Liquid Chromatographic Determination of Vitamin C in Aquatic Organisms

H.J. Nelis* and **A.P. De Leenheer†**
Laboratoria voor Medische Biochemie en voor Klinische Analyse, Harelbekestraat 72, B-9000 Gent

G. Merchie, P. Lavens, and **P. Sorgeloos**
Laboratory of Aquaculture & Artemia Reference Centre, Rozier 44, B-9000 Gent, Belgium

Abstract

A reversed-phase ion-pair liquid chromatographic method using electrochemical detection and internal standardization with isoascorbic acid (IAA) is described for the determination of ascorbic acid (AA) and dehydroascorbic acid (DHAA) in aquatic organisms. Several extractants are compared with regard to recovery and stability of AA and DHAA, including 5% metaphosphoric acid (MPA)–0.54mM ethylenediaminetetraacetic acid (EDTA), 1% acetic acid (Hac)–1mM EDTA, 1% Hac–0.1% MPA–1mM EDTA, 2mM homocysteine–1mM EDTA, and water–methanol (70:30, v/v) containing 0.7mM EDTA. The two nonacidic extractants afford insufficient protection of AA (water–methanol–EDTA) or DHAA (homocysteine–EDTA). High ionic strength acidic mixtures (e.g., 5% MPA) may be associated with a negative drift in the retention times of AA and IAA. Hac (1%)–EDTA (1mM) yields lower recovery except when supplemented with MPA. A mixture of 1% Hac–0.1% MPA–1mM EDTA is useful in extracting AA from the brine shrimp *Artemia, Brachionus*, fish eggs, fish larvae, and postlarvae of shrimp as part of its routine high-performance liquid chromatographic determination in these matrices.

Introduction

The biological relevance of vitamin C in fish and shrimp is well-documented (1,2). This nutrient is essential for the formation of cartilage and bone, probably plays a role in reproduction, and reportedly improves the resistance of fish against stress and infectious diseases. Vitamin C deficiency leads to the typical symptoms of fish scurvy including spinal deformities, hemorrhages, and hyperplasia of gills (1).

As part of studies in our laboratories on the biological effects of vitamin C in fish and shrimp, there has been a need for an analytical method to quantitate ascorbic acid (AA) and its oxidative metabolite, dehydroascorbic acid (DHAA). This method should reconcile the requirements of good analyte recovery and stability with those of sensitivity, speed, and particularly routine applicability.

Many nutritional studies on vitamin C in fish have relied on spectrophotometric analysis using the dinitrophenylhydrazine method (3–5), which is subject to interferences (1). A fluorometric method, originally developed for foods (6), has also been commonly applied to fish (7). Numerous high-performance liquid chromatographic (HPLC) methods have been published for the determination of vitamin C in biological fluids, foods, various animal tissues, and fruit juices (for reviews, see references 8 and 9). In contrast, only a few have been reported specifically for the quantitation of vitamin C in whole aquatic organisms, including fish and crustaceans (10–15). Among these, the method of Wang and Seib (13) based on reversed-phase ion-pair chromatography and electrochemical detection has found the widest application in fish nutrition studies (16–20). Sample pretreatment in HPLC procedures for vitamin C consists of a simple extraction with metaphosphoric acid (MPA) (13,14), trichloroacetic acid (15), perchloric acid (10,11), or MPA–acetic acid (Hac) (12), followed by the direct injection of an aliquot of the extract, as it is or after dilution (13).

However, strong acids with high ionic strength may interfere with the chromatography of AA, causing a drift in its retention time, baseline problems, or extraneous peaks.

In this paper, we report milder methods for the extraction of AA in connection with its quantitation in *Artemia, Brachionus*, fish, and shrimp by liquid chromatography (LC) with electrochemical detection and internal standardization using isoascorbic acid (IAA).

Experimental

Reagents

AA, IAA, and DHAA were from Sigma (St. Louis, MO), Janssen Chimica (Beerse, Belgium), and ICN (Costa Mesa, CA), respectively. Dodecyltriethylammonium phosphate (DTAP)

*Present address: Laboratorium voor Farmaceutische Microbiologie, Harelbekestraat 72, B-9000 Gent, Belgium.
† Author to whom correspondence should be addressed.

came from Regis Chemical (Morton Grove, IL). Stock solutions of AA and IAA were prepared in water containing 2mM homocysteine and 1mM disodium EDTA (further referred to as EDTA).

