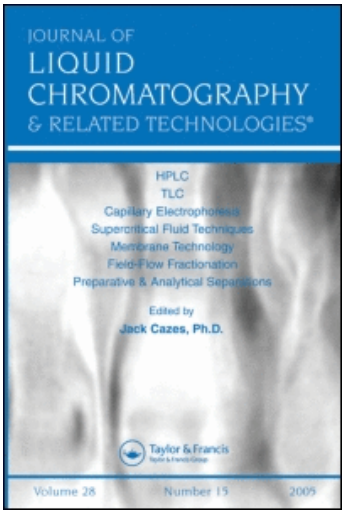

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A. M. Hapette[a]; S. A. Poulet[a]
[a] Station Biologique (C.N.R.S. UPR 4601 and Université Paris 6), Roscoff, France

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APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO THE DETERMINATION OF ASCORBIC ACID IN MARINE PLANKTON

A. M. HAPETTE AND S. A. POULET
Station Biologique
(C.N.R.S. UPR 4601 and Université Paris 6)
Place Teissier, 29211 Roscoff, France

ABSTRACT

Reversed-phase and anion-exchange high performance liquid chromatography, which offer a great potential for the measurement of vitamin C in plankton, were compared using different columns and reagents. A method using a polystyrene divinyl benzene column is described that has been utilized for rapid separation and quantification of L(+)-ascorbic acid in marine plankton.

INTRODUCTION

Research on ascorbic acid (vitamin C) content and metabolism in marine plankton, as well as its ecological importance, has been hindered by the lack of a sufficiently sensitive assay for this ubiquitous micro nutrient.

In the past two decades several methods have been described, ranging from conventional techniques such as titration (1), spectrophotometry (2), polarography (3), microfluorometry (4) and colorimetry, (5) to more recent high-performance

liquid chromatographic methods using anion-exchange (6-7-8) reversed-phase (9), ion-paired reversed-phase (10-11-12-13) and bonded-phase (11) separation techniques, coupled either to electrochemical (7-14-15), fluorescence (16) or UV (6-11-12) detection. High-performance liquid chromatography (HPLC) appears to be one of the most appropriate method for the determination of vitamin C in plankton, even though some methods are limited by interference from sugar, sulfur dioxide and colored material in the sample (17). Furthermore, HPLC methods designed for the measurements of vitamins in tablets or in vitamin-enriched food products (17-18), may not be transferable to plankton, due to extraction and detection limitations. Methods with low sensitivity, which require large amounts of sample (e.g. a g, or more) are not applicable because of the limited amount of sample often available (e.g. mg or µg scale).

This paper compares several ascorbic acid analyses performed with reversed-phase and anion-exchange liquid chromatography with UV detection, using different mobile phases and extraction solvents, which have been used previously (6-7-11), in order to determine the best conditions for the measurement of vitamin C content in a variety of marine planktonic organisms.

EXPERIMENTAL

APPARATUS

A Milton Roy liquid chromatograph system, equipped with a multiple solvent delivery pump (CM 4000), a programmable wavelength detector (SM 4000, including a 14 µl detection chamber), a Rheodyne injector valve (Model 7125) with a sample loop of 20 µl, and a CI-10 integrator, was used.

Three stainless steel columns (250 x 4.6 mm I.D.) were compared; one anion-exchange type - Synchropak AX 300 (300 Å, 6.5 µm; Synchrom Inc.), and two reversed-phase types - Nucleosil C-18 (120 Å, 5 µm; Hichrom) and a PLRP-S (100 Å, 5 µm; Polymer Laboratories). Details on the columns are given in Table I.

Distilled, deionized water was used in the preparation of some extraction solvent and mobile phases. Five different extraction solvents (3% metaphosphoric acid with 8% acetic acid; 100% methanol; 100% ethanol; 100% acetonitrile

TABLE I. CHROMATOGRAPHIC DETAILS FOR EACH COLUMN.

	COLUMN		
	REVERSED-PHASE		ANION-EXCHANGE
	PLRP-S	C-18	SYNCHROPAK AX 300
DEAD TIME: to (min)	1.78	1.53	1.87
RETENTION TIME: tr (min)	1.97	1.87	9.97
CAPACITY FACTOR (K')	0.11	0.22	4.33
PEAK EFFICIENCY (N)	50000	60000	45000
MAXIMUM OPERATING PRESSURE (atm)	200	400	400
SURFACE AREA (m^2/g of resin)	550	200	200

(Aldrich, Chemical) and 100% distilled water) were tested with each column, under two different chromatographic conditions (i.e. sodium acetate or hexane sulfonic acid as mobile phases).

