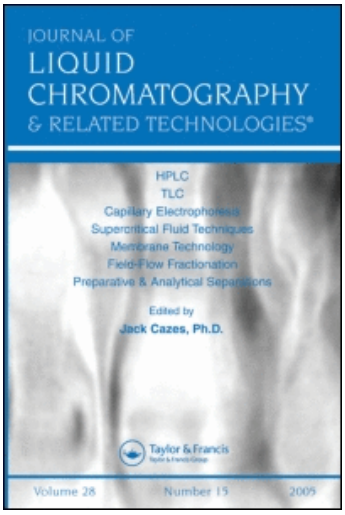

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Non—Ion-Pairing HPLC Method for Measuring New Forms of Ascorbate and Ascorbic Acid

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To cite this Article Felton, S. P. , Grace, R. and Halver, J. E.(1994) 'A Non—Ion-Pairing HPLC Method for Measuring New Forms of Ascorbate and Ascorbic Acid', Journal of Liquid Chromatography & Related Technologies, 17: 1, 123 — 131
To link to this Article: DOI: 10.1080/10826079408013439
URL: http://dx.doi.org/10.1080/10826079408013439

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(1), 123-131 (1994)

A NON-ION-PAIRING HPLC METHOD FOR MEASURING NEW FORMS OF ASCORBATE AND ASCORBIC ACID

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ABSTRACT

A new high-performance liquid-chromatography (HPLC) method separates vitamins L-ascorbic acid (C_1) and L-ascorbyl-2-sulfate (C_2). Previous HPLC reverse phase methods utilized ion-pairing reagents and resulted in co-elution with other substances. Vitamin C_1 preceded C_2 in retention time.

In this new method, which deletes the ion-pairing reagent, vitamin C_2 precedes the elution of vitamin C_1. This new direct method allows for a better separation from other conflicting metabolites and constituents of extraction mixtures. In addition, this method provides a way of identifying ascorbyl-2-monophosphate (C_3), a form of vitamin C that may be found in animal diets.

Examples will be shown of HPLC profiles of extractives from tissue and diets known to contain these new forms of vitamin C.

INTRODUCTION

There have been recent advances in the development of more stable forms of L-ascorbic acid, bringing increased demands for more precise and direct methods of analysis for these esters. A number of methods, which are available in the literature, offer reliable assays for vitamins C_1 and C_2 if the mixtures are not too complex (1,2,3,4,5). A recent comparison found positive and negative aspects in three different methods for the measurement of C_1 and C_2 in shrimp tissue (6).

There are a number of vitamin C_1 methods that employ ion-pairing reagents (IPR) to obtain delayed retention times. Schüep et al. (4) demonstrated very effectively the influence that IPR (1,5-dimethylhexylamine) exerted upon retention times of vitamin C_2 on an ODS Hypersil column. Using a concentration range from 0.2 to 1.0 ml/L of IPR in the mobile phase, the retention times ranged from 5.62 min. to 22.58 min.

Between 1985 and1989, this laboratory used two 10-micron C_{18} µBondapack columns(250mm x 4.6mm) in tandem. The solvent system used was a 0.1 M sodium acetate at pH 5.0 with an n-octylamine concentration of 0.17 ml/L plus 200 mg/L disodium ethylene-diamine tetra-acetic acid (EDTA). A flow rate of 1.25 ml/min was maintained. Utilizing this protocol, it was possible to maintain consistent retention times for vitamins C_1 and C_2. In a 1987 publication (3), a method was reported using the above protocol for the separation of C_1 and C_2. The retention times for that method were 6.0 min. for C_1 and 11.0 min. for C_2. In contrast, two years later, retention times were 4.8 min. for C_1 and 8.0 min. for C_2, as reported at a meeting in New Orleans (7). These retention times remained consistent for a period of about two years.

