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# HPLC-UV determination of total vitamin C in a wide range of fortified food products

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**Abstract**

A HPLC method for the quantification of total ascorbic acid (AA) and isoacorbic acid (isoAA) in fortified food products, premixes and duomixes has been developed. The method is based on the acidic extraction of AA in the presence of reducing agent Tris [2-carboxyethyl] phosphine (TCEP), which maintained AA in its reduced form. The separation was performed on a $C_{18}$ column with a sodium acetate eluent (pH = 5.4) containing TCEP and decylamine as ion pairing agent. The limit of detection was estimated at 0.1 mg/100 g and the recoveries were between 93% to 105% when spiking various food products with different amounts of AA. The intra-assay coefficient of variation value was 4.6% ($n = 8$) for infant formula and 0.8% ($n = 9$) for the premixes. The relative standard deviation reproducibility values obtained by 9 different laboratories ranged between 2.0% and 8.0% ($n = 10$). Application of the method to the analysis of 25 fortified food products, different premixes and duomixes revealed similar results to those found by the AOAC official titrimetry method.

The present work shows that the proposed method can be applied to the determination of AA in different food type products and can be recommended for checking the presence of isoAA in fortified food products.
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*Keywords:* Ascorbic acid; Isoascorbic acid; Premixes; Duomixes; HPLC

## 1. Introduction

Ascorbic acid (AA) and its oxidized form dehydroascorbic acid (DHA) play an important role in the oxidative stress reactions (Hagen et al., 1999). Generally, the total AA is defined as the sum of both AA and its oxidized form. DHA is present in food matrices but its quantification remains difficult because of the instability of the compound. The quantification of DHA is usually performed after its conversion into AA in the presence of reducing agents (Brause, Woollard, & Indyk, 2003). Isoascorbic (isoAA) also called D-ascorbic acid or erythorbic acid is legally used as antioxidant food additive but has only 5% of the antiscorbutic effect of AA. Consequently, the differentiation between AA and isoAA is a prerequisite to any reliable vitamin C or AA determination method in food products.

Methods based on visual end point indophenol titration or that involve the conversion of AA to DHA followed by its reaction with *o*-phenylenediamine to the fluorescent quinoxaline derivatives have been approved by the Association of Official Analytical Chemist (AOAC) for the determination of AA in food products. The main drawback of these official methods is that they can only estimate the total content of vitamin C without discriminating between the AA and isoAA. Alternative approaches to the official methods such as HPLC techniques were investigated. HPLC with a different combination of pre- or post-column derivatization were used to quantify individually AA and DHA (Ali & Phillippo, 1996; Bognar & Daood, 2000; Kall & Andersen, 1999). These approaches require a derivatization step, which makes the method time consuming and the degradation

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of AA can occur during the procedure. The most frequently utilized HPLC methods are based on a separation on a reversed phase column using ion exchange or ion-pair procedure (Eitenmiller & Landen, 1999). However, prior to the LC-analysis, AA is often extracted into buffers that contain compounds such as metaphosphoric or trichloroacetic acid. It has been reported that these acids could coelute or interfere with AA or isoAA and thus generating inconsistency in their retention times (Brause et al., 2003). A recent HPLC method was developed for the determination of total vitamin C in fruit juices and on related products (Brause et al., 2003). With this method, AA and isoAA peaks were barely resolved and their identification or quantification in complex food samples remains problematical. Reductant agents such as cystein (Comité Européen de Normalisation, 2003), homocystein (Behrens & Madere, 1987; Brause et al., 2003) and dithiothreitol (DTT) are frequently used to convert DHA into its reduced form and to stabilize AA. Amongst these chemical compounds, DTT is the most common used for the LC-analysis (Brause et al., 2003; Margolis, Paule, & Ziegler, 1990). Recently, it has been demonstrated that TCEP offers a more efficient reduction of DHA at low pH compared to that of DTT (Lykkesfeldt, 2000).

