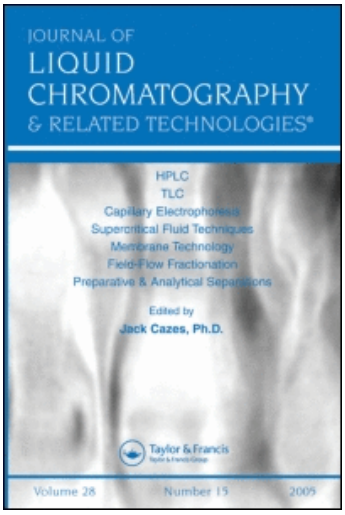

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COMPARISON OF THREE HPLC METHODS FOR THE ANALYSIS OF VITAMERS OF ASCORBIC ACID IN SHRIMP TISSUE

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ABSTRACT

Several high performance liquid chromatography (HPLC) methods have been developed for the analysis of L-ascorbic acid (C1) and two of its more stable analogs dipotassium ascorbyl-2-sulfate dihydrate (C2), and L-ascorbyl-2-phosphate sodium (C3). Three of these methods were evaluated for the analysis of the three vitamers, and three procedures for the extraction of the vitamers from shrimp (Penaeus Setiferus) tissues were compared.

INTRODUCTION

Ascorbic acid is essential in the diets of most species of fish (1 - 3). Since ordinary, unprotected L-ascorbic acid is relatively unstable under

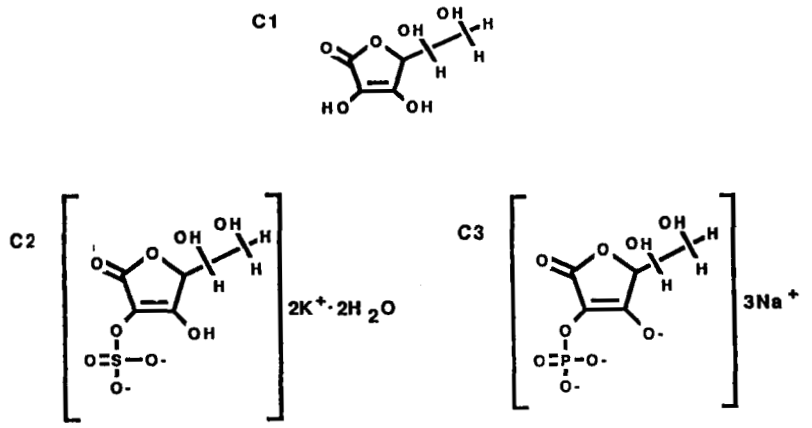

FIGURE 1

C1 = L-ascorbic acid
C2 = Dipotassium ascorbate-2-sulfate dihydrate
C3 = Trisodium ascorbate-2-phosphate

normal conditions of light, temperature, and moisture, it is of value to the aquaculture industry to find a more stable form of vitamin C (2, 3). Of the forms of vitamin C presently commercially available, three were evaluated in this study: ethyl cellulose coated ascorbic acid (C1), dipotassium ascorbyl-2-sulfate dihydrate (C2), and trisodium L-ascorbyl-2-phosphate, commonly known as L-ascorbyl-2-monophosphate sodium (C3). (Figure 1)

Vitamin C is present in all species of plants and animals. Biological activity of vitamin C has been linked to the synthesis of collagen, but there are many other biological mechanisms in which vitamin C plays a role.

Vitamin C deficiency in shrimp is readily noticeable. Impaired collagen formation resulting from the dietary deficiency of vitamin C was shown in shrimp Penaeus Californiensis by Lightner et al (4). In this study, lack of collagen synthesis affected juvenile shrimp by causing "black death," melarized lesions in the collagenaceous tissue beneath the exoskeleton. Deshimaru and Kuroki (5) reported symptoms of decoloration and the occurance of an abnormal milky-gray color on the underside of the lower abdomen and tips of the walking legs. Shiqueno and Itoh reported symptoms similar to "black death" in Penaeus Japonicus (6).

Although most animals are able to synthesize their dietary requirements of ascorbic acid, species such as certain primates, guinea pigs, bats, birds, insects, fish and shrimp cannot because they lack L-gulonolactone oxidase which is the common enzyme required for the synthesis of L-ascorbic acid (7). Thus, shrimp which are not able to synthesize vitamin C must acquire it from their diet.