However, in 1990, when this system was put into use again for C_1 and C_2 analyses, the µBondapack column no longer produced the same results. Two additional C_{18} type columns were tried, also with varying results. Since 1990, when using n-octylamine as an IPR and C_{18} reverse-phase types of columns, this laboratory has experienced variability of retention times for C_1, and particularly C_2. Using a concentration of 0.17 ml/L and a static equilibration period of one night, as well as a mobile equilibration of 2.5 hours, the retention times varied from 5.75 min. to 6.75 min. for C_1 and from 7.5 min. to 14.5 min. for C_2. The result was an overlap of peaks, making it more difficult to quantify. The purpose of this paper is to present a way to circumvent some of the difficulties encountered when using this type of technology.

A new development in column technology eliminates the need for ion-pairing reagents, offering the possibility of an HPLC method to separate smaller, less complex molecules. In addition, this column retains many of the characteristics of existing C_{18} reverse-phase columns.

METHOD AND MATERIALS

The analytical column used is an Alltima (Alltech Associates) 5 µm C_{18} reverse-phase column, 250 mm x 4.6 mm. A hand-packed Alltima guard column is used to protect the analytical column.

The HPLC equipment used is a Perkin-Elmer Corporation(P & E) model 250 isocratic pump with an in line P&E solvent filter, a P&E model 290 UV/Vis detector, set at 254 nm, and a Bioanalytical Systems Inc. (BAS) electro-chemical detector, set at +0.72 volts. The data system is linked to a PE Nelson model 950 interface and an Epson III+ computer. The software used was the PE Nelson 2100.

Special chemicals used were n-octylamine and sweet potato acid phosphatase enzyme (Sigma Chemical Co.).

Tissue was homogenized with a Brinkmann polytron homogenizer and centrifuged with an Eppendorf minifuge. The extracts were denatured by microwave (MW) with a Panasonic commercial 600-watt oven and with trichloroacetic acid (TCA).

Two solvent systems were utilized: (1) 0.10 M sodium acetate pH 5.0 with 200 mg of sodium EDTA per liter, (2) 0.10 M ammonium acetate pH 5.0. For comparative purposes, n-octylamine at 0.17 ml/L was added to solvent (1). The first solvent was utilized whenever the need was only for quantification, whereas the second solvent was used when further purification of the peaks was desired in addition to quantification. The flow rate was 0.75 ml per minute at a pressure of 2200 psi. Flow rate should not exceed 1.0 ml/min.

Tissue extracts were made as follows: (1) The MW extract was made by homogenizing 1 volume of tissue to 2 volumes of glass distilled (GD) water for 30 sec. The homogenate was placed in a sealed Teflon tube and microwaved for 1 min. at a power setting of med-low, and then at medium setting for another 30 sec. The denatured extract was diluted to a total volume of 10 ml, re-homogenized for 1 min., and centrifuged for 4 min. at 15,000 RPM. Supernatant solution was decanted and filtered through a 0.45 μm syringe filter. The filtered solution was used for the HPLC determination. (2) The TCA extract was made by homogenizing 1 vol. of tissue to 4 vols. of GD water for 30 sec., and while homogenizing, 5 vols. of 10% TCA was added and homogenizing was continued for another 60 sec. This extract was centrifuged and filtered as above and then injected into HPLC.

RESULTS AND DISCUSSION

Three very significant results were obtained with this new method:

(1) Very consistent retention times, as well as a rapid equilibration time, were found for vitamins C_1 and C_2. Table 1 compares the retention and equilibration times between the Alltima and conventional C_{18} reverse-phase columns. The day-to-day retention times varied

TABLE 1

Equilibration time comparisons of Vitamins C_1 and C_2

STANDARD C_{18} COLUMN

HPLC Run	#1	#2	#3	#4	#5	#6	#7	#12
VIT C_1	5.6	5.7	5.75	5.78	5.8	5.83	5.92	5.95
VIT C_2	6.0	6.2	6.5	6.62	6.73	6.9	7.06	7.40

ALLTIMA COLUMN

HPLC Run	#1	#2	#3	#4	#5	#6	#7	#8
Vit C_2	4.71	4.72	4.72	4.72	4.71	Equil.	Equil	Equil.
Vit C_1	6.2	6.23	6.22	6.20	6.21	Equil.	Equil.	Equil.

