA, ppm		AsAS, ppm	
		Tekmar	Tekmar & Elvehjen	Tekmar	Tekmar & Elvehjen
rainbow trout liver	1	84	84	21	21
	2	83	90	22	20
	3	85	88	21	21
	Ave.	84	87	21	21
catfish meal	1	24	23	8	8
	2	24	24	6	8
	3	24	21	8	7
	Ave.	24	22	7	8

Table 5. Recovery of AsA and AsAS from Spiked Samples of Fish Feed, Fish Meal, and Fresh Fish Tissue Using the Solvolysis Assay Procedure. Data Are Given in AsA Equivalents.

Sample	MC, %	Form of Ascorbate Added	Ascorbate Added, ppm	Ascorbate Recovered, ppm	Recovery, %
Trout starter feed	10	AsA	25	24	95
Oregon soft moist pellet	31	AsA	50	49	98
Freeze-dried, whole-body RBT	5	AsA	100	95	95
Rehydrated RBT meal[a]	90	AsA	25	25	100
Catfish fillet[b]	70	AsA	100	95	95
Trout starter feed	10	AsAS	25	25	100
Oregon soft moist pellet	31	AsAS	50	51	101
Freeze-dried, whole-body RBT	5	AsAS	100	100	100
Rehydrated RBT meal[a]	90	AsAS	25	24	96
Catfish fillet[b]	70	AsAS	100	94	94

a, 0.2 g freeze-dried, whole-body RBT (MC < 5%) mixed with 1.8 mL water.
b, Purchased from supermarket.

Table 6. Comparison of AsA and AsAS Levels in Selected Rainbow Trout (RBT) Tissues Determined by Three Assay Methods. Data Are Reported in AsA Equivalents.

Sample	Assay Method	AsA, ppm	Total Ascorbate, ppm	AsAS, ppm
RBT liver	solvolysis & LC-EC[a]	123	139	16
	direct assay using LC-EC[b]	87	-	<25[c]
	DNPH	174	(305)[d]	(131)[d]
	DNPH, lit[e]	150	262	112
RBT kidney	solvolysis & LC-EC[a]	92	92	trace
	direct assay using LC-EC[b]	73	-	<25[c]
	DNPH	258	(330)[d]	(72)[d]
	DNPH,lit[e]	220	222	2
RBT skin	solvolysis & LC-EC[a]	95	77	trace
	direct assay using LC-EC[b]	105	-	<25[c]
	DNPH	107	(106)[d]	trace
	DNPH,lit[e]	16	39	23

a, Mobile phase used in LC: a 19/1 (v/v) mixture of methanol and 0.08 M acetate buffer (pH 4.5) containing 0.1 mM EDTA and 1.75 mM TBAP.
b, Mobile phase used in LC: a 9/1 (v/v) mixture of methanol and 0.08 M acetate buffer (pH 4.5) containing 0.1 mM EDTA and 1.75 mM TBAP.
c, Level of AsAS in the sample is below the sensitivity of assay method (25 ppm in a sample).
d, Absorbance can not be accurately determined due to cloudiness of the reaction mixture.
e, Data was reported by Tucker and Halver (1984).

Table 7. AsA and AsAS Levels in Selected Fish Tissues and Brine Shrimp Cysts. Data Are Reported in AsA Equivalents.

Sample of tissue	Ascorbate Added to Diet	Tissue Levels				
		Wet Basis			Dry Basis	
		AsA, ppm	Total AsA, ppm	AsAS, ppm	AsA, ppm	AsAS, ppm
freeze-dried, whole-body rainbow trout	AsPP	20	51	31	21	32
	AsA	9	24	15	9	16
	Blank	trace	trace	trace	trace	trace
fresh rainbow trout liver	AsPP, 100ppm	123	139	16	535	70
	AsPP, 60ppm	106	112	6	455	26
	AsPP, 40ppm	60	74	14	255	60
	AsPP, 20ppm	40	45	5	178	22
fresh rainbow trout kidney	AsPP, 80ppm	92	92	trace	418	trace
	AsPP, 60ppm	86	89	3	382	13
	AsPP, 20ppm	48	48	trace	223	trace
fresh rainbow trout skin	AsA[a]	95	77	trace	183	trace
freeze-dried whole-body catfish	AsPP, 100ppm	24	31	7	25	7
	Coated AsA, 100ppm	10	14	4	11	4
	AsA[a]	8	11	3	8	3
wet catfish fillet[b]	-	10	10	trace	33	trace
dry brine shrimp cysts[b]	-	trace	405	405	trace	452

a, Level unknown.

b, Samples were purchased from supermarket.

