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Journal of Chromatography A, 803 (1998) 141–145

JOURNAL OF CHROMATOGRAPHY A

Assay of L-ascorbic 2-monophosphate in aquaculture feeds by high-performance anion-exchange chromatography with conductivity detection

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Received 23 September 1997; received in revised form 28 November 1997; accepted 28 November 1997

Abstract

L-Ascorbic acid 2-monophosphate (AsMP) was extracted from aquaculture feeds using deaerated, cold water, and the cold extract was subjected immediately to high-performance anion-exchange chromatography with conductivity detection (HPAEC–CD) using gradient elution. The chromatograms of the extracts from three aquaculture feeds showed baseline resolutions of AsMP and recoveries of added AsMP of more than 95%. The detection limit was about 2 ng AsMP (free acid form), which was equivalent to a level of AsMP in feed of 5 mg/kg. The assay for AsMP was direct, rapid, simple, and accurate. © 1998 Elsevier Science B.V.

Keywords: Aquaculture feed; L-Ascorbic acid 2-monophosphate

1. Introduction

Fish and shellfish are unable to produce L-ascorbic acid (AsA) because they lack L-gluconoactone oxidase in their renal and hepatic organs [1]. Feeds formulated for those animals under culture must contain vitamin C; however, AsA is unstable during the processing and storing of aquaculture feeds [2]. On the other hand, the 2-phosphate or 2-polyphosphate esters of AsA are one to two orders of magnitude more stable than AsA and 100% bioavailable to guinea pigs, monkeys, and fish [2].

The 2-polyphosphorylated ester of L-ascorbate is a variable mixture of mostly L-ascorbate 2-triphosphate (AsTP), 2-diphosphate (AsDP), and 2-monophosphate (AsMP) [3]. Direct assays of AsTP, AsDP, and/or AsMP in a feed are difficult because of their low concentrations and the frequent presence in feed of substances that interfere with detection. Recently, two direct assay methods for AsMP have been published based on high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection. Sakai et al. [4] extracted fish tissue with 5% metaphosphoric acid, then separated AsMP using a reversed-phase column with a mobile phase at pH 2.2 containing phosphoric acid and octylamine. Those workers reported baseline resolution of AsMP and a detection limit of 0.1 μg/g tissue. Khaled [5] used 1% metaphosphoric acid containing 0.2% dithiothreitol to extract AsMP from an aquatic feed, and separated AsMP on two reversed-phase columns with a mobile phase of 0.1 *M* acetate buffers (pH 5.0) containing *n*-octylamine and ethyl-

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0021-9673/98/$19.00
PII S0021-9673(97)01230-2

enediaminetetraacetate. Baseline resolution of AsMP was achieved.

High-performance anion-exchange chromatography with conductivity detection (HPAEC–CD) with chemical suppression is a direct and sensitive technique to assay ionic molecules [6–9]. The objective of this study was to develop a method to separate and quantify AsMP by HPAEC-CD and to apply the method to three aquaculture feeds.

2. Experimental

2.1. Materials

Deionized water, purified with a Mill-Q system (Millipore, Bedford, MA, USA), was used for the preparation of eluting solutions, standard solutions, and feed sample extractions. Deaerated cold water was prepared by boiling for 5 min and quickly cooling to 4°C; it was used immediately. An oxygen-specific electrode (Orion, model 97-08-00) showed that the deaerated, cold water contained ~3.1 ppm oxygen compared to 9.0 ppm for untreated water. When the boiled water was purged with nitrogen gas during cooling, the water at 25°C contained 1.5 ppm of oxygen.

All chemicals were analytical-reagent grade. Sodium hydroxide (50% solution) was from Fisher Chemical Company (St. Louis, MO, USA); AsMP magnesium salt (minimum 85% purity), L-ascorbate 2-sulfate (AsS) dipotassium salt, and potato acid phosphatase (5.1 units/mg solid) were purchased from Sigma Chemical Company (St. Louis, MO, USA). The calculated concentrations of AsMP throughout this work are in its free-acid form.

Three aquaculture feeds fortified with L-ascorbate 2-polyphosphate concentrate ('Rovimix STAY-C', Roche, Basel, Switzerland) were obtained from a commercial feed mill. The feeds were ground for 30 s in a small attrition mill at 20 000 rpm and were found to contain 5.5–8.1% moisture content when dried at 105°C for 3 h. Wheat and corn flours were purchased from a local supermarket.

