# An Improved Method for Assay of Vitamin C in Fish Feed and Tissues

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## Key Words

Column liquid chromatography
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## Summary

A method was devised to assay four forms of vitamin C: L-ascorbic acid (AA), dehydroascorbic acid (DHA), ascorbate-2-mono- and polyphosphate (AMP, APP), as well as ascorbate-2-monosulphate (AMS), in sample series of different fish tissues and feed. Direct and indirect detection were combined. Sample extractions were carried out with 0.2 mol $L^{-1}$ sodium acetate buffer (pH 4.8) and extracts were deproteinized after different chemical or enzymatic reactions, with perchloric acid. The DHA was reduced to AA with dithioerythritol (DTE). Ascorbate oxidase enzyme was used for the detection of background and an acidic phosphatase enzyme for the hydrolysis of different phosphate esters. Ascorbate-2-sulphate was detected directly with help of coinjection of the compound. Chromatographic analysis was carried out with a single column isocratic reverse phase method. The mobile phase was an aqueous buffer of 0.04 M sodium-acetate, 0.05 mM EDTA, 0.5 mM tetrabutylammonium dihydrogen phosphate (TBA) adjusted to pH 3.76 with 85 % $H_3PO_4$ and with 24 mL methanol added to 1000 mL. C-18 columns were used with 0.6 mL $min^{-1}$ flow rate at 23 °C. The vitamin C forms were detected by UV absorption at 250 nm. The determination limit was 1.0–5.0 µg $g^{-1}$ in AA equivalent. The standard deviations were between 1–6 % and depended on the concentrations of vitamin C forms and tissues. Recoveries were between 90–96 % in samples.

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## Introduction

Since the antiscorbutic effects of L-ascorbic acid were first discovered [1] the physiological role and requirement of this micro-nutrient vitamin has been studied extensively. Ascorbic acid or, as it was named by Szent-Györgyi and Haworth [2] vitamin C, has a key role in protecting living organisms from the dangerous action of free radicals [3] and it is an essential co-factor for optimal activation of enzymes involved in various hydroxylation reactions [4]. Since a requirement of vitamin C was first demonstrated for fish [5] several studies have been proved that vitamin C is essential to most fish [6–9] due to the lack of gulonolactone oxidase enzyme. Therefore most fish require vitamin C when kept in artificial conditions. Fish can store a part of this micronutrient in chemically stable forms [10] but controversy surrounds the storage formula in different tissues of fish [11].

Because L-ascorbic acid is unstable in storage [12] and dissolves almost immediately, especially in marine water [13], different stable derivatives (ascorbate-2-monosulphate, ascorbate-2-monophosphate, ascorbate-2-polyphosphate and palmitate) are used in commercial diets. It is questionable whether fish species are necessarily able to digest all chemically stable forms.

Numerous methods have been published for the assay of AA and its different derivatives but in most cases these relate to the analysis of AA in foods for human use, especially fruits, fruit juices and vegetables, which contain high levels of vitamin C. An analytical review was published by Pachla et al. [14], and included spectrophotometric, electrochemical, enzymatic and chromatographic methods of ascorbic acid determination. A further review considers HPLC methods for water soluble vitamins and describes various samples and preparations, columns, buffers and detectors [15].

A complete methodology for application in fish studies has not yet been standardised and, often, the reported methods do not define the vitamin C forms correctly. For example if the extraction is carried out with dithioerythritol or derivatives (DTT, DTE), peak C1 means AA + DHA [16], whereas, in the absence of DTE or



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other reductive compound, the C1 includes L-ascorbic acid only [17]. For this reason comparisons of data on AA concentrations in different tissues are difficult and according to Dabrowski [18] many studies on ascorbate concentration have given results of questionable value. Different direct and indirect methods have been described for the determination of AA and its derivatives. Based on the method of Carr et al. [19] indirect colorimetric 2,4-dinitrophenylhydrazine (DNPH) methods have been described to determine the vitamin C derivatives AA, DHA and AMS transformed to DHA [20], AMP and APP [21]. A further indirect assay, the fluorimetric determination with *o*-phenylenediamine (OPDA), was used for total ascorbate analysis [22]. Numerous HPLC determinations have been published on the direct analysis of AA and derivatives: AA and DHA [19] AA and AMS [17], AA, AMS and AP [23], AA [24]. The methods differ in sample preparation, column, mobile phase and/or flow rate and/or detection. Several indirect methods have been found for the HPLC assay of vitamin C and/or derivatives with fluorimetric detection for AA and DHA [25], for AA, DHA and total bound derivatives (Lackner, personal communication), or with enzyme shifting and UV detection for AA, AMS and AMP [16].

