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Capillary zone electrophoretic determination of the four vitamin C esters L-ascorbyl-2-phosphate, L-ascorbyl-2-sulfate, L-ascorbyl-2-diphosphate and L-ascorbyl-2-triphosphate in fish feed, plasma and tissue

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Abstract

A new and fast method for the determination of L-ascorbyl-2-phosphate, the 2-diphosphate, 2-triphosphate and the 2-sulfate ester in feed, fish plasma and tissue samples by capillary zone electrophoresis is described. The substances were extracted with a 200 m*M* potassium phosphate buffer pH 4.0. Separation was achieved using a 50 μm uncoated fused-silica bubble capillary of 40 cm length to the detector, which was set at 254 nm, and a 80 m*M* tricine buffer at pH 9.2. Repeatability checked on a trout feed sample revealed a migration time variation of 1.2% ($n=24$) and an area calculation variation of 2.8% ($n=24$). Detector response for L-ascorbyl-2-phosphate was linear up to at least 100 $\mu g\,ml^{-1}$. After administration of a high dose of L-ascorbyl-2-phosphate to rainbow trout, the compound was found in the stomach and intestine but not in plasma, proving a fast conversion to vitamin C.

Keywords: Vitamin C; Ascorbic acid esters; Ascorbyl-2-sulfate; Ascorbyl-2-phosphate

1. Introduction

L-Ascorbyl-2-phosphate (AAMP) and L-ascorbyl-2-sulfate (AAS) are the two main ascorbic acid derivatives used for supplementation of fish and shrimp feed because of their better stability compared to the free acid. Fish, among other animal species, do not synthesize their own vitamin C and thus depend on a dietary source of L-ascorbic acid [1]. Both, fish and shrimp are scurvy susceptible and show on ascorbic acid deficiency signs of skeleton deformations, gain weight slowly and have high morbidity [1,2]. Ascorbyl-2-phosphate is also supplemented to broiler, piglet and guinea pig feed. L-ascorbyl-2-polyphosphate (APP), one of the products on the market, is a mixture of several phosphorylated compounds. The main products in the synthesis of APP are AAMP, L-ascorbyl-2-diphosphate (AADP) and L-ascorbyl-2-triphosphate (AATP), as described by Wang et al. [3].

The stability of analytes in the course of an analytical procedure, e.g. the chromatographic conditions, is crucial for a reliable quantitation. Furthermore the compounds themselves should be stable in

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the field of application to ensure adequate supplementation. Austria et al. investigated the behaviour of magnesium ascorbyl-2-phosphate and ascorbyl-6-palmitate in standard solution [4]. AAMP proved to be very stable in phosphate buffer solution at pH 4 over several months. They also compared the stability of the two compounds for their use in cosmetic products.

Gabaudan et al. and also Gadient and Fenster examined the stability of calcium AAMP regarding fish feed processing and during storage thereof [5,6], as did Schüep et al. as well as De Antonis et al. with dipotassium AAS in pelleted fish feed [7,8]. Both products showed a remarkable retention, proving their stability as feed supplements.

Esterification of ascorbic acid in the 2-position prevents the breakup of the enediol system and leads to the improved stability. On the other hand, the esters can be cleaved by phosphatases resp. a sulfohydrolase in the animal digestive tract, releasing the needed free ascorbic acid [9–12]. The conversion of AAMP and AAS to ascorbic acid in Penaeus monodon has been studied by Kittakoop et al. with HPLC and colorimetry [13]. The bioavailability of vitamin C from AAS seems to be significantly lower than from AAMP in channel catfish, salmon, rainbow trout and shrimp [14–18], but was reported to be almost equal in tilapia [19]. For the understanding of the physiological processes regarding the conversions of the different ascorbic acid esters into vitamin C a reliable and reproducible analysis method is necessary for these compounds.