CHROMATOGRAPHIC CONDITIONS

The 0.07 M sodium acetate mobile phase was made of sodium salt trihydrate (100%) adjusted to pH 5.20 with acetic acid diluted in water (50:50). It was degassed with helium for 15 min at 0.3 bar before use. The flow rate was 1.5 ml/min. The absorbance at 266 nm (i.e. the wavelength of maximum absorption of vitamin C under the analytical conditions) was monitored at a chart speed of 10 cm/min.

Another potential mobile phase, hexane sulfonic acid, (18) was compared to sodium acetate eluant, under similar conditions using the same PLRP-S column.

SPECIFIC ANALYTICAL CONDITIONS

At the end of each run and before the next injection, the HPLC column was rinsed with mobile phase for 8 min at a constant flow rate of 1.5 ml/min, until absorbance background levels returned to zero. Column cleaning was achieved after long usage ($\sim$1 month; or $\sim$ 200 samples), with a 0.1% trifluoroacetic acid 10% methanol mixture for 10 min at 1.5 ml/min. The sample loop was cleaned before each run with 200 µl of extraction solvent.

CHOICE OF EXTRACTION SOLUTION

The results in Tables II and III show that similar measurements could be obtained with acetonitrile or metaphosphoric acid as extraction solutions, while results with the 4 other solutions were not as good. Although measurements of vitamin C contents were often at time similar for the two best extraction solutions with acetonitrile giving slighly higher results, metaphosphoric acid was selected because of reduced absorbance with acetonitrile (Fig.1; A & B-1,2 : 8 times lower than metaphosphoric acid). Thus, metaphosphoric acid is recommended in plankton research, where sensitivity is crucial.

TABLE II. COMPARISON BETWEEN THE MEASUREMENT OF VITAMIN C CONTENT IN ZOOPLANKTON SAMPLES, ORIGINATING FROM THE SAME STOCK OF CALANUS HELGOLANDICUS (COPEPODA, CRUSTACEA), USING DIFFERENT COLUMNS AND EXTRACTION SOLVENTS. THE MOBILE PHASE WAS SODIUM ACETATE, pH 5.20. UNIT OF VALUES: RELATIVE RESPONSE, SETTING PLRP-S RESULTS WITH ACETONITRILE EQUAL TO 1.

	COLUMN		
	REVERSED-PHASE		ANION-EXCHANGE
SOLVENT OF EXTRACTION	PLRP-S	C-18	SYNCHROPAK AX 300
METAPHOSPHORIC ACID + ACETIC ACID	0.85	0.26	0.08
METHANOL	0.23	0.36	0.15
ETHANOL	0.34	0.28	0.07
ACETONITRILE	1.00	0.16	0.06
DISTILLED WATER	n.d.	0.36	0.09

n.d. : not detectable

TABLE III. COMPARISON BETWEEN MOBILE PHASES AND EXTRACTION SOLVENTS WITH REFERENCE TO THE MEASUREMENT OF THE ASCORBIC ACID CONTENT IN ZOOPLANKTON SAMPLES (SAME STOCK OF CALANUS HELGOLANDICUS), ANALYSED WITH THE SAME PLRP-S COLUMN. UNIT OF VALUES: SAME AS IN TABLE I.

MOBILE PHASE	EXTRACTION SOLVENT					
	METAPHOSPHORIC ACID	METHANOL	ETHANOL	2-PROPANOL	ACETONITRILE	DISTILLED WATER
SODIUM ACETATE	0.85*	0.23*	0.34*	0.31	1.00*	n.d.*
HEXANE SULFONIC ACID	0.41	d.d.	0.27	0.14	n.d.	n.d.

*: Same as in Table 1
n.d. : not detectable
d.d. : not determined (e.g. poor resolution of peak)