No HPLC method has been found for the detection of all four forms in fish tissues. The aim of this study was to develop a comprehensive HPLC method to determine the four forms of vitamin C using the same extraction of different tissues and feed.

# Experimental

## Reagents

Ascorbic acid, dehydroascorbic acid, ascorbate-2-monosulphate (sodium salt), ascorbate oxidase, phosphatase enzyme (acidic, LOT N° 72H7150) and HPLC grade methanol, were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA) and the tetrabutylammonium dihydrogen phosphate, EDTA, sodium acetate, 1,4-dithioerythritol (DTE) from Aldrich-Chemie GmbH (Steinheim, Germany) in analytical purity grade. The HPLC quality water was obtained from Carlo Erba Co. (Milan, Italy) or produced with NORGANIC cartridges (Millipore Co., Bedford, USA) and was used for the standards and reagents. Ascorbate-2-monophosphate (magnesium salt), "PHOSPITAN C®" (95 % pure) were purchased from Sintofarm, Italy, and ascorbate-2-polyphosphate, "ROVIMIX STAY®-C 25" from Roche, Basel, Switzerland.

## Standards and Working Solutions

A stock standard of 1000 mg $L^{-1}$ L-ascorbic acid (AA) was prepared daily in 0.2 mol $L^{-1}$ sodium acetate (NaAc) containing 0.2 % DTE, pH 4.8. Dehydroascorbic acid (DHA) standard solution contained 25 mg $L^{-1}$ DHA in 0.2 mol $L^{-1}$ NaAc pH 4.8 without DTE.

Ascorbate-2-monophosphate (AMP) and monosulphate (AMS) standards were prepared as a stock standard at a concentration of 1000 mg $L^{-1}$.

A solution of 40 mg of phosphatase enzyme (acidic) was prepared by dissolving it in 1 mL 0.2 M sodium acetate buffer containing 0.2 % DTE pH 4.8.

## Sample Collection and Preparation

The fish were killed immediately after collection by a blow to the head. Tissue and feed samples were weighed and analysed immediately or stored at –80 °C. Fresh or frozen tissues were homogenised in ice cold 0.2 mol $L^{-1}$ NaAc buffer, pH 4.8. The extracts were immediately separated into two parts (Figure 1).

One portion (1000 µL) of the first part was treated with ascorbic acid oxidase (30 min) for the detection of the background (Series B). Another 1000 µL was used for the determination of L-ascorbic acid without treatment (Series A). The second part of the extract was treated with 4% DTE solution to give a final concentration of 0.2 % DTE. The total vitamin C (AA+DHA) concentration was determined 30 minutes after the treatment with DTE (Series C). Series D was used only with tissue samples for the qualitative detection of the presence of the ascorbate-2-phosphate esters. Ascorbate-2-phosphate esters were hydrolysed with acidic phosphatase enzyme solution to AA for quantitative determination of AMP and APP (Series E). Ascorbate-2-monosulphate was detected in sulphate form using coinjection of AMS standard (Series F).

Each subsample was deproteinized after chemical or enzymatic reaction with 5 % $HClO_4$, centrifuged at 12000 g and filtered through a 0.45 µm membrane (Millipore) before injection to HPLC.

## HPLC Conditions

The mobile phase was an aqueous buffer of 0.04 M Na-acetate, 0.05 mM EDTA, 0.5 mM tetrabutylammonium phosphate adjusted to pH 3.76 with 85 % $H_3PO_4$ and with 24 mL methanol then mixed with 1000 mL of it. If the separation was less than optimum some further drops of methanol were added to it. The solutions were filtered through 0.45 µm membranes (Whatman or Millipore).