Since the introduction of ascorbic acid esters, a large number of HPLC methods for their determination have been described. These include enzymatic methods, where the released ascorbate is quantitated [20,21], as well as direct HPLC methods with UV [7,22–32], electrochemical [8,33] and mass spectrometric detection [34,35]. An indirect colorimetric method for the assay of AAS in tissues also has been described [36].

Several new methods for the determination of ascorbic acid were introduced during the last ten years with the emerging technique of capillary electrophoresis (CE). CE offers many advantages for the separation of ionic substances, because resolution is mostly affected by the difference in charge to mass ratio of the compounds to be analysed. A group around P.G. Righetti determined total ascorbic acid in fruits using a coated capillary [37]. Lin Ling et al. also proposed a separation with a coated capillary, which was applied to fruit juices and to a pharmaceutical formulation [38]. Isoascorbic acid was used as an internal standard for the measurement of ascorbic acid in biological fluids and fruit juices by another group, who worked with a bare fused-silica capillary [39]. In all three studies, capillary zone electrophoresis (CZE) methods were employed.

A series of micellar electrokinetic capillary chromatography (MECC) methods for ascorbic acid analysis have also been published. MECC has already been employed for the analysis of water-soluble vitamins and multi-vitamin preparations [40,41], for the separation of water- and fat-soluble vitamins [42] and for the determination of total ascorbic acid in beers, wines and fruit drinks [43].

This paper describes a new and fast capillary zone electrophoresis method for the determination of four ascorbic acid esters, namely AAMP, AADP, AATP and AAS, in fish and broiler feed. The method was also applied to the analysis of trout plasma and tissue samples.

2. Conditions

2.1. Reagents

Calcium L-ascorbyl-2-phosphate, trihydrate (98%) and dipotassium L-ascorbyl-2-sulfate, dihydrate were products from F. Hoffmann–La Roche Ltd. Tetracyclohexylammonium L-ascorbyl-2-diphosphate and pentacyclohexylammonium L-ascorbyl-2-triphosphate were kindly given by Dr Paul A. Seib, Kansas State University, USA. Tricine ({N-Tris(hydroxymethyl)methyl}glycine) (≥99.5%), 1 *M* sodium hydroxide solution (HPCE grade) for capillary conditioning and phosphoric acid (puriss. p.a.) were purchased from Fluka, potassium dihydrogenphosphate (p.a.), 1 *M* sodium hydroxide solution for buffer preparation and ethanol (absolute p.a.) from Merck, acetonitrile (HPLC grade S) from Rathburn Chemicals Ltd., Walkerburn, Scotland and 1,5-dimethylhexylamine (99%) from Sigma–Aldrich. Double distilled, deionized water from a Millipore MilliQ plus system was used to prepare all solutions.

2.2. Instrumentation

2.2.1. CE

A Hewlett Packard HP 3D CE system (Hewlett-Packard GmbH, Waldbronn, Germany) was used for this study, with the UV detector operated at 254 nm and with a band width of 16 nm. The capillary was of unmodified fused-silica with 50 μm I.D. and length to the detector was 40 cm (total length 48.5 cm). For the detection, the capillary used had an extended light path of approx. 150 μm (bubble capillary). The capillary was conditioned by rinsing with 1 *M* sodium hydroxide for 5 min, water for 5 min, and separation buffer for 5 min. Quantitative determinations were made by comparing the peak areas of the samples with those given by known concentrations of standards. Corrected areas (area/migration time) were used to calculate results.

2.2.2. HPLC

A stainless-steel column (250 mm×4.0 mm) filled with ODS Hypersil, 5 μm (Stagroma, Wallisellen, Switzerland) was used together with a precolumn (17 mm×4.0 mm) packed with the same stationary phase. Column and precolumn were held at 25°C using a column oven Model 4100 (Innovativ–Bischoff AG, Wallisellen, Switzerland). A L-6200A pump (Merck–Hitachi, Germany) was used to pump the mobile phase through the system. The injection was performed by a 717 plus Autosampler (Waters Associates, Inc., Milford, MA, USA). The detector was a Severn Analytical SA 6504 programmable UV absorbance detector (Linear Instruments Corp., Reno, Nevada, USA) set at 254 nm. A VG-Multichrom Chromatography System (VG Laboratory Systems, Cheshire, UK) in combination with a VAX-computer (Digital Equipment Corporation (DEC), Zürich, Switzerland) was used for the integration. Quantitative determinations were made by comparing the peak areas of the samples with those given by known concentrations of standards.