In the present work, a new HPLC method for the identification and quantification of AA and isoAA acids in fortified food products was developed. TCEP was used as reducing agent and decylamine as pairing agent in order to improve the retention of AA isomers on a $C_{18}$ column. The performances of the method were evaluated by analyzing a wide range of fortified food products, premixes and duomixes that consist of a mixture of vitamins and/or minerals. In addition, the comparison of the HPLC results with those found by the official titrimetry method was performed.

## 2. Experimental

### 2.1. Chemicals

Ascorbic acid, acetonitrile trichloroacetic acid, sodium acetate trihydrate and phosphoric acid were obtained from Merck (Geneva, Switzerland). Tris [2-carboxyethyl] phosphine hydrochloride, isoascorbic acid, orotic acid, uric acid, decylamine and taka-diastase were purchased from Fluka (Buchs, Switzerland). Water was purified using a Milli-Q system from Millipore (Le Mont-sur-Lausanne, Switzerland).

### 2.2. Samples and reference materials

The samples used for this study included: one certified reference material (milk powder, SRM/RM 1846) from National Institute and Standard Technology (NIST, Gaithersburg, MD, USA), a dietetic milk powder used as Nestlé reference sample (NRS 2/2002); hypoallergenic infant formulas, soya-based infant formulas; breakfast cereals, Cereal based infant formula; adult nutrition; fortified soup; fortified juice; health care products, apple and apricot compotes, premixes and duomixes that consist of a mixture of vitamins and minerals.

### 2.3. Solution and mobile phase preparation

The Tris (2-carboxyethyl)-phosphine hydrochloride (TCEP · HCl) solution at 250 μg/ml was prepared by dissolving in a volumetric flask, 125 mg of TCEP with 500 ml distilled water. The trichloroacetic acid (TCA) solution 1% was prepared by dissolving in a volumetric flask 5.0 g of TCA with 500 ml distilled water.

The LC-mobile phase was prepared as follow: 1.6 g of decylamine, 80 ml of acetonitrile, 100 ml of sodium acetate solution (0.25 M) pH 5.4 and 820 ml of distilled water were introduced into a 1000 ml flask; then the pH of the solution was adjusted to 5.4 with phosphoric acid 85% and 50 mg TCEP · HCl were added to the final solution.

### 2.4. Sample preparation

Ten grams of liquid or solid were weighted into a 100 ml volumetric amber glass flask, then 40 ml of TCEP · HCl solution at 250 μg/ml were added, the suspension was thoroughly mixed to obtain homogeneous slurry and the volumetric flask was made up to the mark with TCA solution 1%. The resulting solution was shake for about 1 min and filter through a folded filter paper S & S 597 1/2. Aliquots of the solution were diluted with the mobile phase prior the injection on to the HPLC system. For starch containing samples, 10 g of liquid or solid were weighted into a 100 ml volumetric amber glass flask, then 40 ml of TCEP · HCl solution at 250 μg/ml and 10 mg of taka-diastase were added and the suspension was incubated at 42 °C for 30 min before filling the volumetric flask to the mark with the TCA solution. For the duomixes and premixes, 100 mg or 1 g of sample depending on the vitamin C content was weighted into a 100 ml volumetric amber glass flask and the solution was handled as described above.

### 2.5. Description of the official titrimetry method

The titrimetry method (potentiometric titration) used in this study is based on a stoichiometric oxydo-reduction reaction between vitamin C (AA and isoAA) and a mild oxidizing agent (2,6-dichlorophenol-indophenol). The end point is detected by a potential variation of a combined platinum electrode. The electrode is connected to a potentiograph, which draws the titration curves, automatically finds the end point and calculates

the vitamin C content of the sample. When the resin was used, 1 g of cation-exhange resin (Bio Rex 70, 50–100 mesh, sodium form, Bio-Rad, Reinach, Switzerland) was added to the sample before the titration.