A Hewlett Packard HPLC 1050 system was used with a Rheodyne injector (20 µL loop volume), and a diode array detector (DAD) with HP Chemstation software. The vitamin C forms were detected at 250 nm. The mobile phase was degassed using helium. A Vydac $C_{18}$ column, (5 µm, 4.6 mm × 25 cm) was used. The flow rate was 0.6 mL $min^{-1}$ at 23 °C. The method was also adapted to a Waters HPLC system: Waters 510 pump, Rheodyne injector (20 µL loop volume), 490 multiwave length UV detector, Maxima 820 software and a Nova-Pak $C_{18}$ column (5 µm, 3.9 mm × 3.0 cm).

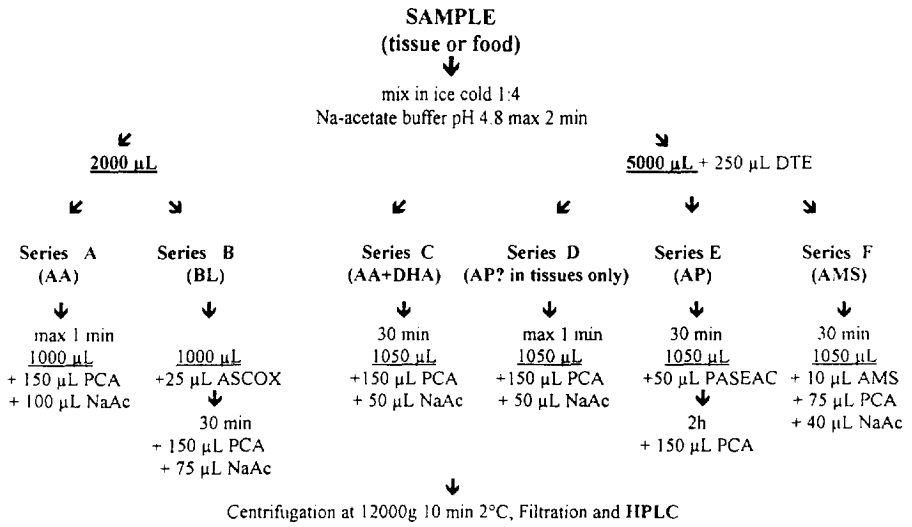


**Figure 1**

Sample preparation for determination of total vitamin C concentration. PCA = 5 % perchloric acid; PASEAC = phosphatase enzyme; NaAc = 0.2 m $L^{-1}$ pH 4.8 sodium acetate; ASCOX = ascorbate oxidase enzyme; AP = ascorbate-2-mono/poly phosphate; AMS = ascorbate-2-monosulphate; DTE = 4 % dithioerithreitol.

Peak purities of the vitamin C forms were determined by detection of the total spectra of peaks using the DAD detector.

### Calculations

All four forms were calculated in ascorbic acid form and equivalents using external standards for the quantitative, and using coinjection with standards for the qualitative determinations. Standard curves for L-ascorbic acid, ascorbate-2 monophosphate and ascorbate-2-monosulphate were obtained in the range of 1 µg $mL^{-1}$ to 100 µg $mL^{-1}$.

Recoveries were detected by addition of known amounts of standards (AA, DHA, AMP, AMS) to tissue and feed samples before extraction. The vitamin C levels were compared with the levels in the same amounts of samples.

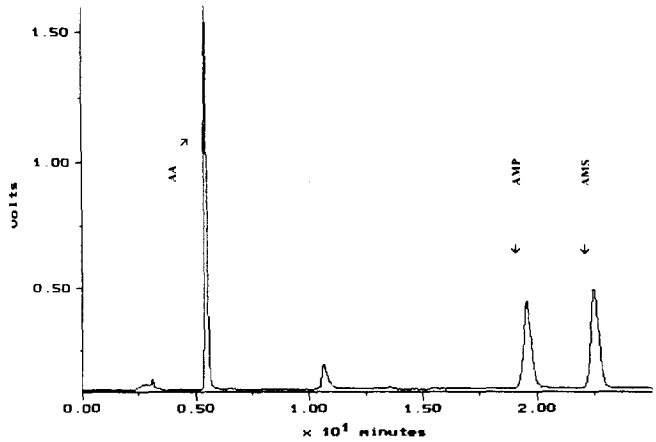


**Figure 2**

Standard chromatogram of different vitamin C forms. L-ascorbic acid = AA, ascorbate-2monophosphate = AMP, ascorbate-2-monosulphate = AMS Nova-Pak $C_{18}$ column (5 µm, 3.9 mm × 3.0 cm, Waters instrument).