Columns were eluted with 0.1 M sodium acetate pH 5.0 with 200 mg/L EDTA. The standard C_{18} column required an added 0.085 ml/L of n-octylamine as an IPR. The Alltima column did not require the IPR addition. Note in the standard C_{18} column an additional 7 runs were required before equilibrium was achieved (#6 to #12). Time in minutes.

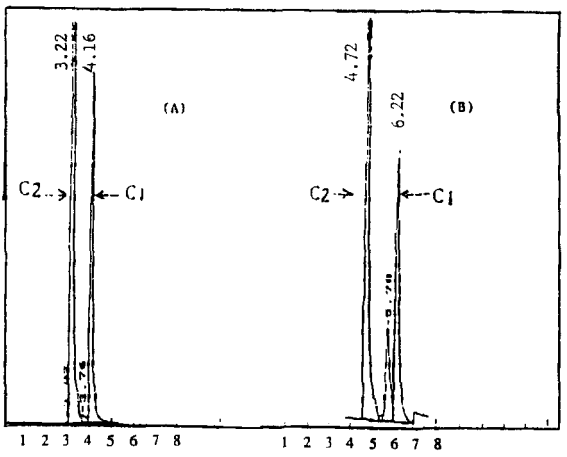

Figure 1. A. L-ascorbyl-2-sulfate and L-ascorbic acid eluting with ammonium acetate solvent. Time scale in minutes. B. Same standards eluting with sodium acetate solvent.

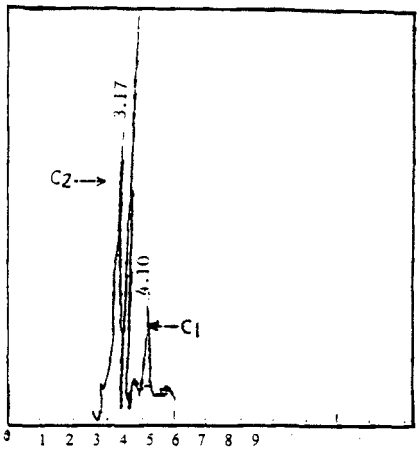

Figure 2. Detection limits for L-ascorbyl-2-sulfate and L-ascorbic acid eluted with ammonium acetate solvent (2 ng and 1 ng, respectively). Time scale in minutes.

less than 0.02 min. for both vitamins on the Alltima column. Figures 1A and 1B illustrate the retention times for the two vitamin standards in two different solvent systems on the Alltima column. Figure 2 represents the detection limits of vitamins C_1 and C_2 eluted from the Alltima column with ammonium acetate solvent (C_1 = 1 ng; C_2 = 2 ng).

(2) Using the Alltima column, vitamin C_2 elutes almost immediately after the injection volume has eluted, as compared with the longer retention time when using the IPR, n-octylamine, and the standard C_{18} column. This shorter retention time for C_2 allows for a reduced chance of co-elution with other substances. Vitamin C_1 unlike C_2 can be easily verified by electro-chemical detection as well as UV. Therefore C_1's later elution presents a lesser problem. Figure 3 illustrates the early elution of vitamin C_2 in a typical microwave extract of rainbow trout muscle.