### 2.6. Liquid chromatography

The LC-analyses were performed with a Merck Hitachi LaChrom system (Merck Geneva, Switzerland) equipped with a UV detector system (Merck Geneva, Switzerland). Sample injections of 20 μl were made from a Hitachi LaChrom auto-sampler (Merck Geneva, Switzerland) on a LiChrospher RP-18; 5 μm; 4.6 × 250 mm column. The LC-analyses were performed under isocratic mode at a flow rate of 1 ml/min with detection at 265 nm.

### 2.7. Statistics

The precision data of the method were evaluated using an in-house statistical program making use of the robust-statistics concept.

## 3. Results and discussion

### 3.1. Identification of AA and isoAA by HPLC-UV

The present method is able to discriminate between AA and isoAA after their separation by HPLC on a reversed phase column with detection at 265 nm. The retention time, the spiking experiments with both AA and isoAA in different food samples as well as the spectra of the standard compounds were used for their identification (Fig. 1(a)).

In the food products (namely, dietetic milk powder), the peak with a retention time of 15.5 min was identified as orotic acid (OA) based on its UV spectra (Fig. 1(b)) and on the spiking of OA in the sample. Under our experimental conditions OA peak was not constant as it varies between 13 and 15.5 min but OA did not interfere with AA or isoAA peak. Moreover, it was also possible to separate uric acid from AA and isoAA in fortified food products tested (Fig. 1(b)).

### 3.2. Quantification of AA and isoAA in fortified food products

The quantification was focused on vitamin C, as we did not found any trace of isoAA in the tested food products. AA or total vitamin C in different food samples was quantified by means of an external calibration curve in the concentration range from 1 to 100 μg/ml of AA. Minimums of five AA concentrations were used to generate the calibration curve that was linear ($r^2 = 0.9999$). The limit of detection (LoD) of the method for AA was calculated as 3 × SD, where SD is the standard deviation of background noise of the standard diluted in the same buffer as the food sample. The LoD