## Results and Discussion

### Linearity and Reproducibility of Standards

Vitamin C forms (AA, AMP, AMS) were well separated in standard chromatograms (Figure 2).

In the standard solutions the linear range was 1 µg $mL^{-1}$ to 50 µg $mL^{-1}$ ($r^2$ = 0.994) for L-ascorbic acid and 5 µg $mL^{-1}$ to 100 µg mL ($r^2$ = 0.989) for the ascorbate-2-monophosphate and monosulphate. The minimal detection limits in the standards were 0.2 µg $mL^{-1}$ AA and 2.5 µg $mL^{-1}$ AMP or AMS. The average relative standard deviation of the AA standard was 1.99 % (0.5–6.5 %), being higher in the lower concentrations.

### Extraction and Chromatography

Extraction with the pH 4.8 NaAc buffer allows the complete oxidation of L-ascorbic acid to dehydroascorbic acid with ascorbate oxidase enzyme as well as stochiometric reduction of DHA to AA with DTE (Figure 3). Enzyme shifting with phosphatase led to complete loss of the ascorbate-2-phosphate ester peaks (ascorbate-2-mono- or ascorbate-2-diphosphate from ascorbate-2-polyphosphate) in the standard solution and most of the tissue samples, but coelution of other compounds was found in some food and fish liver samples (Figure 4). Therefore the AMP and APP were determined quantitative after enzymatic hydrolysis in L-ascorbic acid form.