Vitamers C2 and C3 have been shown to be bioavailable forms of vitamin C for trout, channel catfish, and penaeid shrimp (2, 6, 7). Tucker and Halver added C1 and C2 to separate batches of feeds at a level of 25 mg/kg. Pools of trout fed from each of the batches over a period of time

exhibited normal growth and development, and there was no evidence of vitamin C deficiency (7). Lovell fed pools of channel catfish from batches which contained 11, 22, 44, or 132 mg/kg of C1, C2, or C3. C1 and C3 feeds led to normal growth and development at all levels. Fish fed C2 at levels lower than 132 mg/kg showed signs of scurvy. At a level of 132 mg/kg, the fish exhibited normal growth. Shigueno and Itoh fed test groups of Penaeus Japonicus diets containing 1087, 430, 215, 43 and 0 mg/kg of C3 (6). The pools fed the lowest two doses had high mortality rates. The other three pools developed normally indicating that C3 is a viable alternative to C1 in shrimp diet.

In some species of fish, sulfonation of C1 with ascorbic acid-sulfotransferase results in the formation of C2, and in the presence of an excess of C1, C2 will readily form (7). C2 has been observed in storage in the skin and tissues where it is available until it is needed (7). To fully understand the activity of C1, C2, and C3, good methods of analysis are needed to identify and quantify these vitamers in shrimp. The important requirements for a reliable method are adequate sensitivity and recovery, and the ablity to detect clearly and differentiate between the vitamer levels present in healthy and scorbutic shrimp.

Of several HPLC methods that have been developed, three that appear to be most useful for the studies of vitamers of ascorbic acid in shrimp

were evaluated. The methods differ in column, mobile phase and/or flow rate used. In addition, three different procedures were compared for the extraction of the vitamers from shrimp matrices. Large shrimp (Penaeus Setiferus) grown in maturation units under the same environmental conditions and fed the same diet of squid, oysters and bloodworms were used as the model.

MATERIALS

Apparatus

Chromatographic analysis was performed using an M6000 pump (Waters Division of Millipore, Milford, MA), a manual injector valve (Rheodyne, Cotati, CA) with a 6 µl sample loop, a model 510 UV detector set at 254 nm (Waters Division of Millipore, Milford, MA), and an SP-4270 recording integrator (Spectra Physics Analytical, San Jose, CA). A reverse phase C_{18} column (Waters Division of Millipore, Milford, MA, Novapak, 30 cm X 3.9 mm I.D.) with tetrabutyl ammonium phosphate as the ion pairing reagent (RP-TBA) was used in the first chromatographic method. A reverse phase C_{18} column (Waters Division of Millipore, Milford, MA, Novapak, 30 cm X 3.9 mm I.D.) with n-octylamine (RP-OA) in the second method, and a weak ion exchange column (Shodex, Tokyo, Japan, NHpak 15 cm X 4.6 mm I.D.) (IE) in the third method.

Chemicals

Three commercially available forms of vitamin C were used as references in this study. Ethyl cellulose coated L-ascorbic acid (C1) (97% pure, 3% ethyl cellulose) was obtained from Hoffman LaRoche Inc. (Nutley, NJ). Dipotassium ascorbyl-2-sulfate dihydrate (C2) (97% pure, 48% ascorbic acid equivalence) was obtained from Showa Denko K.K. (Minato-Ku, Tokyo, Japan). L-ascorbyl-2-monophosphate sodium (C3) (95% pure, 46% ascorbic acid equivalence) was obtained from Pfizer, Inc. (Groton, CT).

The following chemicals were reagent grade quality and were used without further purification. Acetic acid (HAc) was purchased from Mallinckrodt (St. Louis, MO). Ammonium chloride (NH_4Cl), meta phosphoric acid (mPA), and sodium acetate (NaAc) were purchased from Fisher Scientific Co. (Pittsburgh, PA). Dithiothreitol (DTT) was purchased from Eastman Kodak Co. (Rochester, NY). Disodium ethylenediaminetetraacetate (EDTA), n-octylamine (OA), tetrabutyl ammonium phosphate (TBA), and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphoric acid (PA) was purchased from Matheson, Coleman and Bell (Norwood, OH), and HPLC grade methanol (MeOH) and potassium phosphate monobasic (KH_2PO_4) were purchased from Fisher Scientific Co. (Pittsburgh, PA).

Doubly distilled and deionized water (DDI water) was prepared in the laboratory and used in all sample solutions and mobile phases.

METHODS

Reference Preparation

For the standard solution of the three vitamers, DDI water was used as the diluent. A solution containing all three vitamers was used to check the efficiency of the column and the separation conditions. Separate solutions of each vitamer were used for peak identification. The standard solutions were prepared by dissolving 5 to 10 mg of C1, C2, or C3 in 5 ml of DDI water. For samples containing all three vitamers, 10 µl of each standard solution was added to 1 ml of water.