Figure 1. LC-EC chromatograms of extracts of samples assayed for AsA: (A) native AsA (10 ppm) in fish feed, (B) AsA (25 ppm) added to catfish feed, (C) native AsA in freeze-dried, whole-body rainbow trout (RBT), (D) native AsA in fresh RBT liver. Chromatogram B, C, and D showed a peak also for uric acid (UA).

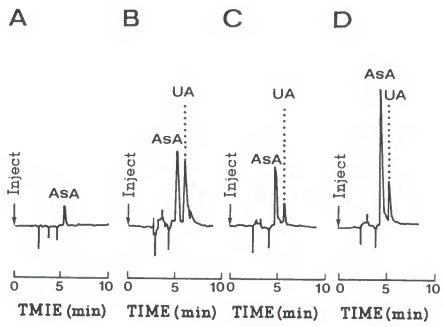

A
B
C
D
Inject
AsA
UA
0
5
10
TMIE (min)
TIME (min)

Figure 2. LC-EC chromatograms of extracts of freeze-dried, whole-body rainbow trout assayed for AsA using two different mobile phases isocratically. (A) Mobile phase: a 19/1 (v/v) mixture of methanol and 0.08 M acetate buffer (pH 4.0) containing 0.1 mM EDTA and 1.0 mM TBAP, (B) mobile phase: a 19/1 (v/v) mixture of methanol and 0.08 M acetate buffer (pH 4.5) containing 0.1 mM EDTA and 1.75 mM TBAP.

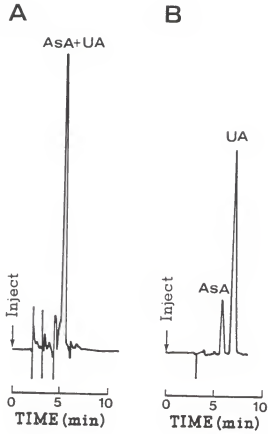

A
AsA+UA
Inject
0
5
10
TIME (min)
B
UA
AsA
Inject
0
5
10
TIME (min)

Figure 3. Typical standard curves for AsA assay in a fish feed, fish meal or fresh fish tissue. (▲) AsA dissolved in 6% aqueous metaphosphoric acid, (●) AsA in a freeze-dried fish meal, (△) AsA in a fresh fish tissue, (○) AsA in a feed.

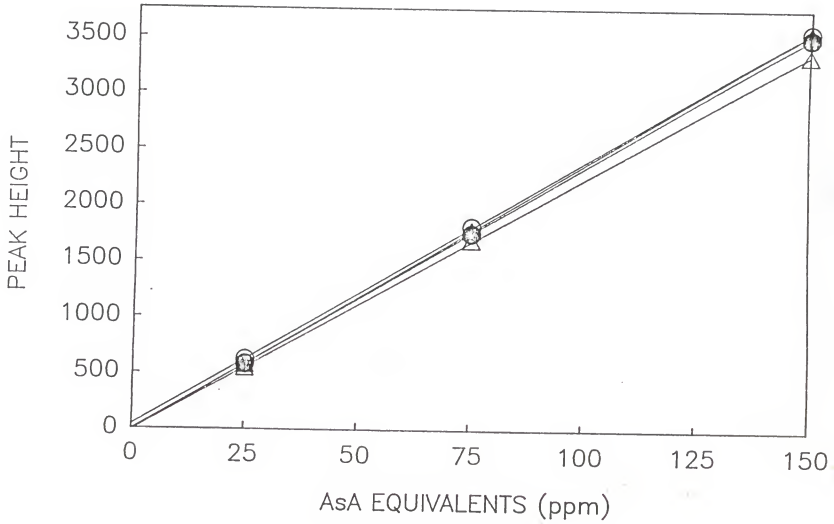

PEAK HEIGHT
3500
3000
2500
2000
1500
1000
500
0
0
25
50
75
100
125
150
AsA EQUIVALENTS (ppm)

Figure 4. Assay procedure for free AsA in a fish feed, fish meal or fresh fish tissue.