2.3. Procedures

2.3.1. Sample preparation

Feed samples were prepared by extracting a portion of 5 g ground feed with 50 ml of a 200 m*M* potassium phosphate buffer pH 4.0 by stirring for 20 min at room temperature. A portion of approx. 10 ml was centrifuged at 1500×*g* for 10 min and then prepared further as follows.

For CE, fish feed samples were first filtered through a 0.45 μm cellulose filter disk and then by centrifugation at 11,000 g for 6 min using Microcon 30 Microconcentrators (molecular weight cut-off 30 000; Amicon Inc., Beverly, USA). All other feed samples were filtered only through a 0.45 μm cellulose filter disk before injection. Standard solutions were prepared in 200 m*M* potassium phosphate buffer pH 4.0.

For HPLC, the extractant was a 400 m*M* potassium phosphate buffer pH 3.0. After extraction and centrifugation, the samples were filtered only through a 0.45 μm cellulose filter disk before injection. Standard solutions were prepared in 400 m*M* potassium phosphate buffer pH 3.0. Plasma and tissue samples were kept at −25°C until analysis. To 100 μl of a plasma sample, 60 μl of a 200 m*M* potassium phosphate buffer pH 4.0 were added, the mixture was vortexed and filtered with Microcon 30 Microconcentrators for deproteinization. Tissue samples (approx. 0.5 g or 1.0 g) were homogenized with a Polytron in 2 ml or 4 ml 200 m*M* potassium phosphate buffer pH 4.0, respectively, and centrifuged at 4200×*g* at 5°C for 10 min. The supernatant was filtered with Microcon 30 Microconcentrators before injection.

2.3.2. CE method

The separation buffer was prepared by dissolving 7.17 g of tricine in 400 ml of water, adjusting the pH to 9.2 with 1 *M* sodium hydroxide and making up with water to the final volume of 500 ml, giving a concentration of 80 m*M* tricine. Tricine buffers with different concentrations were prepared in an analogous way. The separation buffer and all samples were filtered through a 0.45 μm cellulose filter disk before injection. Samples were injected by hydrodynamic injection with 50 mbar for 2 s. Separation voltage was set at +30 kV and temperature at 40°C. Between successive electrophoretic runs, the buffer vials were refilled with fresh separation buffer and the capillary was rinsed sequentially with 1 *M* sodium hydroxide, water and with separation buffer for 2 min each. Before injecting the sample, the buffer filled capillary was preconditioned by apply-

ing a voltage of +30 kV for 2 min [44], generating a current of approx. 100–150 μA, depending on the buffer concentration (80–100 m*M*).

2.3.3. HPLC method

Mobile phase A and B were pumped in the ratio of 3:7 into the HPLC system. Mobile phase A consisted of a solution of 10.8 g potassium dihydrogen phosphate dissolved in 900 ml of water, with 2 ml 1,5-dimethylhexylamine added, pH adjusted to 3.0 and made up to 1000 ml. Mobile phase B consisted of 900 ml of mobile phase A mixed with 140 ml of an acetonitrile–ethanol mixture 7:3. The mobile phase was pumped through the system at a constant flow-rate of 0.8 ml min^{-1}. 10 μl of each sample were injected.

3. Results and discussion

First experiments using a broiler feed showed that with the electroseparation method, the majority of the matrix signals appeared between 1.4 min and approx. 4 min, leaving open a large window for the separation of the investigated compounds after 4 min migration time. The separation conditions employed based on a method used for the measurement of ascorbic acid in biological fluids and fruit beverages [39].