Samples prepared for the linearity and recovery studies using RP-TBA and RP-OA were dissolved in the respective mobile phase. For IE, these samples were dissolved in water. Approximately 20 mg of each of the vitamers was dissolved in 10 ml of solvent and diluted to the range of 0.0002 to 0.2 mg/ml (2 - 2000 ppm).

Sample Preparation

To insure that a representative sample was obtained, whole frozen shrimp (which weighed more than 20 g) were ground with dry ice in a

Camfield mixer to a homogeneous mixture in which none of the shrimp parts were recognizable.

The vitamers were extracted from approximately 500 mg of ground shrimp tissue using each of the extraction techniques described in the next section. The remaining ground shrimp was stored in dry ice, where it could remain for up to 10 hours, until the next sample was needed. Bioextracts were prepared to determine the effectiveness of the extraction techniques and to determine how much of each of the vitamers is endogenously present in shrimp tissue. To 1 ml of each of these extract samples was added 10 µl of a reference sample containing each of the three vitamers. This spiked bioextract sample was injected immediately after its respective non-spiked bioextract sample for peak identification.

Spiked bioextracts were prepared to examine linearity and recovery in each of the three HPLC methods. For the reverse phase methods, the mobile phase was used as the extraction solvent, and for the ion exchange method, DDI water was used as the extraction solvent. Approximately 10 g of the ground whole shrimp was homogenized in 100 ml of the extraction solvent using an Omni International 5000 Homogenizer. The extract solution was then centrifuged in a Damon/IEC Division Clini-Cool refrigerated centrifuge for approximately 15 minutes at 7000 rpm and

filtered through a 0.45 μm MSI syringe filter. To 10 ml of this solution was added 20 mg of each of the vitamers. Serial dilutions were done using the remaining shrimp extract solution to give 0.0002 to 0.2 mg/ml of the vitamers.

Linearity and recovery data were generated. Correlation coefficients were determined using the entire range of the vitamers. Recovery was calculated by comparing the samples from the shrimp extract with the references.

Extraction Techniques

Three techniques were used to extract the vitamers from the shrimp matrix. Prior to extraction, the frozen shrimp was ground dry in dry ice to a powder. In the first technique, the ground tissue sample (500 to 1000 mg) was homogenized in an aqueous solution of 0.2% DTT and 6% mPA (5 to 10 ml) (10, 11) using the Omni homogenizer at maximum speed for approximately five minutes (or until the sample appeared homogeneous). To prevent sample warming, which will increase the rate of sample degradation, the sample container was cooled in an ice bath during the homogenization. Cleland's reagent was used to slow the oxidation of the vitamers, and 6% mPA was used to precipitate the proteins and provide a

cleaner sample (10). The homogenized sample was then centrifuged in the Clini-Cool refrigerated centrifuge (again the sample was kept cold during centrifugation to reduce sample degradation) at 7000 rpm for approximately 15 minutes. The samples must be centrifuged for this length of time in order to remove all of the fats and proteins which were not removed by the mPA. The supernatant was filtered through a 0.45 μm MSI syringe filter and injected on the HPLC.

In the second technique, 500 to 1000 mg of the dry, ground shrimp samples were homogenized first in 2.5 mL of DDI water to lyse the cells. After approximately 1 minute of grinding, 2.5 ml of 10% TCA solution (8) was added to the solution to precipitate the protein. The solution was ground for approximately 1 more minute and centrifuged in the cold at 7000 rpm for approximately 10 minutes. The solution was kept cold during homogenization and centrifuged as described in the first technique. Clear supernatants were produed using this technique, indicating good precipitation of fats and proteins. It was filtered through the syringe filter and an aliquot was injected on the HPLC.

The third technique was the same as the first except that the mobile phase for the chromatographic method being used was used as the extraction solvent. For example, if RP-TBA was the chromatographic

method being used (see chromatographic methods section), the mobile phase for RP-TBA was used as the extraction solvent. The mobile phase was used as an extraction solvent because of its simplicity and the fact that some chromatographic artifacts (which are the result of the interaction of the mobile phase and the sample medium) are minimized when the extract solution and the mobile phase are the same. In addition, neither the extraction solvent nor the mobile phase can cause precipitation of the proteins on the column.

Chromatographic Methods

The chromatographic methods were evaluated under similar environmental conditions and on the same equipment. Methods RP-TBA and RP-OA used ion-pairing with the reversed phase mode of HPLC, whereas IE used a weak ion exchange mode (see Table 1).

