Agricultura 4: 23-26 (2006)

Determination of vitamin C in lemon, grapefruit juices and in dried carrots by liquid chromatography

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An isocratic liquid chromatographic method for the determination of ascorbic acid (vitamin C) is described. The separation was performed on a C18 reversed-phase column with isocratic elution using phosphate buffer / methanol as a mobile phase. The photodiode array detector used was set at a wavelength of 245 nm. The limit of detection and determination was estimated at 0.23 mg/L and 0.46 mg/L per 50 µL injection. Ascorbic acid contents of freeze dried carrots, fresh squeezed lemon and grapefruit juices were analysed comparatively by liquid chromatography and by the AOAC official titrimetric method. Both methods gave similar results for each of the matrices analysed.

Keywords: ascorbic acid, liquid chromatography

INTRODUCTION

Ascorbic acid (AA) is an organic acid with antioxidant properties. The L-enantiomer of ascorbic acid (1-keto-1-threo-hexono-g-lactone-2,3-enediol) is commonly known as vitamin C. It is the micronutrient most readily associated with vegetables and fruit that provide more than 90% of the vitamin C in human diets. Vitamin C is essential for nutrition and recently there has also been an interest in the possibility of AA reducing the oxidative damage caused by free radicals (McCall and Frei 1999). It is also known that vitamin C has many biological functions in collagen formation, reduction of plasma cholesterol level, enhancement of the immune system and inhibition of nitrosamine formation. Vitamin C is also a sensitive and appropriate marker for monitoring quality change of fruit and vegetables during storage, processing and transportation since it is highly water soluble and vulnerable to enzyme and chemical oxidation (Favell 1998).

The sample extraction is very important when analysing vitamin C in complex samples such as fruits and vegetables. It is essential to achieve efficient AA extraction by preventing AA oxidation and by inactivating ascorbic acid oxidase (AAO, EC 1.10.3.3) that is thought to be the major enzyme responsible for the enzymatic degradation of AA. In the literature different extraction methods were reported using acidic extraction solvents such as aqueous solutions of metaphosphoric acid, citric acid, acetic acid, oxalic acid and ortophosphoric acid (Franke et al. 2004; Kabasakalis et al. 2000, Kall and Andersen 1999).

For the determination of ascorbic acid in foods various methods have been reported including titration (AOAC International 1995), spectrophotometry (Güçlü et al. 2005, Arya et al. 2001), fluorometry (Chung and Ingle 1991), voltammetry (Ahmed et al. 2005), electrophoresis (Zinellu et al. 2006) and liquid chromatography (Fontannaz et al. 2005; Margolis and Schapira 1996).

This paper reports a liquid chromatographic (LC) procedure for the determination of AA in lemon, grapefruit juices and in carrots. Taking into account that there is no appropriate reference material containing AA in samples analysed, the comparison of the LC results with those found by the official titrimetric method was performed.

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EXPERIMENTAL

REAGENTS

Methanol was of HPLC-grade from Riedel-deHäen. All the others applied chemicals were of analytical reagent grade and included: L-ascorbic acid (99.5%, Sigma), metaphosphoric acid (MPA) [33.5-36.5% $(HPO_3)n$, Aldrich], acetic acid (Fluka), sodium dihydrogen phosphate dihydrate (Fluka), phosphoric acid (85%, Fluka), 2,6-dichloroindophenol sodium salt hydrate (Sigma), sodium hydrogen carbonate (Fluka), thymol blue (Riedel-deHaën), methylene blue (Fluka) and indigo carmine (Fluka).

Deionised water of 18 MΩ/cm resistivity purified with Milli-Q system (Millipore, Bedford, USA) was employed throughout.

SOLUTIONS

The extraction solution was prepared by dissolving 15 g MPA in 40 mL acetic acid and diluting to 500 mL with deionised water as described in literature (AOAC International 1995). The solution was filtered and stored in refrigerator.