2.2. HPAEC–CD operating parameters

The system was equipped with a gradient pump (GMP-2), a pulsed electrochemical detector in the conductivity mode (Dionex, Sunnyvale, CA, USA), an injector valve (Rheodyne 7010, Cotati, CA) with a 20-μl volume, a Dionex AS-11 analytical column (4×250 mm), an AG-11 guard column (4×50 mm), and an anion self-regenerating suppressor (ASRS-1). An anion trap column (ATC-1, 9×24 mm) from Dionex was installed between the pump and injection valve to minimize interference from anionic impurities in the eluting solution.

Anions were eluted at 2 ml/min with a linear gradient of 20–80 m*M* NaOH in 10 min, which was generated from 200 m*M* NaOH and water. Immediately after gradient elution, the columns were flushed with 100 m*M* NaOH for 5 min and then equilibrated with 20 m*M* NaOH for 10 min before injection. The sensitivity of the conductivity detector was set at 5 μS.

2.3. Preparation of feed extract

An aquaculture feed (1.0 g, dry basis) was extracted in deaerated, cold water (30 ml) at 4°C by shaking for 3 min. As rapidly as possible, the pH of the dispersion was adjusted to 4.8 with 0.01 M HCl, and the total weight of the dispersion was made to 50.0 g by adding deaerated, cold water. Then the dispersion was filtered through a 0.25-μm syringe filter (Supor Acrodisc 13, Gelman Sciences, Ann Arbor, MI, USA), and an aliquot (20 μl) of the filtrate injected into the HPAEC system.

2.4. Standard curves and recovery tests

Standard curves for the determination of AsMP in feed samples were established in phosphatase-digested feed extracts. Phosphatase (0.5 mg) was mixed with an aliquot (5 ml) of a feed extract at pH 4.8, and the mixture was incubated for 1 h at 37°C followed by heat treatment at 100°C for 5 min. The AsMP was added to the digested extract to give a final concentration in the range of 0.1–10 μg/ml (free acid form of AsMP). The mixture containing standard AsMP or a blank mixture was filtered through a 0.25-μm syringe filter and injected into the chromatographic system.

The AsMP solution in deaerated, cold water (50 ml containing 2 or 5 μg/ml) was mixed with a feed

sample of 1.0 g, which corresponded to an addition of 100 or 250 mg of AsMP/kg of feed. The assay procedure given above was followed, and recovery (%) was calculated from the AsMP contents in the unspiked and spiked feed samples. All assays were done in triplicate.

3. Results and discussion

3.1. Standard AsMP

The aqueous extract of a feed was adjusted to pH 4.8 because that is the optimum for activity of acid phosphatase. Phosphatase was used to eliminate AsMP in a feed extract as needed (see below).

A preliminary investigation was made on the stability of AsMP at pH 4.8. Fig. 1A shows a chromatogram of a standard solution of AsMP (10 μg/ml) dissolved in deaerated water at 4°C and adjusted to pH 4.8. The peaks with retention times of less than 1.5 min were mainly chloride ions from pH adjustment and possibly other unknown anions contaminating the glassware. The peak at 4.1 min was AsMP, and the minor peak at 2.6 min was orthophosphate.

The AsMP that had been solubilized in deaerated, cold water without pH adjustment showed the same peak responses for AsMP and orthophosphate (data not shown) as the AsMP dissolved at pH 4.8, indicating that the orthophosphate detected in Fig. 1A was not due to hydrolysis of AsMP but instead was a contaminant in the AsMP sample. In addition, the AsMP solution at pH 4.8 did not show any hydrolysis when it was heated at 100°C for 5 min or stored for 2 days in a transparent bottle at room temperature (data not shown).

On the other hand, AsMP that was dissolved in unboiled water did show hydrolysis of about 8, 22, 68, and 100% after storage for 1, 2, 3, and 4 days, respectively, at room temperature (data not shown). These results suggest that the dissolved oxygen in water caused oxidative dephosphorylation of AsMP, and that removal of dissolved oxygen by preboiling the extraction water was needed to prevent loss of AsMP during analysis. Oxidative desulfation of AsS by oxygen has been reported [10], although we found that AsS was stable under the identical conditions where AsMP was partially lost.