In RP-TBA, the analytical column was a Waters Novapak C_{18} (30 cm X 3.9 mm I.D.) with 4 µm particles, and the guard column was C_{18} (50 mm X 4.6 mm I.D.) with 0.05 to 10 µm particles. The mobile phase was an aqueous solution of 0.1 mM EDTA, 1.0 mM TBA, and 0.08 M NaAc. The pH was adjusted to 4.0 with H_3PO_4 (85%.) This solution was diluted with MeOH such that the final solution was a 1:19 (MeOH:buffer)

TABLE 1

Summary of Extraction Techniques and Analytical Methods

Extraction Techniques	1	2	3
Tissue/Solution	500 mg/5 ml	500 mg/2.5 ml	500 mg/ml
Solution Comp.	6% mPA/ 0.2% DTT	DDI water	Mobile phase
Homogenize	5 min. cold	1 min. cold	5 min cold
Add	---	2.5 ml 10% TCA	---
Homogenize	---	1 min cold	---
Centrifuge	7000 rpm 15min.	7000 rpm 15 min.	7000 rpm 15 min.
Filter Supernatant	0.45 um	0.45 um	0.45 um
Analytical Systems	**RP-TBA**	**RP-OA**	**IE**
Analytical Column	Waters NovapakC_{18} 30cmX3.9mm 4 um particle	Waters Novapak C_{18} 30cmX3.9mm 4 um particle	Showa Denko Shodex NHpak 15cmX4.6mm ---
Guard Column	5cmX4.6mm C_{18}	5cmX4.6mm C_{18}	none
Mobile Phase KH_2PO_4	0.08 M NaAc 0.1mM EDTA 1.0 mM TBA pH 4.0 H_3PO_4	0.1 M NaAc 0.6 mM EDTA 1.3 mM OA pH 5.0 HAc	0.07 M 2.0 mM EDTA 0.02 M NH_4Cl none
Flow Rate	1.5 ml/min.	1.5 ml/min.	1.7 ml/min.

solution. The final solution was degassed under nitrogen, and the flow rate was set at 1.5 ml/min.

RP-OA was an adaptation of the method developed by Felton and Halver (8, 9). The analytical column was a Novapak C_{18} (30 cm X 3.9 mm I.D.) with 4 μm particles, and the guard column was C_{18} (50 mm X 4.6 mm I.D.) with 0.05 to 10 μm particles. The mobile phase was an aqueous solution of 0.1 M NaAc, 1.3 mM OA, and 0.6 mM EDTA. The pH was adjusted to 5.0 with acetic acid. This solution was degassed under nitrogen, and the flow rate was 1.5 ml/min.

IE was an adaptation of the method developed by Showa Denko K. K. (10). The analytical column was a Shodex NHpak NH_2-silica gel (15 cm X 4.6 mm I.D.), and there was no guard column. The mobile phase was an aqueous solution of 0.07 M KH_2PO_4, 0.02 M NH_4Cl, and 2 mM EDTA. This solution was degassed under nitrogen. The flow rate was 1.7 ml/min.

RESULTS and DISCUSSION

Extraction Techniques

Each extraction solvent affected the sample differently. When the 5% TCA was added to the sample solution, the proteins and fats precipitated and the supernatant fluid was clear. The 6% mPA was not as effective in

removing the fats and proteins. The solution appeared cloudy until the sample was centrifuged for at least 15 minutes at which time the solution was finally clear. The cloudiness indicated the presence of proteins and fats in the solution. The extract obtained when the mobile phase was used as the extractant gave the same degree of cloudiness as the mPA solution, indicating that neither the mPA nor the mobile phases were as effective as the TCA for precipitating proteins and fats.

The disadvantage of the sample extraction with TCA was that it degraded the vitamers and the biological matrix. The mPA and mobile phase extractions were stable for a longer period of time (several hours to overnight). Therefore, if samples cannot be analyzed immediately, for example, if they must remain in an autosampler for several hours, the TCA method of extraction is not recommended despite the fact that it gives the best precipitation of the proteins and fats. The method may be useful if after the precipitation of the proteins and fats, the solution is either neutralized with base or the TCA is removed from the solution with ether or an amine freon solution. However, these procedures involve extra steps which increases the chance of analytical error as well as the time required for sample preparation.

Chromatographic Methods

Each HPLC method was examined for retention behavior of the vitamers, detection limits, linearity, precision, recovery and mobile phase stability. The samples used for the validation of the RP-TBA and RP-OA methods were prepared using the mobile phases from the respective methods. In the IE method, since the mobile phase had a peak which interfered with C1 and C3, DDI water was used as the extraction solvent instead of the mobile phase.

Retention Behavior

Chromatograms of the extract of the shrimp with and without the three vitamers obtained by the RP-TBA, RP-OA, and IE methods are shown in Figures 2, 3, and 4. In each chromatographic method there were noticeable differences in retention times. Table 2 contains a summary of retention times and capacity factors (k' values) for each of the methods and extraction techniques. The data in the first three columns are for a solution of the vitamers dissolved in water. The data in the other columns are for the vitamers extracted from a spiked shrimp biomatrix. In the columns headed mPA, TCA and MP the vitamers were extracted with mPA, TCA and mobile phase, respectively.