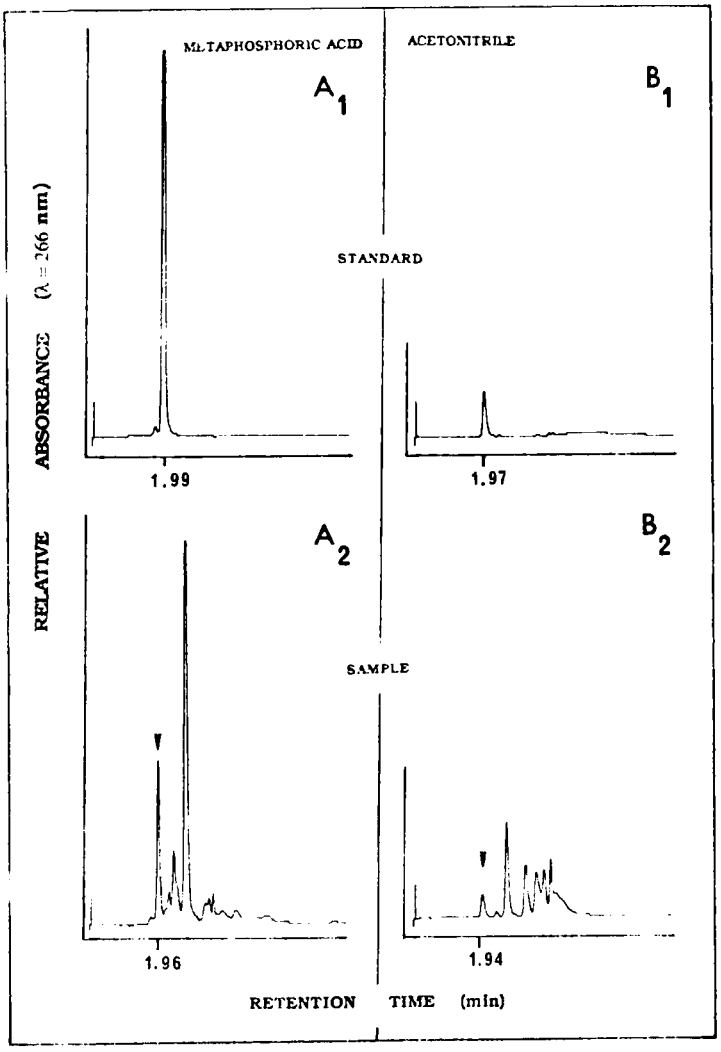

Fig. 1. Chromatograms of ascorbic acid in standards (1), and zooplankton samples (arrow) (2) (same stock of *Calanus helgolandicus*: Copepoda, Crustacea). Comparison of extraction solvent for vitamin C using: (A)-3% Metaphosphoric acid-8% Acetic acid, and (B)-100% Acetonitrile. Standard: 0.2 µg of L(+)-Ascorbic acid /µl (Merk). Sample: 250 mg (wet weight of copepods) in 500 µl of solvent. Reversed-phase PLRP-S column, mobile phase: 0.07 M sodium acetate pH 5.20; flow rate: 1.5 ml/min; and same attenuation (0.01 sensitivity threshold, 1 mv full scale).

The relationship between sample weight, vitamin C content and volume of the extraction solvent seems to be critical, and must be estimated in order to obtain consistent results. This question was considered with sub-samples of constant weight (30 mg, wet weight) extracted with 100-1500 µl metaphosphoric acid. The content of vitamin C varied from 86 to 114 µg/g, between 100 to 500 µl; from 500 to 1500 µl, a stable value of $\sim$ 126 µg vitamin C/g was obtained.

PREPARATION OF STANDARDS AND SAMPLES

Standards of L(+)-ascorbic acid (Merck) were prepared daily and stored at +4°C in the dark. The stability of the standard was checked daily and was found to be stable for $\leq$ 4 days.

Fresh zooplankton samples were weighed, either stored in liquid nitrogen, or quickly (2 min) ground in extraction solution at constant temperature (18°C) in a potter, and centrifuged for 10 min at 10^4 RPM prior to analysis. Phytoplankton samples were collected on glass fiber filters (Whatman GF/F pre-conditionned at 400°C) and biomass was estimated either on the basis of cell number, or chlorophyll *a* per unit volume of seawater, or by cell weight assuming a density of 1. Wet weight was used, instead of a dry weight unit, to avoid the potential degradation of vitamin C by heat.

RESULTS AND DISCUSSION

CHOICE OF COLUMN

The absorbance chromatograms of ascorbic acid standards and zooplankton samples, analyzed under optimized conditions (Fig. 1; A) were compared to chromatograms obtained under different conditions (Fig. 1; B). Results in Table II compare the measurement of ascorbic acid in sub-samples, originating from the same stock, obtained with three different columns and five extraction solvents. Considerable variability was observed among the various techniques. The highest response was obtained with the PLRP-S column for samples extracted with metaphosphoric acid or acetonitrile.

Recovery (known amount of ascorbic acid in standard solutions added to zooplankton samples) was 103-115 % for Synchropak AX 300 and PLRP-S, and only 76% for Nucleosil C-18. Under similar conditions the PLRP-S column demonstrated the highest absorbance, the best reproducibility, corresponding to a relative standard deviation of 0.41%, and showed the most stable retention time, with a drift= 0.40% of the mean. When recovery is not 100%, oxidation of ascorbic acid, into its degradation products (e.g. dehydroascorbic acid, diceto-L-gulonic acid, oxalic acid), is suspected (19). Normaly, the presence of metaphosphoric acid retards the oxidation of ascorbic acid, but addition of sulfite also could suppress vitamin C oxidation. As many as 400 samples could be analysed using the same column whereas the Synchropak AX 300 column deteriorated much sooner ($\sim$ 100 samples), as characterized by increased peak width and decreased retention time. The PLRP-S column appears to be the best column of those investigated for analysing ascorbic acid in plankton.