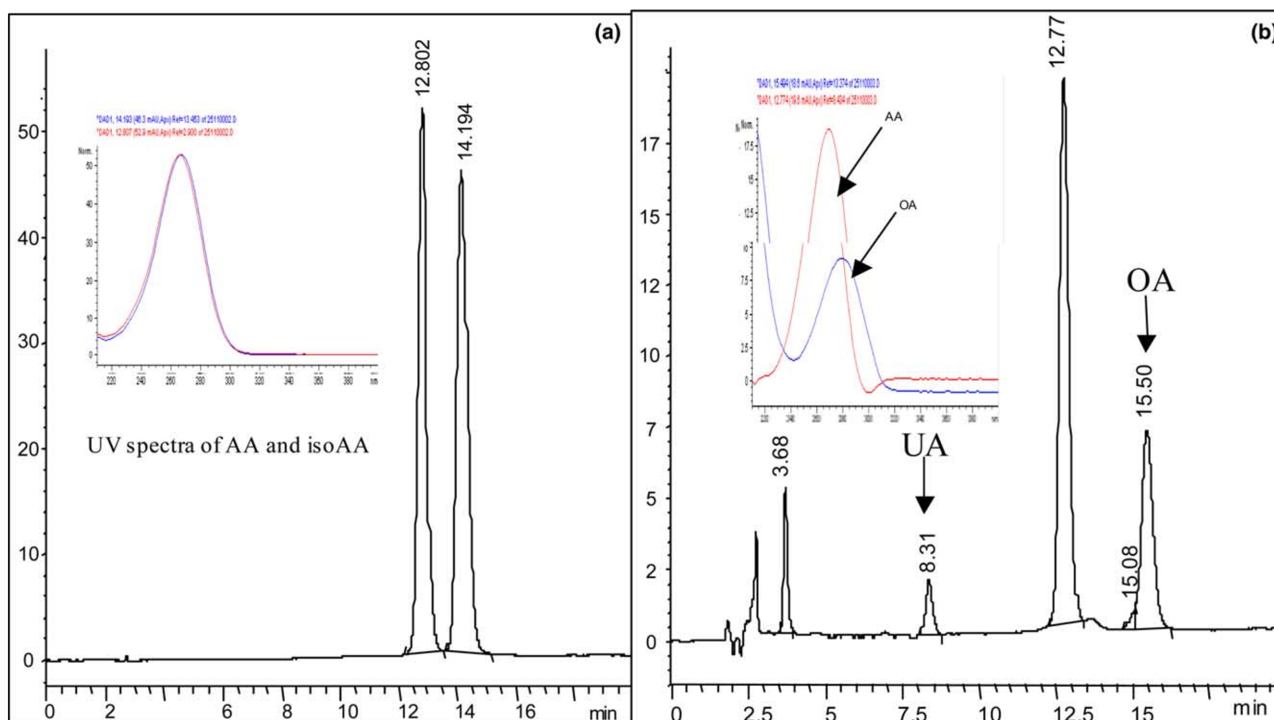


Fig. 1. HPLC-UV chromatograms of (a) AA and isoAA standards and (b) a dietetic milk powder. The retention times of AA and that of isoAA are 12.8 and 14.19 min, respectively. UA, uric acid; OA, orotic acid.

was estimated at 0.1 μg/ml; considering that 10 g of products are diluted in 100 ml, this LoD corresponds to a concentration of 0.1 mg/100 g. The limit of quantification was calculated as 10 × SD and the value found was 0.3 μg/ml, which corresponds to 0.3 mg/100 g. It should be pointed out that the LoD of our method is 50-fold lower than the one found with the official method such as: the titrimetry method.

Under our experimental conditions, AA was stable for a week in solution (data not shown); this is due to the better protection of AA from the degradation confers by the TCEP as it was already mentioned (Lykkesfeldt, 2000). The validation of the method was performed on two milk powders and on one premix. The coefficient of variation (CV) of the within-day precision obtained on a Nestlé reference sample (NRS 2/2002) and on the premix analyzed eight times was below 5% (Table 1). The recovery of the method was carried at two levels of concentrations by spiking AA on NRS and on premixes and the values found were acceptable (Table 1). The accuracy of the method was assessed by analyzing 8 aliquots of a standard reference material (SRM 1846: milk-based powered infant formula) and NRS. The values expressed as mean ± SD are reported in Table 1. The overall relative standard deviation reproducibility values $RSD_R$ obtained on 10 samples analyzed by nine different laboratories ranged between 2.0 and 8.0% (Table 2).

Table 1
Analytical parameters (recovery, repeatability and accuracy) of AA determination

| | Recovery (%) | Repeatability CV (%) | Found values (mg/100 g) | Declared values (mg/100 g) |
|---|---|---|---|---|
| NRS 2/2002 | 99 ± 6 (*n* = 8) | 4.6 (*n* = 8) | 39.5 ± 3.2 (*n* = 9) | 46.2 ± 6 (*n* = 47) |
| Premix 9200 D02 | 100.2 ± 4.7 (*n* = 8) | 0.8 (*n* = 9) | 19.500 | 19.800 |
| NIST 1846 | – | – | 118.3 ± 6 (*n* = 8) | 114.6 ± 6.6[a] |

[a] Certified value.

Table 2
Statistical analysis of AA concentration in different food products by liquid chromatography