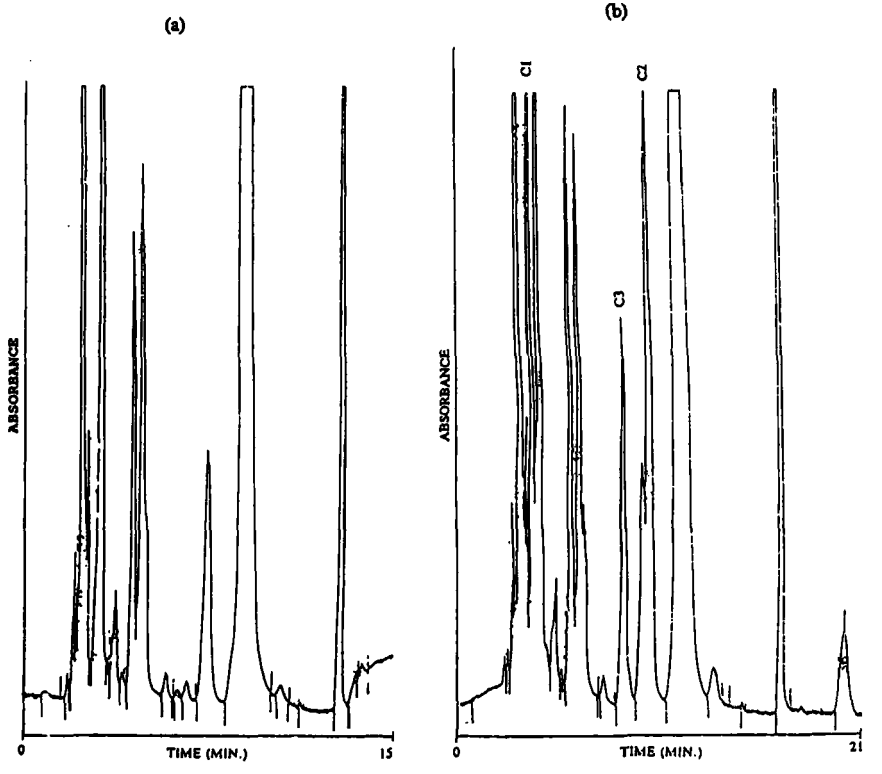

FIGURE 2. Chromatograms (a) and (b) were obtained using the RP-TBA method. The separation was done using a Waters Novapak C_{18} column (30 cm X 3.9 mm ID) with a C_{18} guard column (5 cm X 4.6 mm ID). The mobile phase was an aqueous solution containing 0.08 M NaAc, 0.1 mM EDTA, and 1.0 mM TBA. The pH was adjusted to 4.0 with H_3PO_4. The flow rate was 1.5 ml/min. Chromatogram (a) is the homogenized shrimp sample before the vitamers were added, and chromatogram (b) is the homogenate after the addition of the vitamers.