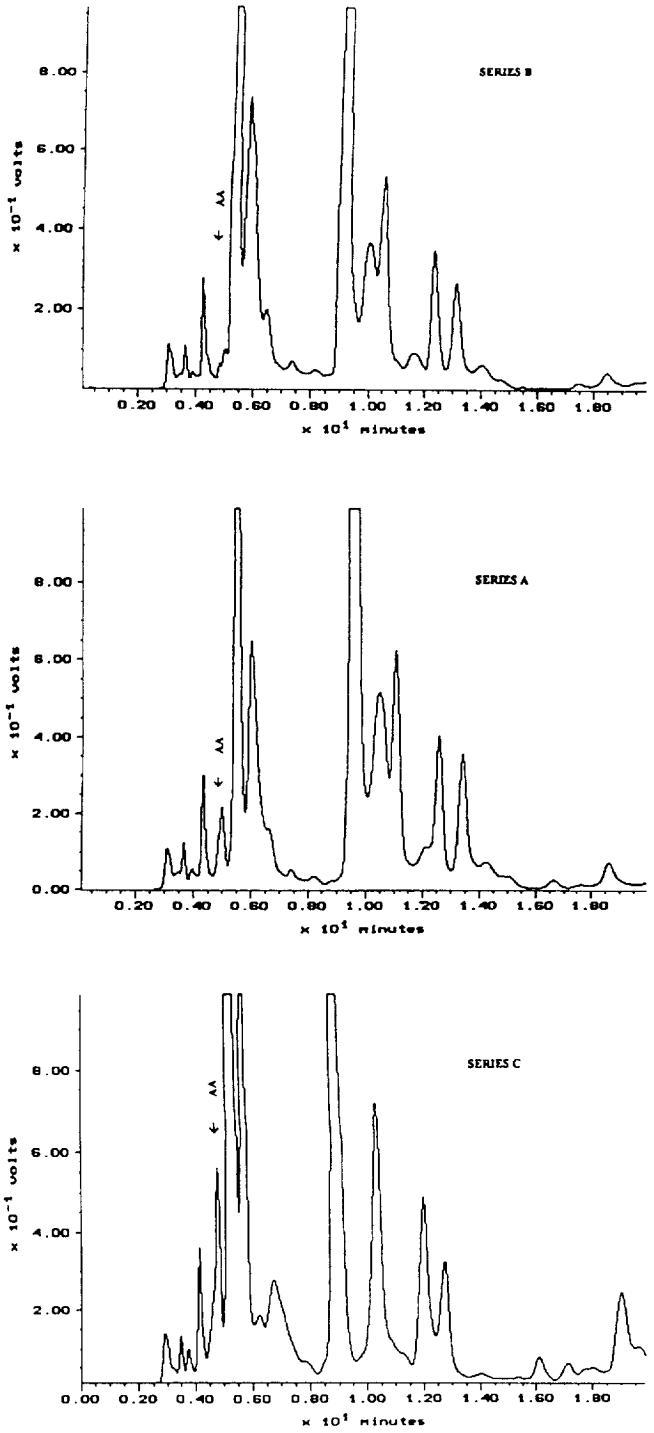


**Figure 3**
Determination of L-ascorbic acid (AA) and dehydroascorbic acid (DHA) in kidney of sturgeon. Series B =chromatogram of background; Series A = chromatogram of L-ascorbic acid (AA), Series C = chromatogram of total vitamin C (AA + DHA). Nova-Pak $C_{18}$ column (5 µm, 3.9 mm × 3.0 cm, Waters instrument).

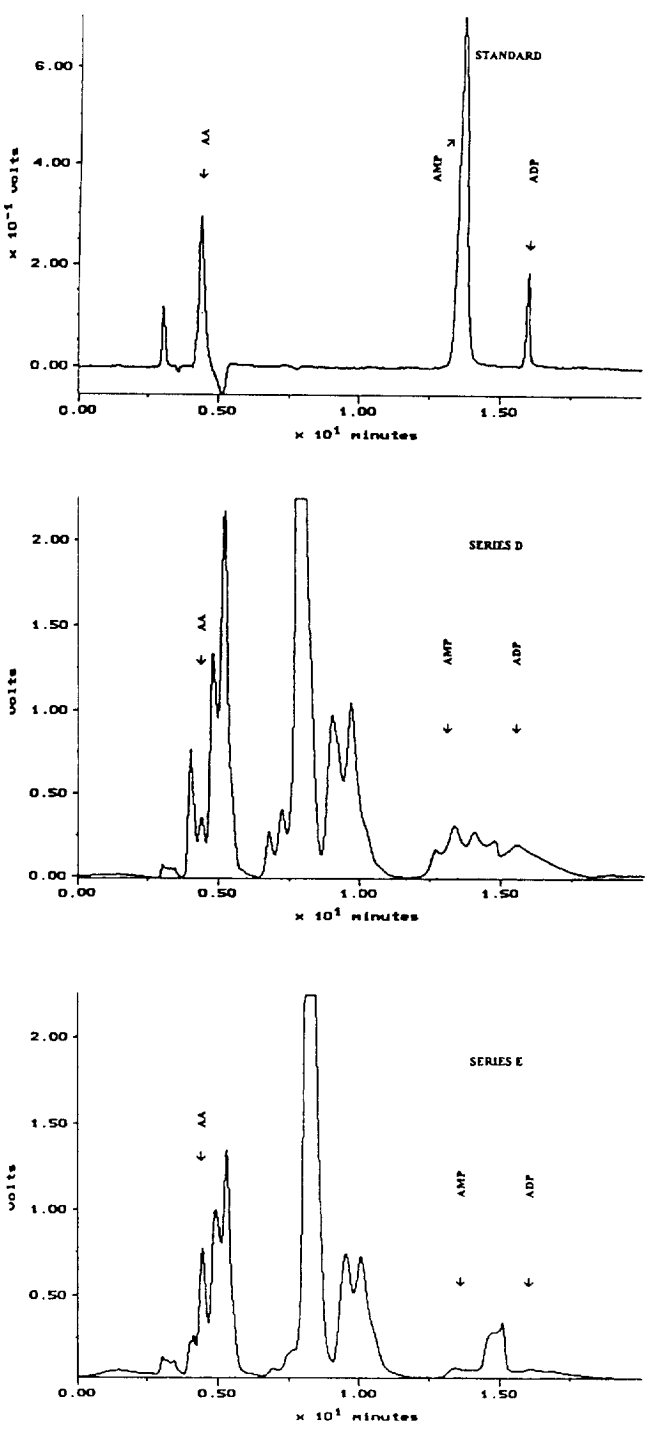


**Figure 4**
Ascorbate-2-polyphosphate in hepatopancreas of European catfish (*Silurus glanis* L.). Standard = Standard mixture of L-ascorbic acid (AA) and ascorbate-2-polyphosphate (AMP + ADP); Series D = chromatogram before reaction with phosphatase enzyme; Series E = chromatogram after reaction with phosphatase enzyme; Nova-Pak $C_{18}$ column (5 µm, 3.9 mm × 3.0 cm, Waters instrument).