Biological samples

Live food organisms (*Artemia* larvae and *Brachionus*) were cultured in the Artemia Reference Centre (Gent, Belgium) according to standard procedures (21). Turbot eggs and hatchery-phase European sea bass were obtained from the commercial hatcheries France Turbot (Ile de Noirmoutier, France) and Cenmar (Zadar, Croatia), respectively. Larviculture of the former species was done in the Artemia Reference Centre (Gent, Belgium). Milkfish eggs and larvae were acquired from the Southeast Asian Fisheries Development Centre (Aquaculture Department, Iloilo, Philippines). European sea bass larvae (36 days old) and white-legged shrimp postlarvae (PL-stage 10) were purchased from the commercial hatcheries of Sepia International (Gravelines, France) and Shrimp Culture Technologies (Ft. Pierce, FL), respectively. Their nursery-phase rearing was performed at the Artemia Reference Centre.

Extraction procedures

Approximately 100 mg (wet weight) of sample was homogenized in 1.5 mL of extractant using a Potter-Elvehjem (Egilabo, Belgium) tube or a Polytron (Kinematica, Lucerne, Switzerland) mixer. The following extractants were used: 5% MPA–0.54mM EDTA; 1% Hac–0.1% MPA–1mM EDTA; 1% Hac–1mM EDTA; 2mM homocysteine–1mM EDTA; and water–methanol (70:30, v/v) containing 0.7mM EDTA. IAA was added as an internal standard. After centrifugation of the homogenate at 1500*g* for 2 min, the supernatant was isolated, and the residue was rehomogenized in 1.5 mL of extractant. The Potter tube was finally rinsed twice with 750 µL of extractant, and all supernatants were combined.

The mixture was centrifuged for 5 min at 12000*g* and passed through a Bond Elut C_{18} cartridge (Varian, Harbor City, CA) that had been preconditioned successively with 1.5 mL each of methanol, water, and the extractant. The cartridge was rinsed with 0.5 mL of extractant, and the final volume of the extract was adjusted to 5 mL in a volumetric flask. If the final solution was turbid, it was filtered over a 0.45-µm pore size Gelman Acro-disc LC1 3PVDF filter (Gelman Sciences, Ann Arbor, MI). A 20-µL aliquot was injected on the column.

For the determination of DHAA, 0.5 mL of the extract in Hac–0.1% MPA–1mM EDTA was mixed with 0.5 mL of a 0.5–3% (w/v) solution of homocysteine. The pH was adjusted to 7.5 with the aid of 1M dipotassium phosphate (dibasic). After 10 min standing at room temperature and adjustment of the pH to 1.6 with 1M phosphoric acid, a 20-µL aliquot was injected.

Chromatography

The HPLC apparatus consisted of a Varian 8500 pump (Varian Associates, Palo Alto, CA), a 7125 Rheodyne valve injector fitted with a 20-µL loop (Rheodyne, Cotati, CA), and an Antec SN20 amperometric detector (Antec, Leiden, The Netherlands). A 5-µm Hypersil ODS column (15 × 0.46 cm) (Shandon, Runcorn, UK) equipped with a 5 × 0.3-cm Chromguard guard column (Chrompack, Middelburg, The Netherlands) was eluted with 0.04M sodium acetate containing 0.54mM EDTA and 1.5mM DTAP at pH 4.8. The flow rate was 1 mL/min, and the temperature was ambient. Detection was carried out in the oxidation mode at an electrode potential of +0.72 V.

Quantitation and method validation

For standardization and the determination of recovery, known amounts of AA were added to homogenates of dry *Artemia* larvae in which all vitamin C had been destroyed by prolonged heating. Standard curves were constructed by plotting peak height ratios (AA versus IAA) against the respective concentration of AA. Levels in samples were calculated per gram of dry mass. To determine the latter, 3–5 additional 200-mg (wet weight) samples were taken simultaneously with

Table I. Reproducibility of the Repeated Determination of AA in *Artemia* Larvae*

Extractant	Mean (µg/g)	SD (µg/g)	RSD (%)	Replicates
5% MPA–0.54mM EDTA	526.8	34.4	6.5	8
2mM homocysteine–1mM EDTA	561.5	24.6	4.4	8
1% Hac–0.1% MPA–1mM EDTA	585.0	50.5	8.6	8
Water–methanol (7:3, v/v)–1mM EDTA	664.6	62.7	9.4	8

* No DHAA detected.