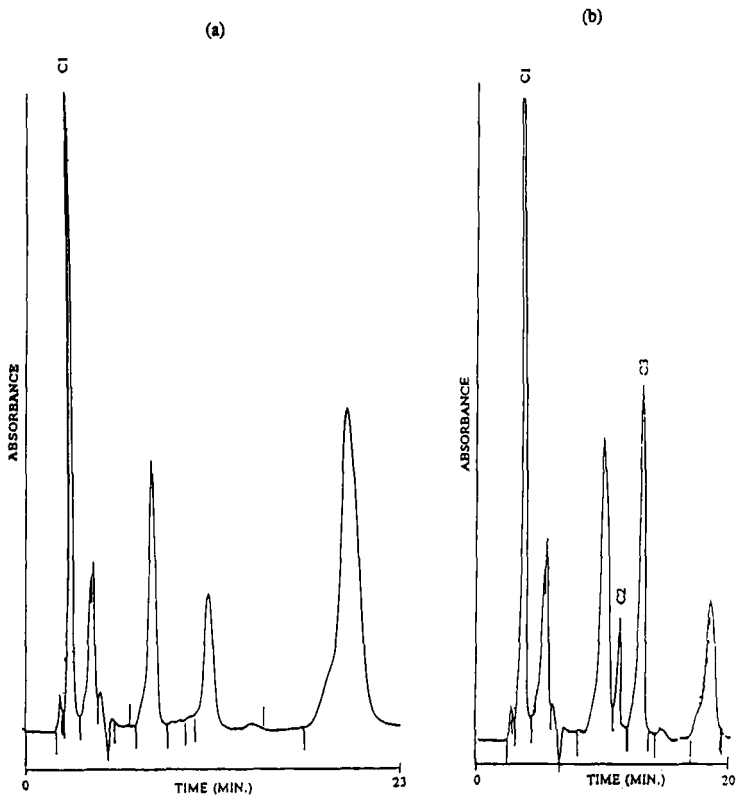

FIGURE 3. Chromatograms (a) and (b) were obtained using the RP-OA method. The separation was done using a Waters Novapak C_{18} column (30 cm X 3.9 mm ID) with a C_{18} guard column (5 cm X 4.6 mm ID). The mobile phase was an aqueous solution containing 0.1 M NaAc, 0.6 mM EDTA, and 1.3 mM OA. The pH was adjusted to 5.0 with HAc. The flow rate was 1.5 ml/min. Chromatogram (a) is the homogenized shrimp sample before the addition of the vitamers, and chromatogram (b) is the homogenate after the addition of the vitamers.

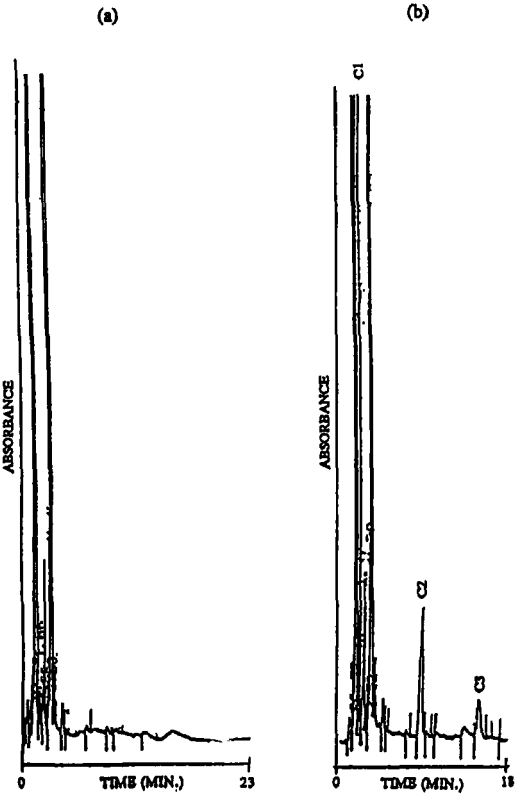

FIGURE 4. Chromatograms (a) and (b) were obtained using the IE method. The separation was done using a Showa Denko Shodex NHpak column (15 cm X 4.6 mm ID). The mobile phase was an aqueous solution containing 0.07 M KH_2PO_4, 2.0 mM EDTA, and 0.02 M NH_4Cl. The flow rate was 1.7 ml/min. Chromatogram (a) is the homogenized shrimp sample before the vitamers were added, and chromatogram (b) is the homogenate after the addition of the vitamers.

TABLE 2

	Retention Time of Standard			Retention Time of Vitamers Extracted From the Shrimp Matrix								
				mPA			TCA			MP		
	C1	C2	C3	C1	C2	C3	C1	C2	C3	C1	C2	C3
RP-TBA	1.99	4.92	4.40	2.00	4.36	4.06	1.98	4.84	4.32	2.02	4.85	4.36
RP-OA	2.38	8.45	11.34	2.39	7.17*	9.57*	2.42	7.95	10.15*	2.38	8.07*	10.35*
IE	1.93	10.40	19.34	1.91	10.08	18.62*	1.91	10.48	18.22*	1.93	9.81	18.06*

	k' of Standard			k' of Vitamers Extracted from Shrimp Matrix								
				mPA			TCA			MP		
	C1	C2	C3	C1	C2	C3	C1	C2	C3	C1	C2	C3
RP-TBA	0.42	2.51	2.14	0.42	2.11	1.90	0.41	2.46	2.08	0.44	2.46	2.11
RP-OA	0.98	6.04	8.45	0.99	4.98*	6.98*	1.02	5.62	7.97*	0.99	5.73*	7.66*
IE	0.61	7.67	15.1	0.59	7.42	14.5*	0.59	7.75	14.2*	0.61	7.18	14.1*

* Peak overlaps with peak in biological matrix or dissolving solvent.

The retention times of C2 and C3 were closer in the chromatograms obtained by the RP-TBA method (Figure 2) than those obtained by the RP-OA method (Figure 3) and IE methods (Figure 4). Using the reverse phase methods, the peaks in the chromatograms of the standards were narrower than those obtained when the endogenous vitamers were extracted from the spiked shrimp matrix. However, the peaks were sharper with the IE method than those obtained by the reverse phase methods (Figures 3 and 4).