(3) Even though their retention times are only 0.2 min. apart, vitamins C_2 and C_3 may be distinguished from one another in this system with the use of sweet potato acid phosphatase. The subsequent measurement of C_3 is indicated by an increase of C_1 concentration. The increase occurs when C_3 is dephosphorylated. This method is useful when analyzing diets containing both vitamins or when determining the stability of vitamins C_2 and C_3. Figure 4A is the HPLC profile of standards C_1, C_2 and C_3 eluted with ammonium acetate as the mobile phase. Figure 4B is the profile of the same standards pre-incubated at

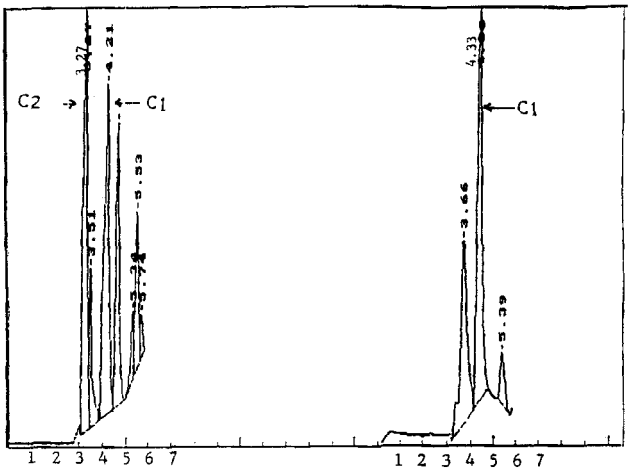

Figure 3. A typical microwave(MW) denatured extract profile(UV detector) from rainbow trout muscle which had been fed a normal vitamin C concentration of 120 mg/Kg; on the right an EC detector profile. Both were eluted with ammonium acetate. Time scale in minures.

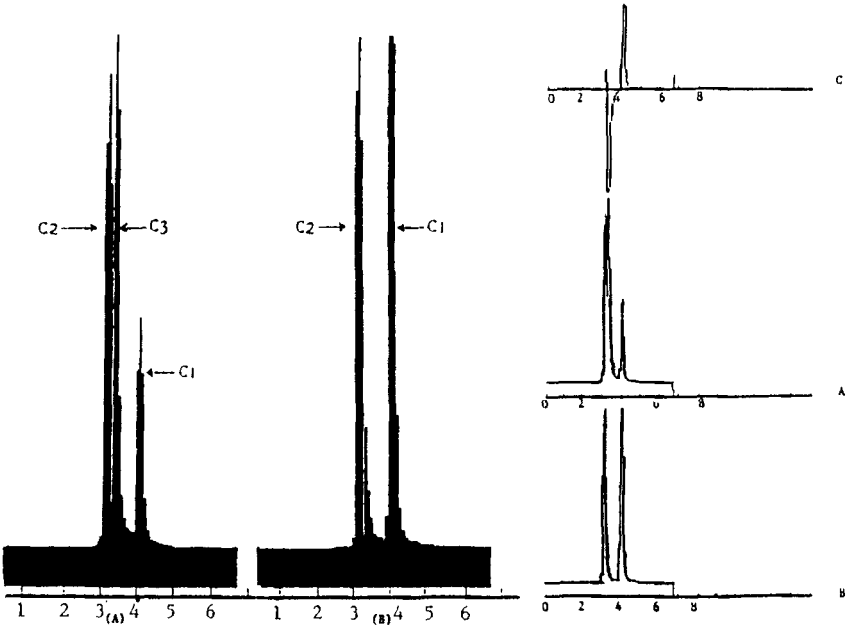

Figure 4. A. The solid profiles of standards L-ascorbyl-2-sulfate, L-ascorbyl-2-monophosphate and L-ascorbic acid eluted with ammonium acetate solvent. B. The same standards after exposure to sweet potato acid phosphatase enzyme (exposure for 30 min. at room temperature).
(Compilation) The same data shown in figure 4 A and 4 B. In the top profile (C) the difference is shown after the enzyme treatment. Note the negative profile which graphically depicts the loss of L-ascrobyl-2-monophosphate. Time scale in minutes.