Table II. Effect of Different Extractants on the Recovery of AA from Spiked *Artemia* Larvae*

Extractant	Recovery (%)	SD (%)	Replicates	*t*-test (probability)
Concentration of AA in larvae: 2.4 µg/mL (except where indicated)				
5% MPA–0.54mM EDTA	82.7	2.0	4	–
1% Hac–0.1% MPA–1mM EDTA	86.2	2.1	9	0.017 (S)
1% Hac–1mM EDTA	76.4	3.8	8	0.012 (S)
2mM homocysteine–1mM EDTA (3.8 µg/mL)	73.0	3.5	6	0.001 (S)
Water–methanol (70:30, v/v)–0.7mM EDTA (4.3 µg/mL)	85.0	3.5	6	0.27 (NS)
Concentration of AA in larvae: 0.47 µg/mL (except where indicated)				
5% MPA–0.54mM EDTA	62.3	3.8	6	–
1% Hac–0.1% MPA–1mM EDTA	68.1	3.3	6	0.009 (S)
1% Hac–1mM EDTA	59.9	16.1	6	0.72 (NS)
2mM homocysteine–1mM EDTA (0.38 µg/mL)	69.3	4.2	6	0.013 (S)
Water–methanol (70:30, v/v)–1mM EDTA (0.43 µg/mL)	59.0	7.0	6	0.94 (NS)

* Significance of difference with reference to the recovery in 5% MPA–0.54mM EDTA. S = Significant; NS = not significant (α level = 0.05).

the ones to be analyzed by HPLC. After drying at 60°C for 24 h in aluminium cups and cooling in a desiccator, the samples were reweighed, and the water content was calculated. For determination of the recovery, absolute peak heights of AA obtained on spiked samples were compared with those provided by blank sample extracts to which the AA was added just before injection. Reproducibility was evaluated by repetitively analyzing fresh *Artemia* larvae from the same batch.

Results and Discussion

The HPLC system in this paper was adopted from Kutnink et al. (22), with the exception of the column. These investigators reported improved compatibility of a mobile phase containing the ion-pairing agent DTAP with the injection of extracts in MPA, one of the most widely used extractants and stabilizers for AA. Wang and Seib (13) and Hoffman et al. (14) found MPA to be a satisfactory agent to extract AA from fish and shrimp tissue, respectively. However, to minimize the undesirable phenomena mentioned earlier, extracts in this high-acidity, high-ionic strength medium are often extensively diluted before injection (13,22,23), thus compromising the sensitivity of the method. The use of 5% MPA–0.54mM EDTA to extract AA from *Artemia* larvae in conjunction with our HPLC conditions afforded good linearity (the regression coefficient was greater than 0.999; range, 0.4–15 µg/mL), recovery (62.3 ± 3.8%, corresponding concentration = 0.5 µg/mL, six replicates; and 82.7 ± 2.0%, corresponding concentration = 2.4 µg/mL, four replicates), and reproducibility (Table I). However, the occasional observation of a negative drift in the retention time of AA (–16% over a period of 6 h or 24 injections) and IAA (–14.9%) caused concern over the routine applicability of this extractant. This prompted us to investigate milder (i.e., less acidic) low-ionic strength mixtures.

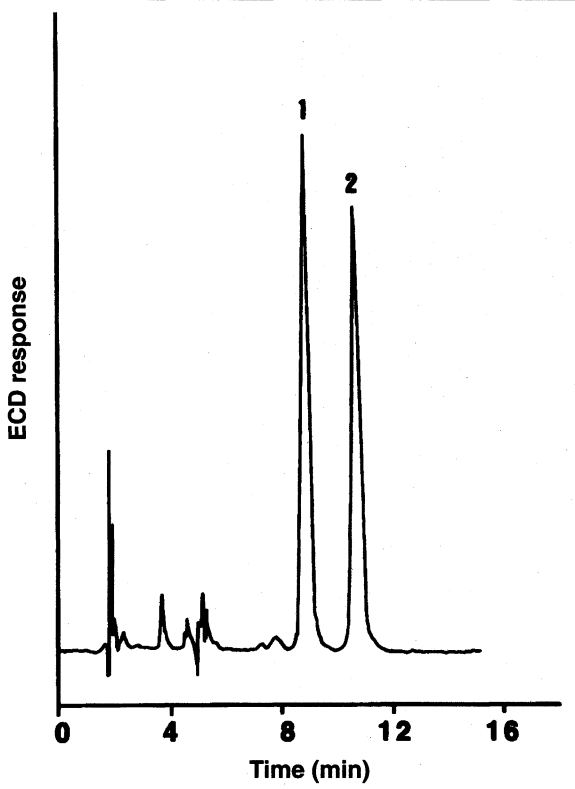

Figure 1. Representative chromatogram of an extract of *Artemia* larvae in 1% Hac–0.1% MPA–1mM EDTA. Peaks: 1, AA; 2, IAA (internal standard). Chromatographic conditions: column, 5-µm 15 x 0.46-cm Hypersil ODS preceded by a 5 x 0.3-cm Chromguard column, eluent, 0.04M sodium acetate containing 0.54mM EDTA and 1.5mM DTAP, pH 4.75; flow rate, 1 mL/min; detection, amperometry at + 0.72 V.