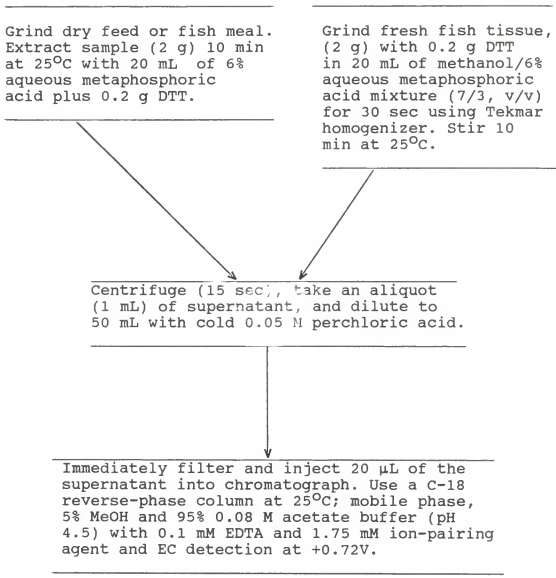

Grind dry feed or fish meal. Extract sample (2 g) 10 min at 25°C with 20 mL of 6% aqueous metaphosphoric acid plus 0.2 g DTT.
Grind fresh fish tissue, (2 g) with 0.2 g DTT in 20 mL of methanol/6% aqueous metaphosphoric acid mixture (7/3, v/v) for 30 sec using Tekmar homogenizer. Stir 10 min at 25°C.
Centrifuge (15 sec), take an aliquot (1 mL) of supernatant, and dilute to 50 mL with cold 0.05 M perchloric acid.
Immediately filter and inject 20 µL of the supernatant into chromatograph. Use a C-18 reverse-phase column at 25°C; mobile phase, 5% MeOH and 95% 0.08 M acetate buffer (pH 4.5) with 0.1 mM EDTA and 1.75 mM ion-pairing agent and EC detection at +0.72V.

Figure 5. LC chromatograms showing release of AsA by acid-catalyzed solvolysis of AsAS. (A) AsAS in 6% aqueous metaphosphoric acid. (B) AsA released from AsAS after acid-catalyzed solvolysis, (C) AsA standard in 6% aqueous metaphosphoric acid, where AsA level is equivalent to AsAS in chromatogram (B).

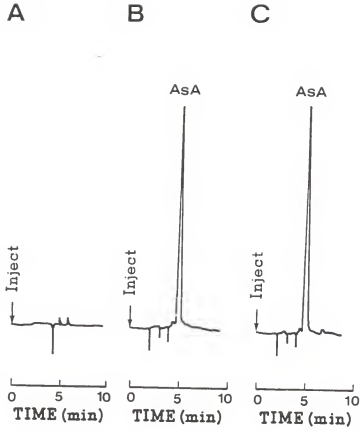

A
B
C
AsA
AsA
Inject
Inject
Inject
0
5
10
0
5
10
0
5
10
TIME (min)
TIME (min)
TIME (min)

Figure 6. Release of AsA by solvolysis of AsAS in 4% aqueous methanol containing 1, 2, or 3% by volume of added acetyl chloride.

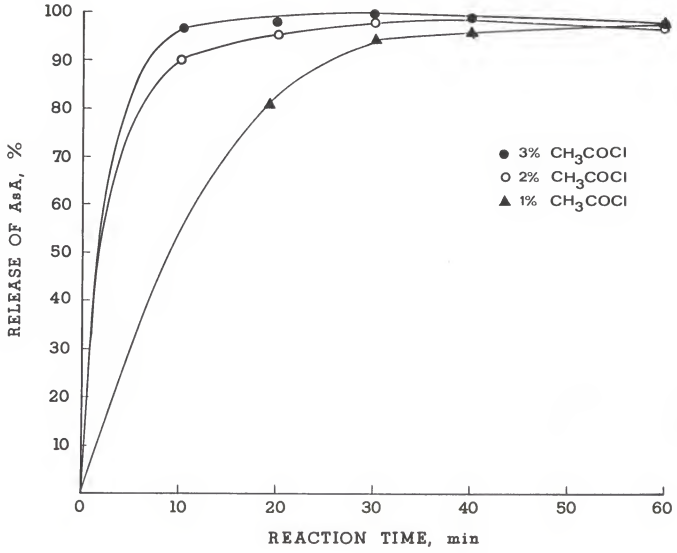

100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
RELEASE OF AsA, %
REACTION TIME, min
● 3% CH3COCl
○ 2% CH3COCl
▲ 1% CH3COCl