The analysis of broiler feed containing AAMP gave migration times around 6 min for the vitamin derivative. Sample preparation in this case included only a filtration through a 0.45 μm filter after the extraction and centrifugation. When fish feed was analysed, migration time for AAMP was in the range of 12–15 min with very broad signals. We therefore introduced another filtration step, using commercial microconcentrators with a molecular weight cut-off of 30 000 to separate most of the proteins. With this additional sample pretreatment, electropherograms similar to the ones from broiler feed analysis were obtained.

Migration time can vary from run to run with capillary electrophoresis. This may be due to fluctuations of the electroendosmotic flow (EOF), especially at higher pH values, where the EOF is less reproducible. The influence on the migration time is generally greater for anions than for cations in the positive polarity mode. In order to improve migration time reproducibility in this assay, a voltage preconditioning technique was applied [44]. By applying a voltage of +30 kV for 2 min to the buffer filled capillary before injection of the sample, the variation in migration time could be improved to 0.5% for successive runs ($n=7$), compared to 2.3% ($n=7$) without preconditioning.

The migration time dependence of the four components AATP, AADP, AAMP and AAS on the electrolyte concentration was investigated. Fig. 1 shows the migration times obtained with concentrations of 75–125 m*M* tricine. With 75 m*M* tricine, AAMP and AAS are not fully separated. With 90 m*M* and ascending concentrations, the difference in migration time of AAS and AAMP grows considerably. AATP, AADP and AAMP are well resolved over the whole concentration range.

An unusual signal distortion (i.e. signals with a foot) was observed in the analysis of fish feed samples with a separation buffer concentration of 100 m*M* tricine, which could not be explained. At 90 m*M* and at 125 m*M* tricine, however, normal signal shapes were obtained. Generally, longer migration times were found with feed samples than with plain buffer solutions. However this did not influence quantitation significantly because corrected areas

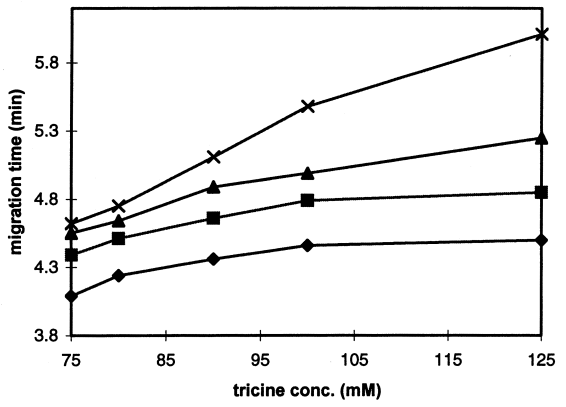

Fig. 1. Migration times of AAMP (▲), AADP (■), AATP (♦) and AAS (×) solutions in 200 m*M* potassium phosphate buffer pH 3.0 at different separation buffer concentrations.

(ratio area/migration time) were measured. The pH values of the extracts of fish feed samples were at approx. 5.4 and were not adjusted. The different pH value together with the much higher buffer capacity coming from the feed samples may have contributed to the observed increase in migration time.

The electropherogram of a commercial fish feed sample spiked with all four compounds is shown in Fig. 2. Signals were ascribed to the individual esters by spiking with the single compounds. For feed samples, resolution of the four vitamin C esters was sufficient with 80 m*M* tricine within only 6 min. Therefore, this concentration was chosen as appropriate for further separations. In Fig. 3, a typical electropherogram obtained from supplemented fish feed is shown.