Phosphatase digestion [3,4,9] was used to identify and to remove the AsMP peak in the HPLC analysis [4,5,11]. We determined that the AsMP dissolved in 5 ml of water at 20 μg/ml was digested completely with 1 mg phosphatase at room temperature within 5 min. Fig. 1B shows complete disappearance of AsMP and release of an equivalent level of orthophosphate in that digest.

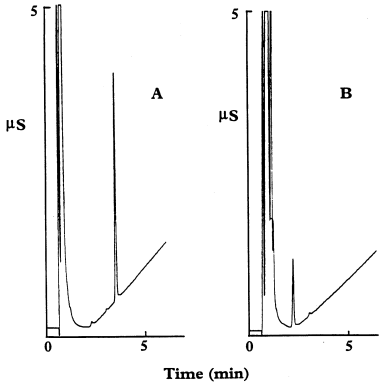

Fig. 1. High-performance anion-exchange chromatograms with conductivity detection (HPAEC–CD). (A) Standard AsMP; (B) standard AsMP after digestion with phosphatase.

3.2. Aquaculture feeds

Fig. 2 shows the chromatograms of the aqueous extracts from three aquaculture feeds. All the chromatograms had a peak at retention time 4.1 min, corresponding to that of AsMP shown previously in

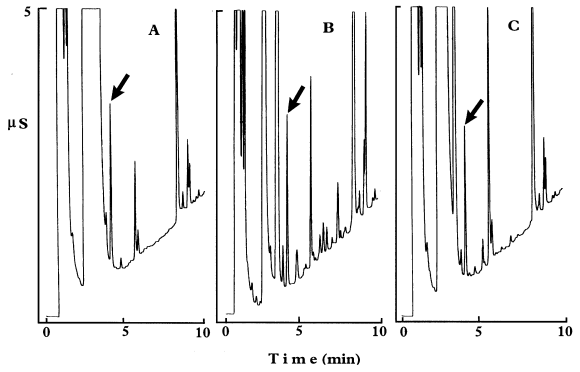

Fig. 2. Chromatograms of the extracts of feed samples A, B, and C. The arrow indicates AsMP.

Fig. 1A. In other feed extracts, should resolution of AsMP not be achieved with the gradient of hydroxide ion used here, a less steep gradient would be advised.

Some aquaculture feeds, especially those containing ingredients from wheat and corn, may retain phosphatase activity in spite of processing. When AsMP at 20 μg/ml (25 ml) was stirred with wheat or corn flour (1.0 g) at room temperature, the AsMP peak disappeared within 5 min of stirring (data now shown). To investigate if the three feed samples contained phosphatase, a chromatographic analysis was made on the extracts. Holding the extracts of the feeds at 37°C for 1 h resulted in no loss of AsMP from feed sample A (compare peaks at R_T=4.1 min in Fig. 2A and Fig. 3A). However, decreases of about 52 and 22% of the peak responses at R_T=4.1 min were observed for feed samples B and C, respectively (compare Fig. 2B and Fig. 3B; and Fig. 2C and Fig. 3C). Therefore, cold water and rapid preparation (about 5 min) of feed extracts were used to limit phosphatase activity.

Fig. 4 shows the chromatograms of the feed extracts after digestion with phosphatase. The peak corresponding to AsMP essentially disappeared in all three chromatograms, suggesting that AsMP in feeds can be assayed with baseline resolution. Other peaks with retention times longer than 4.1 min for AsMP also disappeared or became smaller after phosphatase digestion. Some of the peaks may be higher phosphate esters of AsA [3] or other phosphated ingredients. The retention times of pyrophosphate, tripolyphosphate, AsDP, and AsTP, which are formed during reaction of AsA with sodium trimetaphosphate [3], were about 5.6, 6.4, 6.1, and 7.8 min, respectively [12]. In this study, however, we did not attempt to identify or quantify the inorganic phosphates and phosphate esters of AsA other than AsMP.