A dilute solution of homocysteine in combination with EDTA (pH 4) (24) and mixtures of methanol–water–EDTA (25) has been proposed as a nonacidic extractant for biological materials other than fish or shrimp. Homocysteine effectively prevents the oxidation of AA and, at pH 4, allegedly does not reduce DHAA to AA (24). In our study, no degradation of AA was observed in 2mM homocysteine–1mM EDTA at 0°C over a time period of 6 h. A water–methanol mixture (70:30, v/v) containing EDTA has been purported to maintain AA stability at room temperature for 4 h (25). In this solution containing 0.7mM EDTA, the peak height of AA was found to be reduced by 15% after 6 h of storage in ice. Both nonacidic extractants afforded excellent linearity over the concentration range of 0.4–15 µg/mL, with correlation coefficients exceeding 0.999, as well as satisfactory reproducibility (Table I). However, the recovery of AA from *Artemia* larvae in homocysteine–EDTA, unlike in water–methanol–EDTA, was lower than that in MPA, as shown in Table II. Furthermore, when blank *Artemia* supplemented with a low quantity of DHAA (0.2 µg/mL) in the absence of AA, was extracted with 2mM homocysteine 1mM–EDTA, approximately 13% was converted to AA, despite the unfavorable pH. Many of the analyzed fish and *Artemia* samples did not show an increase in the peak height of AA after the pH of the extract was adjusted to 7.5, indicating the virtual absence of DHAA.

Hac has been advocated as an efficient stabilizer of AA (26). However, the recovery of AA from *Artemia* larvae in 1% Hac was significantly lower than in MPA (76.4% ± 3.8 versus 82.7% ± 2.0, corresponding concentration = 2.4 µg/mL) but increased with the addition of a small quantity (0.1%) of MPA to 86.2% ± 2.1. Table II shows a comparison of the recoveries in 5% MPA, 1% Hac, 1% Hac–0.1% MPA, and in the two nonacidic extractants. EDTA was always added as an extra stabilizing agent, although its chelating activity at acidic pH is weak. For mixtures of Hac–MPA, the recovery of AA remained virtually constant over the concentration range of 0.1–1% MPA, whereas concentrations below 0.1% resulted in decreased values (results not shown). Unlike 5% MPA, the other four extractants left the retention times of AA and IAA virtually unaffected upon repetitive injection. For MPA, the progressive negative drift observed in one experiment could not be reproduced in another one, suggesting that the effect possibly depended on the degree of contamination or the age of the column (or both). AA remained stable both in 1% Hac–0.1% MPA–1mM EDTA and in 5% MPA–0.54mM EDTA for at least 6 h upon storage in ice. The variability (relative standard deviation [RSD]) in peak height ratios over this time period was 5.3% (Hac–MPA–EDTA) and 6.7% (MPA–EDTA), respectively. A

typical chromatogram of an extract of *Artemia* larvae is depicted in Figure 1. Good linearity (regression coefficient greater than 0.999) was demonstrated with this approach over the concentration range of 0.4–15 µg/mL. The reproducibility of the procedure is illustrated in Table I. Based on the near equivalency in performance of 5% MPA and 1% Hac–0.1% MPA, the latter milder mixture was chosen as the standard extractant for routine analyses.

DHAA can be determined indirectly after reduction to AA with homocysteine at pH 7.5 (27). To demonstrate the linearity of this approach, blank *Artemia* larvae were supplemented with AA (0.4–15.8 µg/mL) and DHAA (0.1–2.8 µg/mL) and peak height ratios were plotted versus the concentration of AA (before reduction with homocysteine) and "total" AA (after reduction) to yield correlation coefficients greater than 0.999 in both cases. No DHAA was detected in the *Artemia* larvae that were used for the evaluation of the reproducibility of the AA determination.