Figure 7. Time for 98% release of AsA by solvolysis of AsAS in 0-2% aqueous methanol containing 1, 2, or 3% by volume of added acetyl chloride.

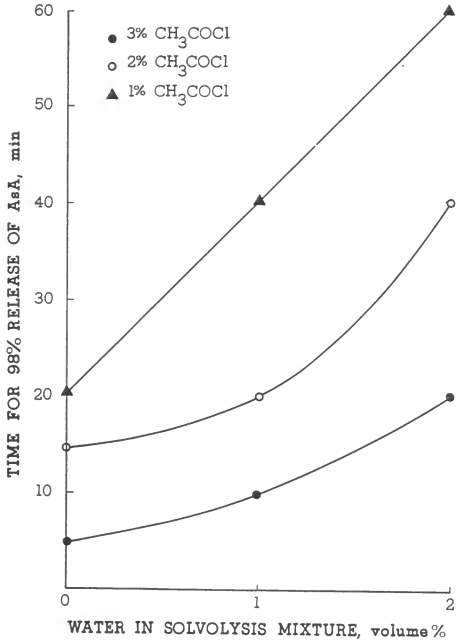

60
50
40
30
20
10
0
1
2
3% CH_3COCl
2% CH_3COCl
1% CH_3COCl
TIME FOR 98% RELEASE OF AsA, min
WATER IN SOLVOLYSIS MIXTURE, volume %

Figure 8. Time for 95% release of AsA by solvolysis of AsAS in 0-8% aqueous methanol with and without addition of trimethyl orthoformate.

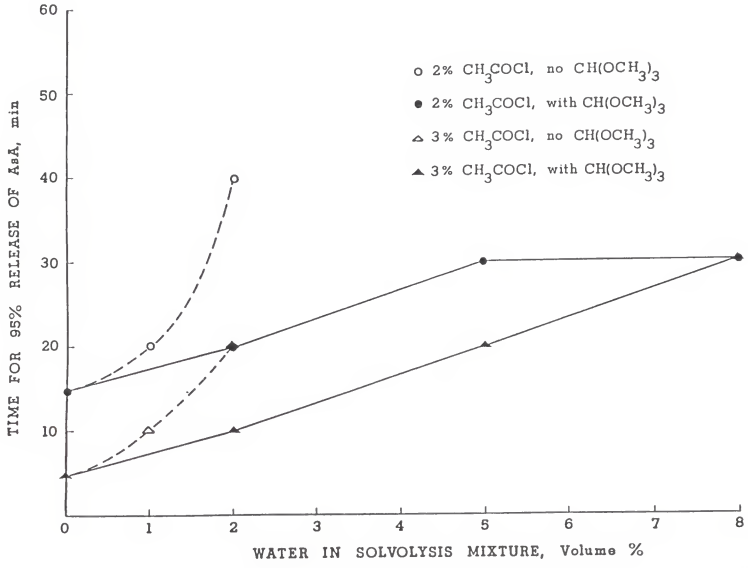

60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
TIME FOR 95% RELEASE OF AsA, min
WATER IN SOLVOLYSIS MIXTURE, Volume %
o 2% CH_3COCl, no $CH(OCH_3)_3$
● 2% CH_3COCl, with $CH(OCH_3)_3$
△ 3% CH_3COCl, no $CH(OCH_3)_3$
▲ 3% CH_3COCl, with $CH(OCH_3)_3$

Figure 9. (A) Chromatogram of the solvolysis reaction mixture containing a high molar ratio (1.6) of trimethyl orthoformate to H_2O (5%), (B) chromatogram of the solvolysis reaction mixture containing a relatively low molar ratio (0.5) of trimethyl orthoformate to H_2O (5%). Peak (X) was assumed to be a formate ester of AsA.