Standard stock solution containing 100 mg/L of AA was prepared by weighting 0.0050 g of AA in a 50 mL volumetric flask and diluting with extraction solution. Standard solutions for calibration curves were obtained from standard

stock solution by appropriate dilution to give the concentration range of 2 - 20 mg/L. All standards were prepared in 5 times diluted extraction solution.

The LC phosphate buffer (25 mM) was prepared as follows: 1.95 g $NaH_2PO_4 \cdot 2H_2O$ was introduced into a 500 mL volumetric flask and diluted with deionised water. Then the pH was adjusted to 2.2 with ortophosphoric acid.

Indophenol solution used for titrimetric determination of AA was prepared according to description in literature (AOAC International 1995).

SAMPLE PREPARATION

Lemons and grapefruit were purchased in local market, hand squeezed and stabilised by addition of equal volumes of extraction solution. Samples were centrifuged (Tehtnica CENTRIC 3000R) at 3500 rpm at 4˚ C for 15 min and filtered through 0.45 μm membrane filter (Chromaphil). Fruit extracts were then diluted twenty times with deionised water and injected into LC.

AA determinations in carrots were performed using lyophilised samples because this is the procedure most frequently described in literature. Carrots were washed, peeled, sliced and frozen in liquid nitrogen. Carrot slices were lyophilised at -53˚ C and 0.060 mBar (Alpha 1-2 LD, Vacuumbrand GMBH, Germany), ground in a mortar and stored in 50 mL polyethylene bottles at -70˚ C until the analyses were carried out. One gram of powdered carrot slices was weighted into 15 mL extraction solution. The mixture was homogenised using an Ultra-turrax high speed blender for 5 min. Extraction was carried out in an ice bath. Sample extracts were centrifuged at 3500 rpm and 4˚C for 15 min, filtered through 0.45 μm membrane filter (Chromaphil) and then they were diluted in such a manner to give a solution with final acidity identical to the standard solutions prepared for LC injection.

APPARATUS AND CHROMATOGRAPHIC CONDITIONS

The LC analyses were performed using a Waters 600E System controller equipped with an autosampler model Triathlon 900 (Spark) and an injection loop of 50 μL, column oven (WAT 062079), pumping system Waters 600E Pump and photodiode array detector (Waters 996). Eluent reservoirs were purged with helium. Separation was performed on a reversed-phase column (Synergi 4μ Hydro-RP 80A, 150x4.6 mm, Phenomenex, USA) with a precolumn (SecurityguardTM C18, 3 mm i.d.x 4 cm). The temperature of the analytical column was kept at 23˚ C. The LC-analyses were performed under isocratic conditions at a flow rate of 0.5 mL/min with detection at 245 nm. The mobile phase was a mixture of phosphate buffer: methanol (95:5).

THE AOAC METHOD FOR THE DETERMINATION OF ASCORBIC ACID

Ascorbic acid standard solution (2 mL aliquot) was transferred in 50 mL Erlenmeyer containing 5 mL of extraction solution. Than it was titrated with indophenol solution until light and distinct rose pink colour appears and persists for more than 5 seconds. Blank solutions (n=3) composed of 7 mL extraction solution and volume water ca. equal to volume indophenol solution used in titration of standard solution were titrated. The indophenol solution was standardised daily with fresh prepared AA standard solution. Sample extract solution (2-5 mL aliquot) was titrated in the same manner as blank solutions (AOAC International 1995).

RESULTS AND DISCUSSION

Ascorbic acid extraction

Prior to chromatographic analysis samples were extracted with a mixture of MPA and acetic acid as recommended in many papers (Franke et al. 2004, AOAC International 1995, Fontannaz et al. 2005). The extraction was performed under conditions described in the section sample preparation. To check the efficiency of AA extraction, to approximately one g of carrot powder 10, 15, 20 and 25 mL of extracting solution was added. There were no significant differences in the values obtained for AA using ≥ 15 mL of extracting solution (Table 1). Fruit extracts were stable for at least a week at 4˚ C. This indicates that the acidic pH of the extract prevents oxidation of AA and that in the fruit examined no compounds are co-extracted that are capable of oxidising AA.