Statistical data including migration time and area

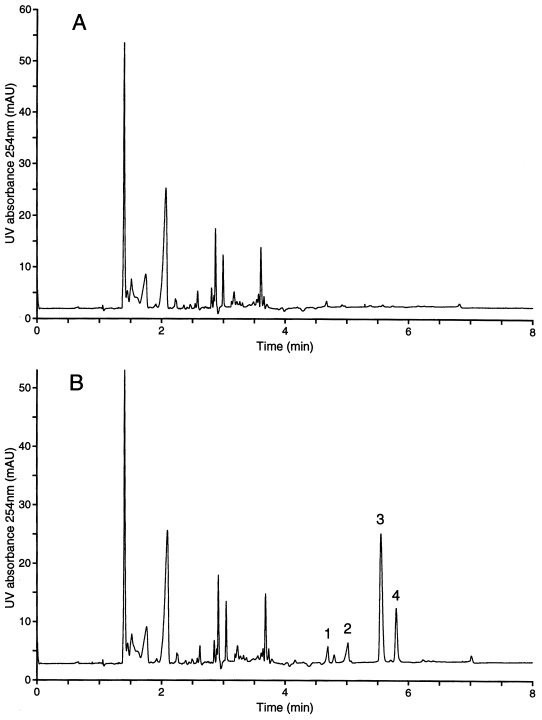

Fig. 2. Electropherogram of (A) an extract of an unspiked fish feed sample, (B) an extract of a fish feed sample spiked with (1) 9.9 μg AATP ml^{-1}, (2) 10.6 μg AADP ml^{-1}, (3) 39.2 μg AAMP ml^{-1} and (4) 11.2 μg AAS ml^{-1}. Buffer: 80 m*M* tricine, pH 9.2, separation voltage +30 kV, T=40°C, sample dissolved in 200 m*M* potassium phosphate buffer pH 3.0.

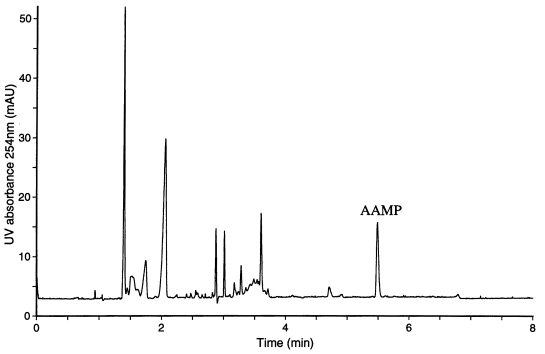

Fig. 3. Electropherogram of an extract of a fish feed sample containing 191 mg AAMP kg^{-1} feed. Buffer: 80 m*M* tricine, pH 9.2, separation voltage +30 kV, T=40°C, sample dissolved in 200 m*M* potassium phosphate buffer pH 4.0.

reproducibility are shown in Table 1. Very good reproducibilities were obtained with a standard solution as well as with commercial broiler and trout feed samples containing supplemented AAMP.

The response for AAMP was linear up to at least 100 μg ml^{-1} (mAU corrected area vs. μg AAMP ml^{-1}: $y=0.0049x-0.0017$, $R^2=0.9999$, 200 m*M* potassium phosphate buffer pH 4.0) and up to at least 50 μg ml^{-1} for the other three compounds (AATP: $y=0.0033x-0.0011$, $R^2=0.9996$; AADP: $y=0.0039x-0.00028$, $R^2=1.000$; AAS: $y=0.0056x-0.0013$, $R^2=0.9998$, each in 200 m*M* potassium phosphate buffer pH 4.0). The limits of detection, where signal height to noise ratio was 3:1, were 0.3 μg ml^{-1} for AAMP, 0.25 μg ml^{-1} for AAS, and approx. 0.4 μg ml^{-1} and 0.5 μg ml^{-1} for AADP and AATP, respectively.

Recoveries of the novel CE method were checked by spiking broiler and fish feed samples with known amounts of standards at three different levels (15, 30 and 45 μg ml^{-1} in broiler feed; 20, 40 and 60 μg ml^{-1} in fish feed) using a separation buffer concentration of 80 m*M* and the extraction buffer adjusted to pH 4.0. Recoveries averaged 94% in the broiler feed, compared to 95% in the HPLC assay, and 93% in the fish feed, compared to 94% in HPLC, respectively.