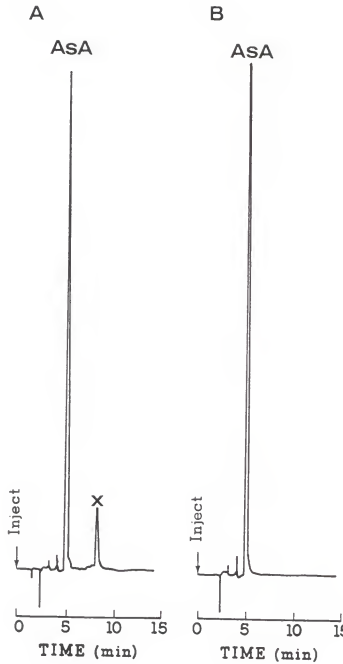

A
AsA
X
Inject
0
5
10
15
TIME (min)
B
AsA
Inject
0
5
10
15
TIME (min)

Figure 10. Assay procedure for total L-ascorbic acid equivalents (AsA + AsAS) in a fish feed, fish meal, or fresh fish tissue.

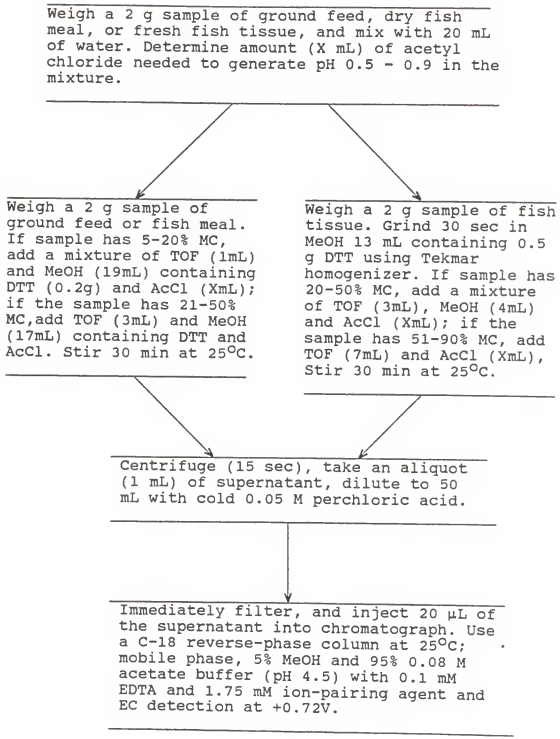

Weigh a 2 g sample of ground feed, dry fish meal, or fresh fish tissue, and mix with 20 mL of water. Determine amount (X mL) of acetyl chloride needed to generate pH 0.5 - 0.9 in the mixture.
Weigh a 2 g sample of ground feed or fish meal. If sample has 5-20% MC, add a mixture of TOF (1mL) and MeOH (19mL) containing DTT (0.2g) and AcCl (XmL); if the sample has 21-50% MC,add TOF (3mL) and MeOH (17mL) containing DTT and AcCl. Stir 30 min at 25°C.
Weigh a 2 g sample of fish tissue. Grind 30 sec in MeOH 13 mL containing 0.5 g DTT using Tekmar homogenizer. If sample has 20-50% MC, add a mixture of TOF (3mL), MeOH (4mL) and AcCl (XmL); if the sample has 51-90% MC, add TOF (7mL) and AcCl (XmL), Stir 30 min at 25°C.
Centrifuge (15 sec), take an aliquot (1 mL) of supernatant, dilute to 50 mL with cold 0.05 M perchloric acid.
Immediately filter, and inject 20 µL of the supernatant into chromatograph. Use a C-18 reverse-phase column at 25°C; mobile phase, 5% MeOH and 95% 0.08 M acetate buffer (pH 4.5) with 0.1 mM EDTA and 1.75 mM ion-pairing agent and EC detection at +0.72V.

Figure 11. LC chromatograms for determination of free AsA and total AsA equivalents (AsA + AsAS) in freeze-dried, whole-body RBT. (A) Free AsA, (B) total AsA equivalents (AsA + AsAS) by solvolysis method, (C) total AsA equivalents when RBT was spiked with AsAS (200 ppm equivalents of AsA).