Conclusion

Using the methods described in this paper, AA and (if applicable) DHAA were routinely quantitated in live food organisms (the brine shrimp *Artemia* and *Brachionus*), various fish species (turbot, European sea bass, milkfish), and postlarvae of white shrimp (21,28, G. Merchie, P. Lavens, J. Verreth, F. Ollevier, H. Nelis, A.P. De Leenheer, V. Storch, and P. Sorgeloos. The effect of supplemental ascrobic acid in enriched live food for *Clarias gariepinus* larvae at startfeeding. *Aquaculture*, in press).

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References

1. J.E. Halver, R.R. Smith, B.M. Tolbert, and E.M. Baker. Utilization of ascorbic acid in fish. *Ann. N.Y. Acad. Sci.* **258:** 81–102 (1975).
2. K. Sandnes. Vitamin C in fish nutrition—A review. *Fisk. Dir. Skr., Ser. Ernaering* **4:** 3–32 (1991).
3. K. Dabrowski and S. Hinterleitner. Applications of a simultaneous assay of ascorbic acid, dehydroascorbic acid and ascorbic sulphate in biological materials. *Analyst* **114:** 83–87 (1989).
4. K. Dabrowski and G. Köck. Absorption of ascorbic acid and ascorbic sulfate and their interaction with minerals in the digestive tract of rainbow trout. *Can. J. Fish. Aquat. Sci.* **46:** 1–6 (1989).
5. K. Dabrowski and J.H. Blom. Ascorbic acid deposition in rainbow trout (*Oncorhynchus mykiss*) eggs and survival of embryos. *Comp. Biochem. Physiol.* **108A:** 129–35 (1994).
6. R.B. Roy, A. Conetta, and J. Salpeter. Automated fluorometric method for the determination of total vitamin C in food products. *J. Assoc. Off. Anal. Chem.* **59:** 1244–50 (1976).
7. R. Waagbø, J. Glette, E. Raa-Nilsen, and K. Sandnes. Dietary vitamin C, immunity and disease resistance in Atlantic salmon (*Salmo salar*). *Fish Physiol. Biochem.* **12:** 61–73 (1993).
8. P.W. Washko, R.W. Welch, K.R. Dhariwal, Y. Wang, and M. Levine. Ascorbic acid and dehydroascorbic acid analyses in biological samples. *Anal. Biochem.* **204:** 1–14 (1992).
9. M.T. Parviainen and K. Nyyssönen. Ascorbic acid. *Modern Chromatographic Analysis of the Vitamins,* 2nd ed. A.P. De Leenheer, W.E. Lambert, and H.J. Nelis, Eds. Marcel Dekker, New York, NY, 1992, pp. 235–60.
10. R.S. Carr and J.M. Neff. Determination of ascorbic acid in tissues of marine animals by liquid chromatography with electrochemical detection. *Anal. Chem.* **52:** 2428–30 (1980).
11. S.P. Felton and J.E. Halver. Vitamin C_1 and C_2 analysis using double column reverse phase high pressure liquid chromatography. *Aquacult. Fish. Manag.* **18:** 387–90 (1987).
12. A.M. Hapette and S.A. Poulet. Application of high performance liquid chromatography to the determination of ascorbic acid in marine plankton. *J. Liq. Chromatogr.* **13:** 357–70 (1990).
13. X.Y. Wang and P.A. Seib. Liquid chromatographic determination of a combined form of L-ascorbic acid (L-ascorbate-2-sulfate) in fish tissue by release of L-ascorbic acid. *Aquaculture* **87:** 65–84 (1990).
14. K.M. Hoffman, P.R. Brown, and P.D. Maugle. Comparison of three HPLC methods for the analysis of vitamers of ascorbic acid in shrimp tissue. *J. Liq. Chromatogr.* **15:** 2581–2610 (1992).
15. S.P. Felton, R. Grace, and J.E. Halver. A non-ion pairing HPLC method for measuring new forms of ascorbate and ascorbic acid. *J. Liq. Chromatogr.* **17:** 123–31 (1994).
16. M.R. Johnson and A.J. Ainsworth. An elevated dietary level of ascorbic acid fails to influence the response of anterior kidney neutrophils to *Edwardsiella ictaluri* in channel catfish. *J. Aquat. Animal Health* **3:** 266–73 (1991).
17. G.O. El Naggar and R.T. Lovell. Effect of source and dietary concentration of ascorbic acid on tissue concentrations of ascorbic acid in channel catfish. *J. World Aquacult. Soc.* **22:** 201–206 (1991).
18. M.H. Li, M.R. Johnson, and E.H. Robinson. Elevated dietary vitamin C concentrations did not improve resistance of channel catfish, *Ictalurus punctatus,* against *Edwardsiella ictaluri* infection. *Aquaculture* **117:** 303–312 (1993).
19. W.G. Mustin and R.T. Lovell. Na-L-ascorbyl-2-monophosphate as a source of vitamin C for channel catfish. *Aquaculture* **105:** 95–100 (1992).
20. M.H. Li and E.H. Robinson. Effect of dietary vitamin C on tissue vitamin C concentration in channel catfish, *Ictalurus punctalus,* and clearance rate at two temperatures—A preliminary investigation. *J. Appl. Aquacult.* **4:** 59–71 (1994).
21. G. Merchie, P. Lavens, P. Dhert, M. Dehasque, H. Nelis, A. De Leenheer, and P. Sorgeloos. Variation of ascorbic acid content in different live food organisms. *Aquaculture* **134:** 325–37 (1995).
22. M.A. Kutnink, W.C. Hawkes, E.E. Schaus, and S.T. Omaye. An internal standard method for the unattended high performance liquid chromatographic analysis of ascorbic acid in blood components. *Anal. Biochem.* **166:** 424–430 (1987).
23. W.A. Behrens and R. Madere. A procedure for the separation and quantitative analysis of ascorbic acid, dehydroascorbic acid, isoascorbic acid, dehydroisoascorbic acid in food and animal tissue. *J. Liq. Chromatogr.* **17:** 2445–55 (1994).
24. C.J. Bates. Use of homocysteine to stabilise ascorbic acid, or to reduce dehydroascorbic acid, during HPLC separation of large volumes of tissue extracts. *Clin. Chim. Acta* **205:** 249–52 (1992).