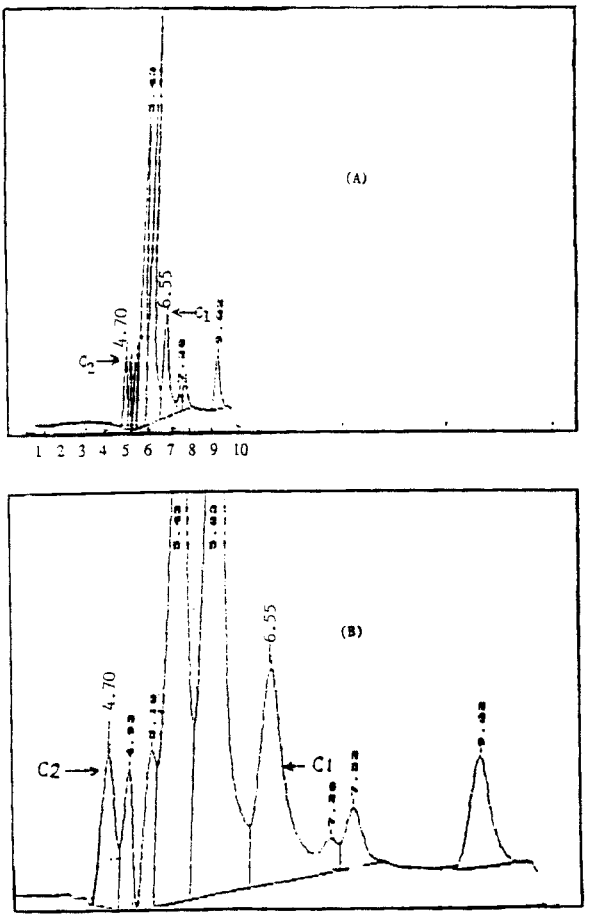

Figure 5. A. A profile of a 5% trichloroacetic acid(TCA) extract from the carcass of a coho salmon smolt fed a diet containing 120 mg/Kg L-ascorbic acid and eluted with ammonium acetate. B. An enpanded portion of the profile showing the vitamin C_2 and C_1 peaks. Time scale in minutes.

room temperature with sweet potato acid phosphatase enzyme. Note that the C_1 component of the profile has increased, whereas the C_2/C_3 double peak has disappeared. Figure 4 (compilation) is a computer-generated profile of the standards shown in the previous Figures 4A and 4B. A ratio differential after the enzyme treatment is shown in the top profile.

Figures 5A and 5B show additional uses of the Alltima column for determining C_1 and C_2 concentrations in fish tissue. Figure 5A represents a typical 5% TCA extract of freeze-dried powder from whole-body tissue of a coho salmon reared on a diet containing 120 mg/kg of C_1. Solvent system was sodium acetate. Figure 5B is a computer expansion profile of the retention time periods of C_1 and C_2. Note that the two peaks of interest are well differentiated.

CONCLUSIONS

(1) Retention times for L-ascorbyl-2-sulfate and L-ascorbate are more consistent when using the Alltima column (without an ion-pairing reagent) than when using a standard C_{18} column (with the reagent).

(2) Equilibrium is more quickly achieved using the Alltima column than when using the standard C_{18} which uses the ion-pairing reagent.

(3) L-ascorbyl-2-sulfate elutes almost immediately after the injection volume has eluted from the Alltima column (as compared with the longer time required when using the ion-pairing reagent of the standard C_{18} column). This C_2 shortened retention time reduces the chance of confusion with other eluted peaks. L-ascorbate unlike L-ascorbate-2-sulfate can be differentiated by dual detection of UV and EC. Therefore, L-ascorbate's longer elution time presents less of a problem.

(4) Salt-free eluates of desired peaks may be obtained with the use of ammonium acetate mobile phase rather than sodium acetate and the ion-pairing reagent. These eluates may then be further purified for more rigorous identification.

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Received: May 27, 1993
Accepted: June 11, 1993