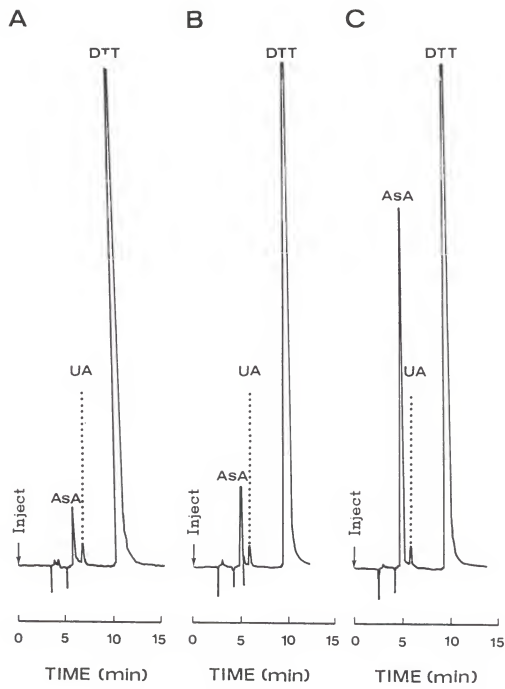

A
B
C
DTT
DTT
DTT
AsA
UA
UA
UA
AsA
Inject
Inject
Inject
0
5
10
15
0
5
10
15
0
5
10
15
TIME (min)
TIME (min)
TIME (min)

Figure 12. Recovery of AsA and AsAS subjected to solvolysis assay method. (●) AsA standard solution in 6% metaphosphric acid, (○) AsA standard subjected to extraction/solvolysis, (△) AsAS subjected to extraction/solvolysis.

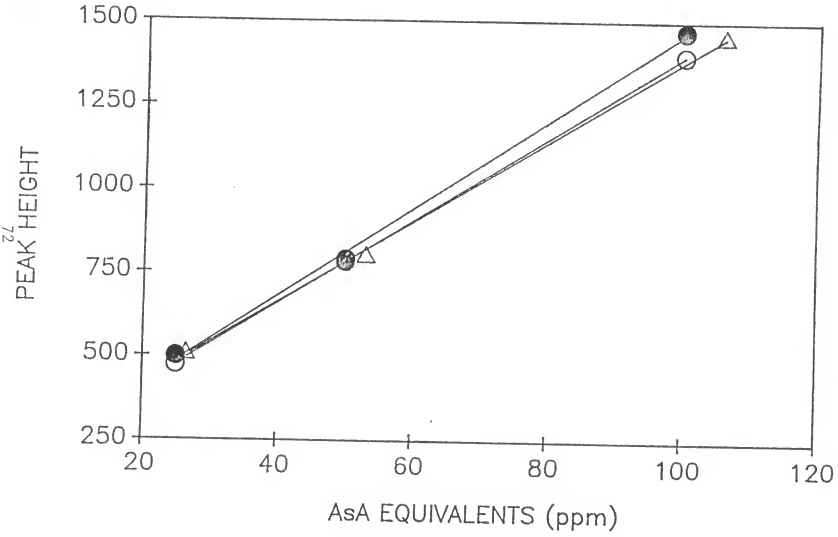

1500
1250
1000
750
500
250
PEAK ^{72} HEIGHT
20
40
60
80
100
120
AsA EQUIVALENTS (ppm)

Figure 13. Typical LC chromatograms of extracts of freeze-dried, whole-body RBT assayed directly for AsAS by LC-EC. (A) 50 ppm AsAS (or 26.5 ppm AsA equivalents) standard in 6% aqueous trichloroacetic acid, (B) extract of freeze-dried, whole-body RBT in 6% aqueous trichloroacetic acid, (C) extract (B) spiked with 100 ppm AsAS (or 53 ppm AsA equivalents), (D) extract (B) spiked with 2,000 ppm AsAS (or 1,060 ppm AsA equivalents).