Table 1. Influence of extraction solvent volume on extraction efficiency of AA from carrot powder.

Volume of extraction solution (mL)	10	15	20	25
Content of AA (mg/kg dry weight)	350	422	424	424

Quantification of AA

AA was quantified by means of an external calibration curve in the concentration range from 2 to 20 mg/L of AA. The calibration curve was generated using seven calibration points. A linear relationship of peak area and concentration of AA over the mentioned concentration range was obtained, respectively. The limit of detection (LOD) and quantification (LOQ) were calculated using the equations proposed by Miller and Miller (1993). They are presented in Table 2 together with other parameters obtained for the calibration curve. Precision was calculated from 6 replicate injections of standard solutions at each concentration level and RSD of 0.7 to 1.7% was obtained. The reproducibility in terms of retention time was good. Day-to-day variations in retention time were found to be lees than 2.1% over a period of one month.

Table 2. The important parameters for the calibration curve in the concentration range 2- 20 mg/L of AA.

Vitamin	y = ax+ b*	r^2 *	S_a*	S_b*	LOD (mg/L)	LOQ (mg/L)
AA	Y=376000-16700	0.9999	12336	1143	0.23	0.46

r^2 correlation coefficient, S_a standard deviation of the intercept, S_b standard deviation of the slope, LOD limit of detection, LOQ limit of quantification.
*Experimentally determined values

Representative chromatograms obtained for AA standard solution (8 mg/L), carrot extract, grapefruit and lemon juice are presented in Figure 1.

To evaluate the effect of sample matrix on the accuracy of analysis standard addition method was applied. Three series at different concentration levels were prepared by adding known amounts of AA standard substance to different sample matrices prior to extraction. Recovery was calculated as the fraction of analyte added to a test sample prior to analysis as follows:

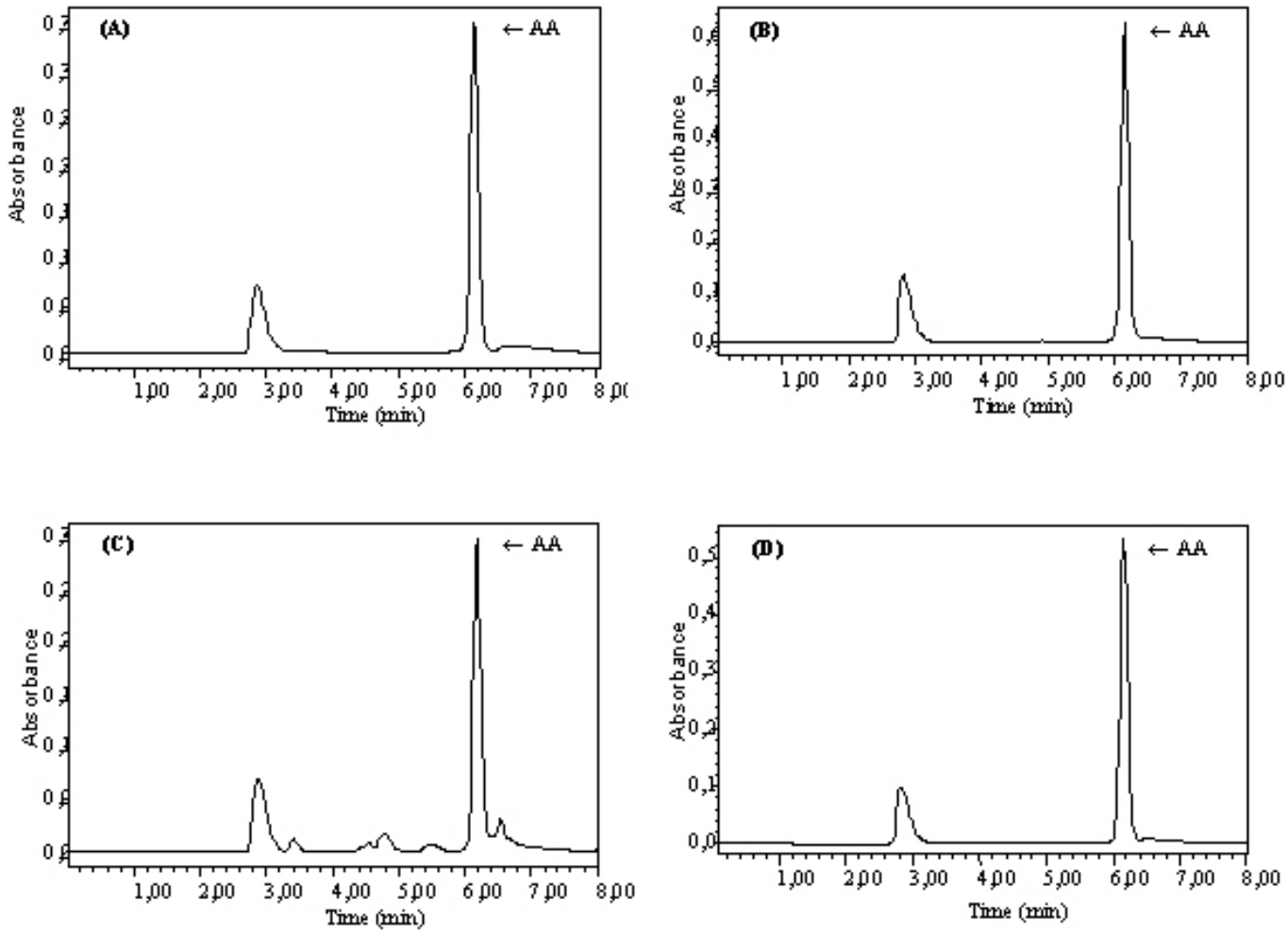

Fig. 1. Chromatograms of AA. (A) Standard solution of 8 mg AA/L, (B) fresh squeezed grapefruit juice, (C) extract of carrot powder and (D) fresh squeezed lemon juice. The retention time for AA is 6.1 min. Chromatographic conditions are as described in Experimental section Apparatus and chromatographic conditions.

$$\%R = \left(\frac{CF - CU}{CA}\right) \cdot 100 \qquad (1)$$

Where CF is the concentration of analyte measured in the fortified sample; CU is the concentration of analyte measured in the unfortified sample; CA is the concentration of analyte added (measured value, not detrmined by method) in fortified sample (Eurachem 1998, http://www.eurachem.ul.pt/guides/valid.pdf). The mean recoveries (Table 3) ranged from 92 to 102% for lemon and grapefruit juices and from 91 to 98% for carrot samples.

Table 3. Mean recovery of AA ± standard deviation (n = 3).

Sample	m (added)[a], µg	m (found)[b], µg	Recovery (%)
Dried carrot	150	139 ± 4	93 ± 2
	300	287 ± 7	96 ± 2
Lemon juice	1200	1165 ± 53	97 ± 5
Grapefruit juice	1200	1238 ±55	98 ± 4

[a]This value represents the amount of AA added into sample before extraction (CA in Eq. 1).

[b]This value represents the difference: CF-CU in Eq.1.

Furthermore, the content of AA obtained by the LC method for carrot samples, lemon and grapefruit juices were compared with those obtained using the AOAC official titrimetric method. Before applying the AOAC method all extracts were tested for the presence of reducing ions Fe(II), Cu(II) and Sn(II) using indicators methylene blue and indigo carmine. Products containing those ions give values in excess of their actual AA content so in such cases the AOAC method is not applicable due to overestimation of the AA content. None of the extracts contained interfering substances and the titrimetric method was applied for the determination of AA. Comparable results were obtained between LC method and the reference titrimetry method (Table 4). The deviations of LC results from those obtained by AOAC method were found to be less than ± 7%.

Table 4. AA contents of selected lemon, grapefruit juices and dried carrot determined by the LC method and AOAC titrimetric method.

Sample	AA (LC method)	AA (AOAC titrimetric method)
Dried carrot	423 ± 5[a]	446 ± 2[a]
Lemon juice	356 ± 3[b]	366 ± 2[b]
Grapefruit juice	417 ± 6[b]	421 ± 3[b]

Mean ± standard deviation (n = 4)
[a] This value represents the amount of AA in mg/kg dry weight
[b] This value represents the amount of AA in mg/L.