Linearity and Detection Limits

Using the RP-TBA method, the concentrations were linear for each of the vitamers in the standard solution in the range of 0.0002 to 0.2 mg/ml. When the endogenous vitamers were extracted from the shrimp matrix, the linear range went down to 0.002 mg/ml of each of the vitamers. The 0.0002 mg/ml sample was not detected due to interferences from the matrix (Figure 2). The correlation coefficients can be found in Table 3.

In the standard solution, the linear range for the RP-OA method was 0.0002 to 0.2 mg/ml for C1, but only 0.001 to 0.2 mg/ml for C2 and C3. Since C2 and C3 are eluted much later than C1, they are broader, and it is more difficult to detect them at low levels. When the vitamers were

TABLE 3

	Linearity w/o Matrix			Linearity w/ Matrix		
	C1	C2	C3	C1	C2	C3
RP-TBA	0.999	0.999	0.999	0.997	0.999	0.999
RP-OA	1.000	0.998	1.000	0.999	0.997	*
IE	0.998	0.999	0.999	1.000	1.000	0.999

* C3 could not be examined for linearity due to an interfering peak from the shrimp matrix which was eluted with C3.

extracted from the biomatrix, the linear range for the vitamers was 0.0002 to 0.2 mg/ml for C1, 0.001 to 0.2 mg/ml for C2, and undetermined for C3. A peak from the shrimp matrix co-eluted with the C3 peak, making it impossible to acquire valid linearity data. (See Table 3 for correlation coefficients.)

Using the IE method, the linear concentration range was 0.0002 to 0.2 mg/ml for C1 and C2 in the standard solution. The linear range for C3 was from 0.001 to 0.2 mg/ml. Since C3 was eluted approximately ten minutes after C2, the C3 peak was broader than that of C2 (Figure 4), causing the lower concentration (0.0002 mg/ml) to be lost in the noise. When the vitamers were extracted from the shrimp matrix, the linear ranges for C1 and C2 were 0.001 to 0.2 mg/ml, and 0.002 to 0.2 mg/ml for C3.

Sensitivity

With the RP-TBA method the lowest concentration of C1, C2, and C3 in the standard solution detected was 0.0002 mg/ml. When the vitamers were extracted from the ground shrimp, the lowest concentration detected was 0.0010 mg/ml of each of the vitamers which corresponds to a level of 10 ppm by weight in the shrimp.

In RP-OA the limit of detection was 0.0002, 0.0010, and 0.0010 mg/ml for C1, C2 and C3 in the standard solution, respectively. When extracted from the shrimp matrix, the detection limit was 0.0002 mg/ml for

TABLE 4

	Reproducibility* (RSD for 6 injections)		
	C1	C2	C3
RP-TBA	3.6%	0.8%	1.7%
RP-OA	1.8	2.4	5.9
IE	15**	2.1	3.5

* Reproducibility samples were aqueous solutions of approximately 0.01 mg/ml of each of the vitamers.

** C1 degraded consistently with time in water. The areas were, in order of injection: 305615, 265771, 236060, 227661, 216091, and 204078. The validation for the other methods was done using the respective mobile phase as the dissolving solvent. Water was used for IE because there is an interfering peak with C3 in the mobile phase.

C1 and 0.001 mg/ml for C2 which correspond to 2 ppm and 10 ppm by weight, respectively. The detection limit for C3 could not be determined due to an interfering peak from the shrimp matrix.

In IE the limit of detection for C1, C2, and C3 in the aqueous solution was 0.0002, 0.0002 and 0.0010 mg/ml, respectively. In the shrimp matrix, the limits of detection for C1, C2, and C3 increased to 0.0010, 0.0010, and 0.0020 mg/ml due to small interferences from the biological matrix. These concentrations correspond to a level of 10 ppm or 20 ppm by weight in the shrimp.

Precision

The three methods were examined for reproducibility. Six injections of a sample at a concentration of 0.01 mg/ml of each of the vitamers in a standard solution was made. The relative standard deviations are listed in Table 4.

The reproducibility of C1 in IE was 15%. This high value is due to the degradation of C1, which is the least stable of the three vitamers and, therefore, the most susceptible to degradation when EDTA is not present. The EDTA chelates with potential C1 oxidizers, preventing the degradation of C1. When samples are prepared in neutral solutions such as water

containing EDTA and mobile phases used in the reversed phase methods, C1 is more stable. The reproducibility of the other vitamers fell in an acceptable range of 1% to 6% RSD.

Recovery of Spiked Samples

For recovery studies, extract samples were prepared from the ground shrimp and each of the three vitamers were added in the range of 0.0002 to 0.2000 mg/ml. Recovery was determined using each of the three chromatographic methods. Recoveries are summarized in Table 5. At levels approaching the detection limit, the percent recovery deviated significantly from 100%. The deviation is due to the difficulty in quantifying accurately the small signal produced.