A weak anion-exchange column PolyWAX LP (100 x 4.6 mm I.D.; 300 Å, 5 µm; PolylC) was also used for the study of different zooplankton samples (20), using the same HPLC conditions as those reported in the legend of Figure 1; A. Results were better than for Synchropak, and were comparable to PLRP-S (Table II). However, the lifetime of this type of column was the lowest ($\sim$ 1 week).

CALIBRATION

Calibration of the method showed that the relationship between vitamin C content in standard solutions and relative peak area was linear from 0 to 0.15 mg vit. C/ml (regression equation: $Y=7.265 \times 10^{-7}X - 0.108$; r=1 ; n=8). The detection limit was 0.108 µg/ml $\pm$ 0.842 ($p \leq 0.01$). From 0.15 to 0.40 mg/ml, linearity was modified, as reflected by the increase of the value of the slope of the regression equation ($a=1.275 \times 10^{-6}$). Beyond this upper concentration threshold, double peaks appeared as a result of column saturation.

CHOICE OF MOBILE PHASE

The effects of two mobile phases on the detection of ascorbic acid (pK_{a1}= 4.17; pK_{a2}= 11.57) in samples was tested using the same PLRP-S column in

conjunction with six different extraction solvents (Table III). We found that peak separation and reproducibility of measurements were optimal with the sodium acetate mobile phase (pH 5.20). The pH was tested in the 2.50-7.00 range: absorbance became stable at pH $\geq$4.80. Similar test were performed with the two other columns, but detection was not satisfactory. It is obvious that the absorbance spectrum of vitamin C is highly pH dependent. It is known that the absorbance corresponding to the isobestic point (λ= 242 nm) is not pH sensitive. However, maximum absorbance obtained at λ= 266 nm corresponds to the reduced form of ascorbic acid; whereas the oxidized form (e.g. dehydroascorbic acid), the maximum absorbance of which is obtained at λ= 230 nm, was not considered in this study.

PRESERVATION OF SAMPLES

The question of the short (days to weeks) and long term (months) preservation of samples stored in liquid nitrogen was considered. Results demonstrated that during a week or a 7 months period, loss of vitamin C was less than 12% of the initial content measured in freshly collected zooplankton samples.

ANALYSIS OF PLANKTON

Results of ascorbic acid determinations in marine plankton, using optimized conditions (Fig. 1; A-1,2) are summarized in Table IV. Quantification of vitamin C in plankton was performed with reference to standards containing known concentrations of L(+)-ascorbic acid. Other chromatographic peaks in A2 and B2 were not identified. Occurrence of vitamin C in zooplankton samples was verified (20), using tandem mass spectrometry (MS-MS) (21) as well as specific HPLC tests, such as sample spiking and double wavelength scanning at wavelengths ranging from 210 to 300 nm. High ascorbic acid concentration found in phytoplankton is likely related to their ability to synthesize vitamin C (22). High contents also were measured in herbivorous and omnivorous zooplankters, which feed on algal cells and presumably accumulate this nutrient. Vitamin C content in carnivorous species was much lower. This result has been confirmed in a larger spectrum of species (Hapette and Poulet, unpublished data). We assumed that it

TABLE IV. MEAN CONTENT OF VITAMIN C IN MARINE PLANKTERS BELONGING TO THREE TROPHIC LEVELS IN THE MARINE FOOD CHAIN.

	PHYTOPLANKTON	ZOOPLANKTON		ICHTHYOPLANKTON
	CHLOROPHYCEAE (Autotrophic)	COPEPOD (Carnivorous)	COPEPOD (Herbivorous)	FISH LARVAE (Omnivorous)
SPECIES	*Dunaliella tertiolecta*	*Anomalocera patersoni*	*Calanus helgolandicus*	*Sprattus sprattus*
VIT. C CONTENT (µg/g wet weight)	79.81 (347 pg/10^3 cells)	11,66	194.68	204.03
	n=4	n=2	n=4	n=5

n : number of samples

should be related to the third rank of carnivorous species in the marine food chain, and also to their hypothetical unability to synthesize this compound (22).

As reported earlier (15), ascorbic acid values obtained for biological samples vary considerably depending on the method employed. Therefore, choice of a method should be made with some caution. Our method is rapid and reliable. A similar column has been used to measure ascorbic acid in fruit juice (24). The sensitivity and extraction efficiency are sufficient for the measurement of vitamin C in marine plankters originating from various trophic levels in the marine food chain, despite their small biomass.

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