Data of some pelleted fish feed analysed with the CE method were also compared with those obtained by HPLC. These data are summarized in Table 2. A

Table 1
Reproducibility of the CE deternibation of L-ascorbyl-2-phosphate[a]

	n	Migration time (min)	±S.D. (min)	R.S.D. (%)
Run-to-run				
Standard solution[b]	28	5.68	0.02	0.4
Trout feed sample[b]	24	5.74	0.07	1.2
	n	Concentration (μg AAMP ml^{-1})	±S.D. (μg AAMP ml^{-1})	R.S.D. (%)
Run-to-run				
Standard solution[b]	28	50.3	1.1	2.2
Trout feed sample[b]	24	8.46	0.24	2.8
Broiler feed sample[c]	6	40.6	1.2	3.0
Trout feed sample[c]	6	36.7	1.3	3.5
Day-to-day				
Broiler feed sample[c]	6	39.5	1.3	3.3
Trout feed sample[c]	6	38.1	0.9	2.4

[a] Solutions/extracts with 200 m*M* potassium phosphate buffer pH 3.0.
[b] Determined at 100 m*M* tricine.
[c] Determined at 80 m*M* tricine.

good agreement between the two methods was found.

The new method was also used to assay AAMP in fish plasma and tissues. As Fig. 4 shows, AAMP can be easily detected in plasma samples. A group of three rainbow trouts was fed AAMP equivalent to 200 ppm ascorbic acid for eight weeks. Another group of two rainbow trouts was force-fed once with a gelatine capsule containing 20 mg AAMP (0.055 mmol). Samples from the latter two animals were taken after 14 h. No AAMP was found in the plasma samples from all five trouts. Figs. 5 and 6 show the electropherograms for liver and muscle where no detectable amount of AAMP could be observed. These results support the former findings of Schüep and Gabaudan [45], who also under stronger extraction conditions were not able to proof the presence of AAMP in these trout tissues.

However, when stomach and intestine samples from the trouts fed with the capsule were examined,

Table 2
Comparison of CE and HPLC results from pelleted fish feed analyses

Sample	HPLC method (mg AAMP kg^{-1})	CE method (mg AAMP kg^{-1})
Fish feed 1	65	62
2	63	63
3	62	65
4	56	60
5	92	88
6	159	155
7	157	158
8	186	196
9	1158	1175
10	190	198

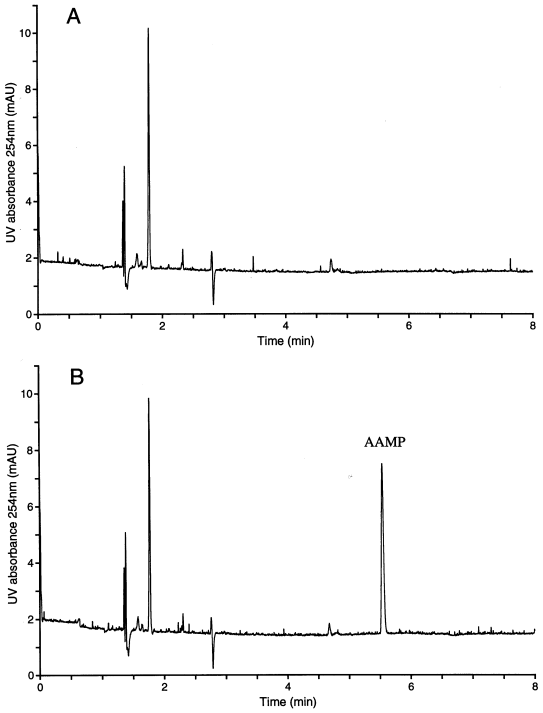

Fig. 4. Electropherograms of (A) a trout plasma sample without AAMP, (B) the same sample spiked with 10.2 μg AAMP ml^{-1}. Conditions as in Fig. 3.