25. P.W. Washko, W.O. Hartzell, and M. Levine. Ascorbic acid analysis using high performance liquid chromatography with coulometric electrochemical detection. *Anal. Biochem.* **181:** 276–82 (1989).
26. M.H. Bui-Nguyén. Ascorbic acid and related compounds. *Modern Chromatographic Analysis of the Vitamins*, 1st ed. A.P. De Leenheer, W.E. Lambert, and M.G.M. De Ruyter, Eds. Marcel Dekker, New York, NY, 1985, pp. 267–301.
27. D.B. Dennison, T.G. Brawley, and G.L.K. Hunter. Rapid high performance liquid chromatographic determination of ascorbic acid and combined ascorbic acid–dehydroascorbic acid in beverages. *J. Agric. Food Chem.* **29:** 927–29 (1981).
28. E.K. Kontara, G. Merchie, P. Lavens, H.J. Nelis, A.P. De Leenheer, and P. Sorgeloos. Improved larviculture outputs of post-larval shrimp *Penaeus vannamei* through supplementation of L-ascorbyl-2-polyphosphate in the diet. *Larvi '95—Fish and Shellfish Symposium*. P. Lavens, I. Roelants, and E. Jaspers, Eds. European Aquaculture Society, Special Publication 24, Gent, Belgium, 1995, pp. 230–34.
29. G. Merchie, P. Lavens, P. Dhert, R. Pector, A.F. Mai Soni, M. Abbes, H.J. Nelis, F. Ollevier, A.P. De Leenheer, and P. Sorgeloos. Live food mediated vitamin C transfer to *Dicentrarchus labrax* and *Clarias gariepinus*. *J. Appl. Ichthyol.* **11:** 336–41 (1995).
30. G. Merchie, P. Lavens, J. Radull, H. Nelis, A.P. De Leenheer, and P. Sorgeloos. Evaluation of vitamin C-enriched *Artemia* nauplii for larvae of the giant freshwater prawn. *Aquaculture Int.* **3:** 355–63 (1995).
31. G. Merchie, P. Lavens, P. Dhert, M. Garcia Ulloa Gomez, H. Nelis, A.P. De Leenheer, and P. Sorgeloos. Dietary ascorbic acid requirements during the hatchery production of turbot larvae. *J. Fish Biol.* **49:** 573–83 (1996).
32. G. Merchie, P. Lavens, V. Storch, U. Ubel, H. Nelis, A.P. De Leenheer, and P. Sorgeloos. Influence of dietary vitamin C dosage on turbot (*Scophtalmus maximus*) and European sea bass (*Dicentrarchus labrax*) nursery stages. *Comp. Biochem. Physiol.* **114A:** 123–33 (1996).

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