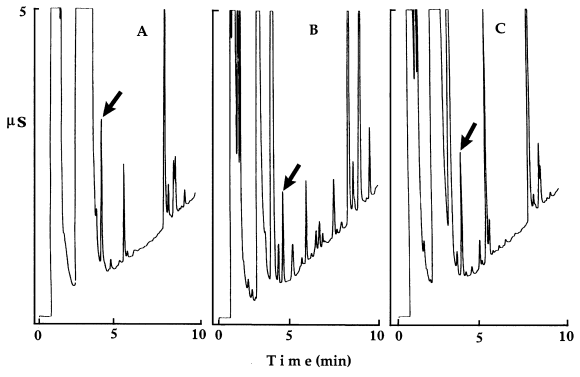

Fig. 3. Chromatograms of extracts of feed samples A, B, and C after holding the extracts for 1 h at 37°C. The arrow indicates AsMP.

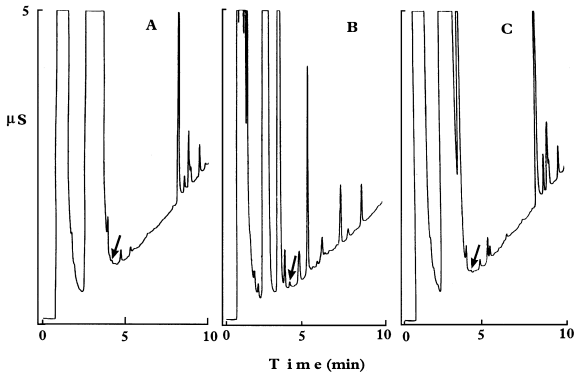

Fig. 4. Chromatograms of extracts of feed samples A, B, and C after digestion with phosphatase for 1 h at 37°C. The arrow indicates AsMP.

3.3. Standard curves and recovery tests

Standard curves were prepared for AsMP to assay its content in the aquaculture feeds. For each feed, the relationship between peak response (height) and AsMP concentration was found to be linear (regression coefficient=0.99) over the 0.1–10 μg/ml concentration range. Using the standard curves, AsMP contents of the feeds were determined (Table 1).

Because the blank aquaculture feeds without addition of phosphorylated AsA were not available,

Table 1
Levels of AsMP in three aquaculture feeds and recoveries of added AsMP

Feed sample	AsMP in feed (mg/kg)		
	As received	AsMP added	Recovery (%)
A	349±10	100	98±4
		250	97±6
B	386±12	100	94±7
		250	96±7
C	313±10	100	101±6
		250	99±6

recovery tests were performed on feed samples by adding known amounts of AsMP (100 and 250 mg/kg of sample). The results in Table 1 indicate that satisfactory recoveries were achieved for the three feed samples. Even though feeds B and C had phosphatase activity as previously shown in Fig. 3B and C, the recoveries of added AsMP from those two feeds were not significantly different than that from feed A, indicating that the use of cold water and rapid extraction and injection circumvented dephosphorylation of AsMP by oxygen and enzyme.

3.4. Detection limit

The signal-to-noise ratio of the conductivity detector was adequate with a 20-μl injection of a standard solution of AsMP in water at a concentration of 0.1 μg/ml. Under the assay conditions used in this study, the detection limit of AsMP in a feed was calculated to be about 5 mg/kg. Thus, the quantification of AsMP by conductivity detection appears to be similar to that by UV detection [4,5,11].

3.5. Separation of AsMP and AsS

A known mixture of AsMP and AsS was separated readily by the HPAEC–CD method, except isocratic elution with 20 m*M* NaOH replaced gradient elution. The retention times of AsMP and AsS were 5.4 and 4.7 min, respectively (data not shown). L-Ascorbate 2-sulfate is a metabolite of AsA that occurs in fish tissue [5], and AsMP also has been reported in tissue [4]. The time to separate pure AsMP and AsS using HPAEC-CD was shorter than the 20 min reported [4] for reversed-phase, HPLC-UV assay of AsMP in tissue. The time for possible simultaneous assay of those two esters in biological samples remains to be determined.

4. Conclusions

An anion-exchange chromatographic method is available to separate and assay AsMP in aquaculture feeds and probably could be used on other feeds and foods. The same chromatographic system gives baseline separations of AsMP and AsS in water.

Acknowledgements

Contribution No. 98-85-J of the Kansas Agricultural Experiment Station.

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