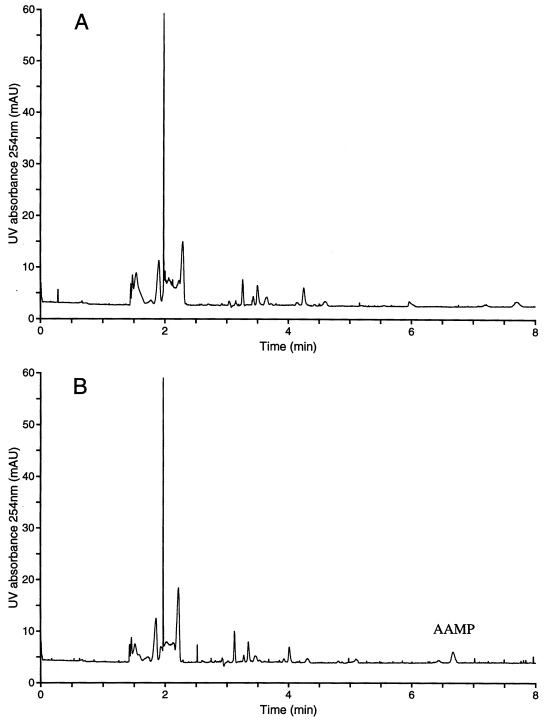

Fig. 5. Electropherograms of (A) a liver sample of a trout fed AAMP equivalent to 200 ppm ascorbic acid for 8 weeks, (B) a trout liver sample spiked with approx. 10 μg AAMP ml^{-1}. Conditions as in Fig. 3.

we found signals corresponding to AAMP. The amount was approx. 10 μg g^{-1} in stomach samples. The electropherograms obtained from the intestine samples showed in one case a signal for AAMP (29 μg g^{-1}), as well as a large and very sharp signal at 2.6 min which was identified to be ascorbic acid (Fig. 7). In the other case, only the signal for ascorbic acid could be found. Quantification of vitamin C could not be performed in an accurate way because the extraction conditions were not adequate enough to stabilize the molecule in its reduced form. These results suggest that AAMP is readily hydrolysed in the intestine and free ascorbic acid is absorbed, which is in accordance with the results found by Dabrowski et al. [10]. To investigate this hydrolase activity, trout intestine homogenate was incubated with a solution of AAMP at pH 5.6 at room temperature and samples were taken at regular intervals. The results of this experiment are given in Fig. 8 and show an exponential decrease of the dissolved AAMP content. After 4 h, more than 90% of the initial AAMP is hydrolysed.

In conclusion, a new and fast capillary electrophoresis method for the determination of several ascorbic acid esters in feed was developed. AAMP, AADP, AATP and AAS can be well separated within short analysis time with minimal interference by matrix signals. The capillary electrophoresis technique has the advantage that no organic solvents are used and samples can be kept at minimal size (injection of nl amounts). Also, the detection limits compare favourably to the ones obtained by HPLC. The new method showed good recovery values in fish feed as well as in broiler feed and excellent linearities were observed for all investigated compounds. In addition, very good reproducibility data were found. Comparison with the HPLC method showed good agreement of the results. AAMP was also determined in trout plasma and tissue samples