The IE method most consistently yielded the best recovery although at the lowest concentration levels, the C1 recoveries are abnormally high due to interference peaks from the biomatrix.

Stability of mobile phase

The mobile phases were examined for long term stability. The vitamers of C1, C2, C3 at approximately 0.0100 mg/ml in the aqueous solutions were injected using a batch of mobile phase on the day of

TABLE 5

Recovery

Conc. (mg/ml)	Vitamer	RP-TBA (%)	RP-OA (%)	IE (%)
0.001	C1	84	258	169
	C2	ND	79	81
	C3	32	*	ND
0.002	C1	79	249	126
	C2	185	79	90
	C3	94	*	110
0.010	C1	69	91	107
	C2	120	169	96
	C3	100	*	97
0.020	C1	74	76	104
	C2	108	139	93
	C3	122	*	89
0.100	C1	80	92	102
	C2	100	91	96
	C3	95	*	92
0.200	C1	90	89	103
	C2	105	97	99
	C3	95	*	96

ND = not detected.

* C3 could not be accurately measured due to an interfering peak from the shrimp matrix.

preparation, after 24 hours and after 1 week. In RP-TBA, the retention times of approximately 2.3, 5.7, and 4.9 minutes for C1, C2, and C3, respectively, remained the same after 24 hours. After 1 week the retention times increased to 2.6, 7.6, and 6.6 minutes because of the evaporation of MeOH from the mobile phase.

With the RP-OA system, the retention times for C1, C2, and C3, which were approximately 2.4, 8.6, and 13.0 minutes, respectively, did not change significantly after one week, indicating minimal change in the mobile phase composition.

With the mobile phase for IE, the retention times of C1, C2, and C3 on day 1 were 1.9, 9.3, and 16.7 minutes, respectively. After 1 week the chromatographic peaks were still sharp, but the retention times had decreased and were 1.9, 7.8, and 13.4 minutes which indicates a change in the mobile phase composition.

CONCLUSION

All methods exhibited good linearity and sensitivity. The detection limit for all vitamers separated by the RP-TBA and IE methods was approximately 0.001 to 0.002 mg/ml for each of the vitamers extracted from the shrimp matrix. These limits correspond to a level of

approximately 10 ppm to 20 ppm by weight. The C1 has a lower detection limit due to the fact that it was eluted first in all methods, and there was no band broadening. C3 could not be accurately measured by RP-OA due to a peak from the shrimp matrix that was co-eluted with C3.

In the aqueous solution of the vitamers, the RP-TBA and IE methods exhibited better selectivity than the RP-OA method, which does not completely resolve C2 from C3. From day to day, these peaks may overlap significantly or not at all, indicating that this method was not reproducible. This phenomenon is exaggerated when vitamers are extracted from the shrimp matrix since the vitamer peaks are broader. In RP-OA, C2 and C3 have a distorted peak shape. They have a slight shoulder on the front of the peak. The IE method gave complete separation of C1, C2, and C3 on a daily basis. In addition, the peak shapes of C1 and C2 in the IE method are much sharper than those in the other two methods. However, it has a higher detection limit than the other methods.

The reproducibility of all vitamers is good in all methods except for C1 in IE an C3 in RP-OA. At 15% RSD, precision is questionable. The samples prepared for IE reproducibility were prepared in water, unlike the RP-TBA and RP-OA samples which were prepared in their respective

mobile phases. The mobile phase appears to have a stabilizing effect on the vitamers, and thus minimizing degradation. As long as the sample prepared in water is injected immediately, the effect of the degradation is minimized to an acceptable level.

Since the shrimp samples were prepared in a neutral water matrix for analysis by the IE method and in an acidic mobile phase for the reversed phase methods, the acidic mobile phases may have broken the protein bonds to substrates such as amino acids. Thus, many of these compounds may now be free, causing interferences in the chromatograms. As a result, the chromatograms generated by IE appear cleaner than those generated by RP-TBA and RP-OA.

At present, the recommended method for use in the analysis of the three vitamers of vitamin C in Penaeus Setiferus is IE. It provides the complete separation of the three vitamers, and there is interference from the shrimp matrix only near the void volume in the chromatogram. The only requirement is that the samples must be injected immediately after preparation to minimize the degradation. However, work is proceeding in our laboratory to determine if the problem of sample stability and reproducibility of C1 values is due to the solvent systems used in the extraction procedure. Therefore, the effect of pH, EDTA, and presence of

ion-pairing agent on the stability of the vitamers as well as precision of the C1 values are being investigated and will be reported in the near future.

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