| No | Sample designation | *n* | *p* | Median (mg/100 g) | $SD_R$ (mg/100 g) | $CV_R$ (%) |
|---|---|---|---|---|---|---|
| 1 | Infant Formula | 9 | 2 | 39.5 | 3.2 | 8.0 |
| 2 | Hypoallergenic infant formula | 9 | 2 | 86.6 | 6.2 | 7.2 |
| 3 | Soya-based infant formula | 9 | 2 | 208.5 | 4.3 | 2.0 |
| 4 | Breakfast cereals | 9 | 2 | 72.3 | 4.7 | 6.5 |
| 5 | Infant formula | 9 | 2 | 62.2 | 2.9 | 4.7 |
| 6 | Adult nutrition | 9 | 2 | 119.1 | 6.8 | 5.7 |
| 7 | Apple and apricot compote | 9 | 2 | 6.2 | 0.5 | 7.5 |
| 8 | Fortified soup | 9 | 2 | 98.4 | 7.3 | 7.5 |
| 9 | Orange Juice | 9 | 2 | 25.6 | 1.7 | 6.7 |
| 10 | Health care products | 8 | 2 | 25.4 | 1.4 | 5.5 |

*n*, Number of participants.
*p*, Number of replicates.

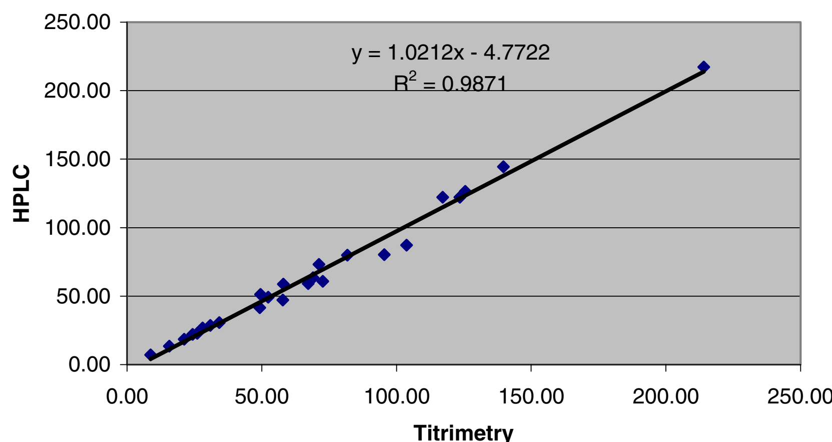


Fig. 2. Comparison between titrimetry and HPLC for the determination of AA in 25 fortified food products. The category of the products tested was as followed: Fortified soup (2), dietetic milk powder (3) soya-based product (1), breakfast cereal (2), SRM NIST 1846, cereal based product (1), hypoallergenic infant formula (5), health care product (2), fortified juice (2), adult nutrition (3), chocolate based product (1) and apple and apricot compotes (2). In brackets, the number of products tested per category.

In order to compare the HPLC method and the official method, 25 fortified samples were analyzed. From Fig. 2, comparable results were obtained between HPLC method and the reference titrimetry method. In addition, a good correlation was found between the HPLC and the reference methods data for vitamin C concentration in premixes (Fig. 3) and duomixes (Fig. 4). It should be also mentioned that there was no interaction between iron and vitamin C during its analysis, as the results found with each type of duomix with low or high content of iron are similar with the both HPLC and titrimetry methods (Fig. 4). The comparable results obtained

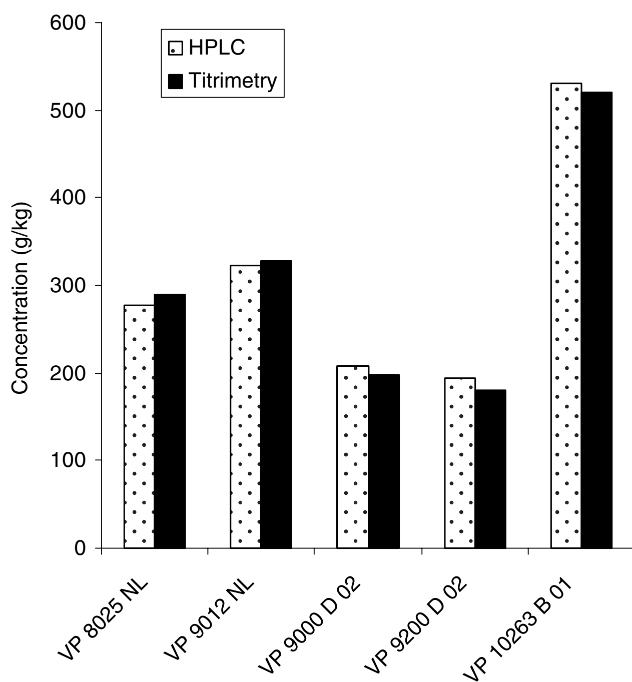


Fig. 3. AA concentration in premixes obtained by HPLC and titrimetry methods.