Ascorbate-2-sulphate was not reacted with sulphatase enzyme.

Hydrolysis of ascorbate-2-monosulphate with $KBrO_3$ was used successfully in a colorimetric assay [20], but this reaction caused a large contamined peak ($^tR$ 4.0–7.8 min). Therefore the ascorbate-2-monosulphate was calculated directly from the AMS peak (Figure 5).

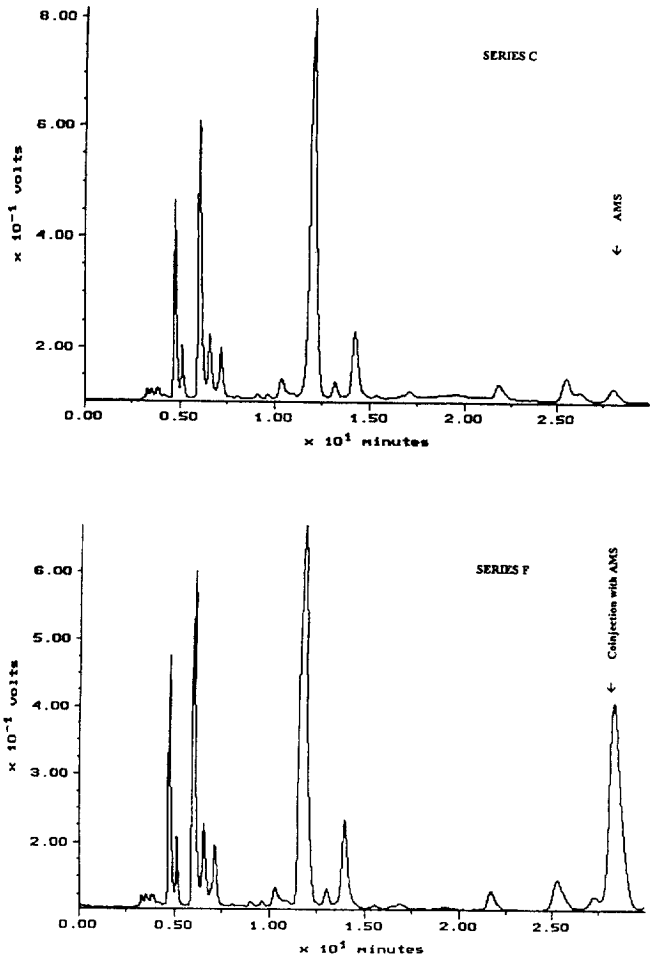


**Figure 5**

L-ascorbic acid (AA), ascorbate-2-monophosphate (AMP), ascorbate-2-monosulphate (AMS) in the intestine of sturgeon. Series C = chromatogram before coinjection with AMS; Series F = chromatogram after coinjection with AMS; Nova-Pak $C_{18}$ column (5 µm, 3.9 mm × 3.0 cm, Waters instrument).

### Recovery, Reproducibility, Sensitivity and Peak Purity in Samples

Extraction with ice-cold NaAc buffer gave adequate protection against rapid degradation of AA. Recovery of 2-ascorbic acid was 93 ± 4.3 %. A 4–5 % concentration loss of the other vitamin C forms was detected after addition of standard solutions to the samples.

The detection limit of AA compares well with the colorimetric methods [19, 20] or with other HPLC methods [16. 26].

Peak purities of L-ascorbic acid, ascorbate-2-monophosphate and ascorbate-2-monosulphate were 92 ± 5 %, 95 ± 6 % and 98 ± 6 % respectively. It is possible that the colorimetric method of Dabrowsky and Hinterleitner [20] could determine the bound (AMP, APP and AMS) vitamin C forms quantitatively, but qualitatively it cannot separate them. Direct determination of vitamin C forms [16] might give quantitative differences because of coeluted peaks. Thus the main advantages of this method lie in the combination of chemical and enzymatic reaction and in the indirect quantification of the vitamin C forms, that it achieves.

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