CONCLUSION

A simple LC method for the determination of AA in selected fruits and vegetable was described. The method offers a relative short analysis time and good precision. The comparable results obtained between LC method and the reference AOAC method demonstrate that the proposed LC method is accurate. Ascorbic acid content in fresh squeezed grapefruit and lemon juices ranged from 353 to 423 mg/L of juice, and the contents of AA in dried carrots ranged from 295 to 600 mg/kg dry weight. From literature it is known that the vitamin C content varies a lot even within-food type due to different reasons, such as genetic conditions, growing location, climatic conditions, harvesting time, ripening degree and storage conditions.

In contrast to other methods this method determined levels of ascorbic acid in its reduced state. We made no attempts to assess of dehydroascorbic acid (DHA). Experiments for determination of DHA are currently underway.

REFERENCES

1. Ahmed R, Qureshi SA, Viqar-Un-Nisa. Studies about the determination of ascorbic acid by differential pulse voltammetry. Journal of the chemical society of Pakistan 2005;27(2):168-173.
2. Arya SP, Mahajan M, Jain P. Spectrophotometric determination of vitamin C with iron(II)-4-(2-pyridylazo) resorcinol complex. Anal. Chim. Acta 2001; 427:245-251.
3. Chung HK, Ingle JD. Jr. Fluorometric kinetic method for the determination of total ascorbic acid with o-phenylenediamine. Anal. Chim. Acta 1991;243:89-95.
4. Eurachem guide, The fitness for purpose of analytical methods. A laboratory guide to method validation and related topics. http://www.eurachem.ul.pt/guides/valid.pdf. Eurachem. 1998.
4. Favell DJ. A comparison of the vitamin C content of fresh and frozen vegetables. Food Chem. 1998;62: 59-64.
5. Fontannaz P, Kilinç T, Heudi O. HPLC-UV determination of total vitamin C in a wide range of fortified food products. Food Chem. 2005;94 (4):626-631.
6. Franke AA, Custer LJ, Arakaki C, Murphy SP. Vitamin C and flavonoid levels of fruit and vegetables consumed in Hawaii. J. Food Compos. Anal. 2004;17: 1-35.
7. Güçlü K, Sözgen K, Tütem E, Özyürek M, Apak R. Spectrophotometric determination of ascorbic acid using copper (II)-neocuproine reagent in beverages and pharmaceuticals,Talanta 2005;65:1226-1232.
8. Kabasakalis V, Siopidou D, Moshatou E. Ascorbic acid content in commercial fruit juices and its rate of loss upon storage. Food Chem. 2000;70:325-328.
9. Kall MA, Andersen C. Improved method for simultaneous determination of ascorbic acid and dehydroascorbic acid, isoacsorbic acid and dehydroisoascorbic acid in food and biological samples. J. Chromatogr. B 1999;730:101-111.
10. McCall MR, Frei B. Can antioxidant vitamins materially reduce oxidative damage in humans. Free Radicals Biology & Medicine 1999;26:1034-1053.
11. Margolis SA, Schapira RM. Liquid chromatographic measurement of L-ascorbic acid and d-ascorbic acid in biological samples. J. Chromatogr. B, 1997; 690:25-33.
12. Miller JC, Miller JN. Statistics for analytical chemistry, Ellis Horwood PTR Prentice Hall, New York London Toronto Sydney Tokyo Singapore,1993 (eds).
13. Official Methods of Analysis, AOAC International, 16th ed., Ascorbic acid in vitamin preparations and juices. Arlington, Virginia,1995:Method 967.21.
14. Zinellu A, Sotgia S, Deiana L. Pre-analytical factors affecting ascorbic and uric acid quantification in human plasma. J. Biochem. Biophy. Methods 2006;67(2-3):95-105.

Received: Avgust 12, 2005
Accepted in final form: September 10, 2006