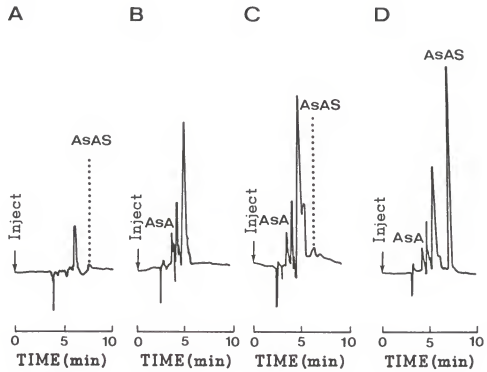
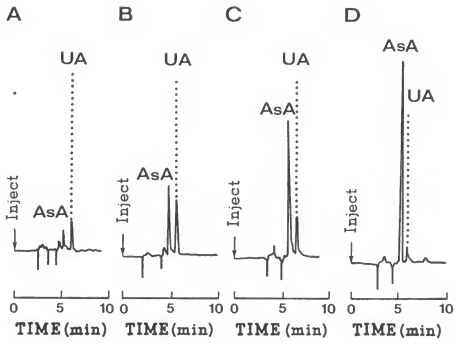

A
B
C
D
AsAS
AsAS
AsAS
AsAS
Inject
Inject
Inject
Inject
AsA
AsA
AsA
0
5
10
TIME (min)
TIME (min)
TIME (min)
TIME (min)

Figure 14. Typical LC chromatograms for assay of AsA and AsAS in fish tissue. (A) Free AsA in freeze-dried, whole-body RBT, (B) total AsA equivalents (AsA + AsAS) in solvolysis reaction mixture on freeze-dried, whole-body RBT, (C) free AsA in fresh RBT liver, (D) total AsA equivalents (AsA + AsAS) in solvolysis reaction mixture on fresh RBT liver.


A
B
C
D
UA
AsA
Inject
0
5
10
TIME (min)

LIQUID CHROMATOGRAPHIC DETERMINATION OF A COMBINED FORM OF L-ASCORBIC ACID (L-ASCORBATE 2-SULFATE) IN FISH FEEDS AND FISH TISSUES BY SOLVOLYTIC RELEASE OF L-ASCORBIC ACID

by

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B.S., WUXI INSTITUTE OF LIGHT INDUSTRY, 1982

AN ABSTRACT OF A THESIS

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Manhattan, Kansas

1988

A new method to determine total L-ascorbic acid (AsA) in aquaculture feed and fish tissue was devised, where total AsA was defined as free AsA plus AsA released by acid-catalyzed solvolysis. The combined form of ascorbate was presumed to be L-ascorbate 2-sulfate, which was shown to be quantitatively converted to AsA by acid-catalyzed solvolysis.

In the new method, a sample was subjected to acid-catalyzed extraction/solvolysis, and total AsA was determined using liquid chromatography under pressure with electrochemical detection (LC-EC). All chromatograms showed base-line resolution of AsA. The level of AsAS in a sample was calculated by the difference between total and free AsA, which was determined in a separate assay using LC-EC.

The new method gave 94-101% recovery of AsA and AsAS (25-100 ppm AsA equivalents) added to fish feed and fish tissue varying in MC 5-90%. Replication of the new assay method on three types of fish feed and fish tissue gave 0.8% coefficient of variation on AsAS levels >25 ppm. The coefficient of variation increased to 7.8% at 8 ppm of AsAS.

Several fish tissues were assayed for free and total AsA. Liver from rainbow trout (RBT) was found to contain 40, 60, 106, and 123 ppm AsA and 5, 14, 6, and 16 ppm AsAS (AsA equivalents on wet basis) when fish were fed diets

with 20, 40, 60, and 100 ppm L-ascorbate 2-polyphosphate (AsA equivalents). The highest level of AsAS (452 ppm AsA equivalents on dry basis) was found in brine shrimp cysts.

Free and total AsA were also determined in fish feed and fish tissue by the modified Roe-Kuether method. Free AsA levels determined by the modified Roe-Kuether and the LC-EC methods were in agreement, but in our laboratory the modified Roe-Kuether method gave high values of total AsA due to opacity in the colored reaction mixture and possibly interference from sugars. Thus, the levels of AsAS in fish tissue estimated by the modified Roe-Kuether procedure were at least one order of magnitude greater than those determined using the solvolysis method.