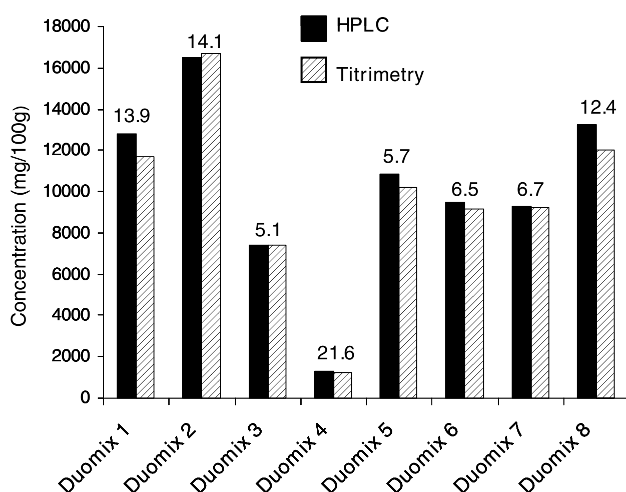


Fig. 4. AA concentration in 8 duomixes obtained by HPLC and titrimetry methods. The number mentioned on the top of each set of histogram represents the ratio VitC/Fe.

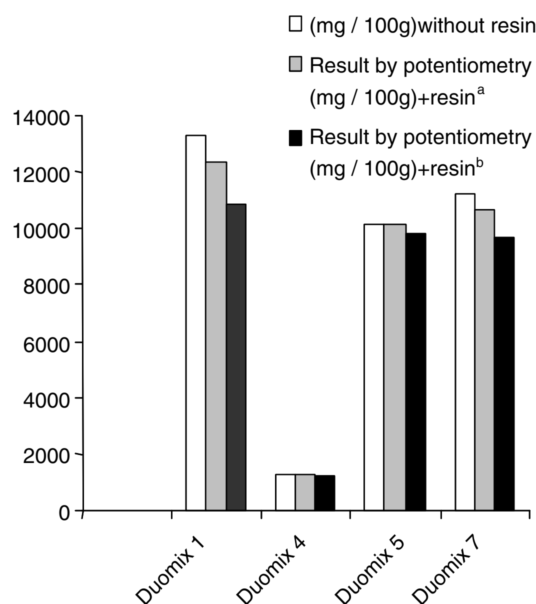


Fig. 5. Comparison of AA concentration in duomixes obtained by titrimetry (potentiometry) method without or with the treatment of sample by the cation exchange resin. [a,b] Results obtained by two independent laboratories.

between our method and the reference method demonstrates that the food products used in this study contain negligible amount of DHA. Therefore, it is not possible to estimate the efficiency of the conversion of DHA into AA as it was shown in biological system. Iron is commonly described as a redox interferent in titrimetry based methods; this was not the case with the titrimetry method that we used as the data found in absence of reductant agent (titrimetry method) or in the presence of TCEP (the HPLC method) are similar. Moreover, the results obtained with the titrimetry method and with our HPLC methods were similar to those found by a titrimetry method that uses cation-exchange resin for the depletion of iron before the titration (Fig. 5).

## 4. Conclusions

The HPLC method offers high accuracy, good repeatability and reproducibility, a relative short analysis time, unambiguous identification of AA and isoAA. Moreover, the use of TCEP, which confers a greater stability to AA, is an advantage over the current official methods. The successful application of the method to several fortified food products should make it highly desirable as a routine method in vitamin laboratories.

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