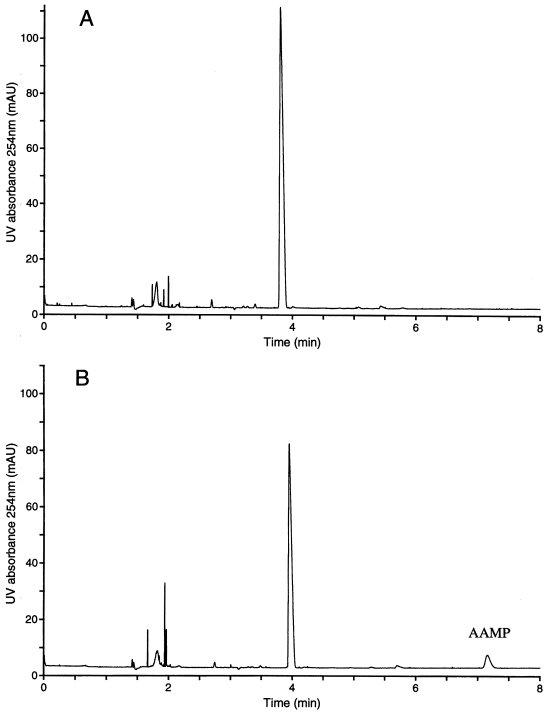

Fig. 6. Electropherograms of (A) a muscle sample of a trout fed AAMP equivalent to 200 ppm ascorbic acid for 8 weeks, (B) a trout muscle sample spiked with approx. 10 μg AAMP ml^{-1}. Conditions as in Fig. 3.

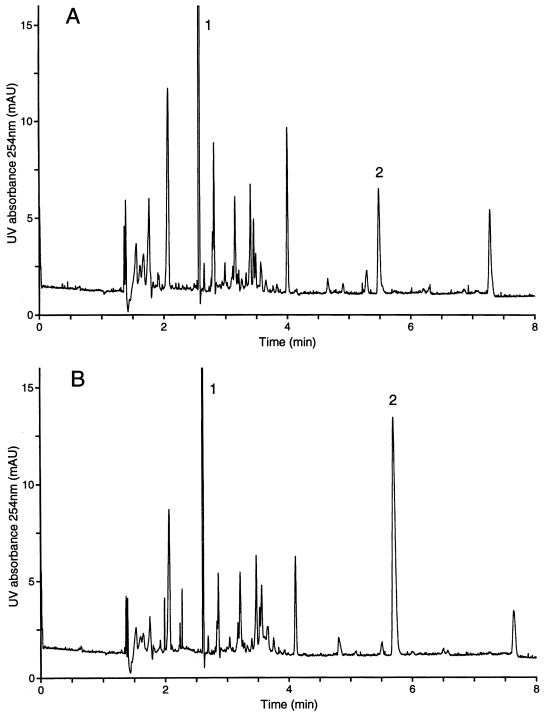

Fig. 7. Electropherograms of (A) the extract of an intestine sample from a trout force-fed a capsule with 20 mg AAMP (0.055 mmol) after 14 h 1=ascorbic acid, 2=AAMP, (B) the extract of the same sample spiked with additional AAMP. Conditions as in Fig. 3.

with the new method. AAMP and also a large amount of ascorbic acid was found in the intestine of trouts fed a high dose of AAMP. This suggests that AAMP is hydrolysed in the digestive tract and the released ascorbic acid is being absorbed. The kinetics of AAMP hydrolysis in trout intestine were also studied. After a few hours, most of the phosphate ester is hydrolysed.

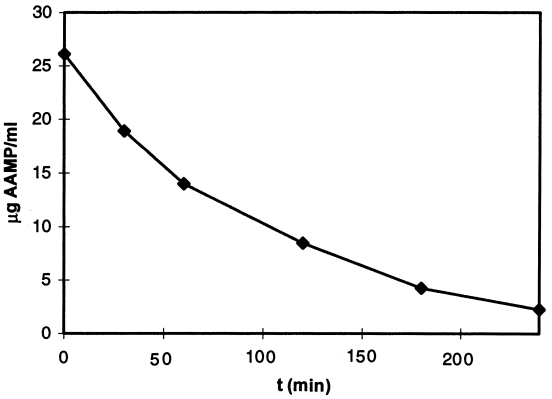

Fig. 8. Time course of AAMP hydrolysis in trout intestine measured at room temperature and at pH 5.6 with capillary zone electrophoresis.

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