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Evaluation of sampling and extraction procedures for the analysis of ascorbic acid from pear fruit tissue

Anna Rizzolo*, Ada Brambilla, Sabrina Valsecchi, Paola Eccher-Zerbini

Istituto Sperimentale per la Valorizzazione Tecnologica dei Prodotti Agricoli—via Venezian, 26—20133 Milan, Italy

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Abstract

A suitable method of extraction of ascorbic acid (AA) and dehydroascorbic acid (DHAA) from Conference pears during storage was developed. Analytes determination was carried out by HPLC with UV detection at 254 nm. Three sampling procedures from fresh fruit and three extraction procedures from frozen tissue were tested. Recovery data were achieved by running at the same time the sample with and without addition of an ascorbic acid standard solution. Results showed that improper sampling procedure from fresh fruit and a storage temperature of frozen samples prior to extraction of −20 °C led to AA degradation in pears. Furthermore, small variations in temperature and light exposure during extraction significantly affected ascorbic acid recovery. The final set up procedure, comprising a specific sampling from fresh fruit, a storage temperature of frozen samples of −80 °C, and an extraction with 6% meta-phosphoric acid on ice, under reduced light and in a cold room at 2 °C, gave the highest AA recoveries. It was easy to perform and had high extraction rates. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Ascorbic acid; Pears; Sampling; Extraction; HPLC-UV detection

1. Introduction

Ascorbate (AA) is a water-soluble antioxidant which is present, along with other oxygen scavenging systems, in plant tissue (Davey et al., 2000; Lee & Kader, 2000) and could play a key role in preventing brown heart and cavities, common disorders occurring in stored pears. Ascorbate disappearance in pear tissue after excessive oxidative stress has been associated with brown heart appearance in 'Conference' fruits (Lentheric, Pinto, Vendrell, & Larrigaudiere, 1999; Veltman, Sanders, Persijn, Peppelenbos, & Oosterhaven, 1999; Veltman & van Schaik, 1997).

The amounts of AA tends to decrease during the storage of fruit and vegetables (Agar, Streif, & Bangerth, 1997; Lee & Kader, 2000). In pear tissue AA level can be very low: for Conference pears grown in the Netherlands from 72 mg/kg fresh weight (FW) at harvest (Veltman, Kho, van Schaik, Sanders, & Oosterhaven, 2000) it decreased to 13 mg/kg FW (Veltmann et al., 1999) or 23 mg/kg FW (Veltman et al., 2000) after 199 days storage in a controlled atmosphere, while for Conference pears grown in Spain, Lentheric et al. (1999) found 26–45 mg/kg FW at harvest. In addition, the pear tissue is a matrix which is characterized by a very high ascorbate peroxidase activity (Lentheric et al., 1999; Veltman, Larrigaudiere, Wichers, van Schaik, van der Plan, & Oosterhaven, 1999), which, therefore, impairs AA determination as it can easily destroy AA during the extraction, unless a suitable extraction procedure is used. To quantify ascorbic acid in pear tissue, Lentheric et al. (1999) followed the protocol of Brubaker, Müller-Mulot, and Southgate (1985), mixing 25 g of sliced pulp with 75 ml of distilled water and 10 ml of 10% metaphosphoric acid solution, then centrifuging at 20,000 g for 25 min and then the supernatant made up to 200 ml with water. Then ascorbic acid was analysed on a μBondapak carbohydrate column, by detecting it at 254 nm according to Wimalasiri and Wills (1983). Veltman et al. (2000) sampled portions of cortex tissue just above the core tissue using a 17 mm diameter cork borer, pooled portions from five fruits, which were immediately frozen in liquid nitrogen and crushed in a kitchen mixer. Then 10 g were diluted with 5 ml of 9.5% oxalic acid solution, 5 ml of methanol and 30 ml Milli-Q water, homogenized and filtered at 5 °C in the dark. Then ascorbic acid was analysed on a Symmetry C-18 column in ion-pair mode using UV detection at 251 nm.

The overall purpose of our work was to study the gradient in ascorbic acid concentration within pear

* Corresponding author. Tel.: +39-02-239557213; fax: +39-02-2365377.

E-mail address: rizzolo.ivtpa@interbusiness.it (A. Rizzolo).

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tissue in single fruits of Conference pears subjected to various storage regimes within the European Project FAIR CT-96–1803 "Quality improvement of pears by predictive and adaptive technology". The previously-mentioned methods used for the same type of fruit as ours had not straight applicability to this project's samples, because the small amount of tissue available made the sample crushing and homogenizing very difficult. In addition we had to detect AA using the UV–visible detector, as other types of detectors, such as the electrochemical and fluorimetric ones, more suitable for detecting the low amounts forseenable in our samples, were not available.

This study aims to find a suitable sampling procedure and an extraction method for pear fruits for the analysis of ascorbic acid baring in mind all the earlier-mentioned limitations. Three sampling procedures from fresh fruits and three extraction procedures from frozen tissues were tested. In addition, we focused on ascorbic acid recovery data, which were achieved by running at the same time the sample with and without addition of an ascorbic acid standard solution.

2. Materials and methods

2.1. Reagents

L-ascorbic acid was purchased from Merck, a 6% (w/v) aqueous solution of metaphosphoric acid (Merck) was used to extract samples and to prepare standards.

2.1.1. Standards

Stock standard solution contained 40 mg of ascorbic acid per 20 ml of 6% metaphosphoric acid. The 80 μg/ml ascorbic acid working standard solution for recovery tests was prepared by diluting 1.00 ml of stock solution to 25 ml with 6% metaphosphoric acid.

2.2. HPLC instrumentation

A Jasco (Tokio, Japan) HPLC system consisting of a PU-980 liquid chromatographic pump, a model AS-1555–10 autosampler and an UV-1575 intelligent UV/vis detector was used. Chromatographic data were stored and processed with a Shimadzu CR6A Chromatopac data processor. Separations were performed on an Inertsil ODS-3 (4.6 mm i.d.×250 mm length, particle diameter 5 μm) GL Science column either at ambient temperature or at 20 °C which was maintained using a Jasco Co-1560 Intelligent Column thermostat. The sample injection volume was 5 μl.

2.3. Ascorbic acid determination

Ascorbic acid was determined using 0.02 M orthophosphoric acid as mobile phase at a flow rate of 0.7 ml/min and UV detection at 254 nm, at 0.002 AUFS. The mobile phase was prepared by diluting 13 ml of 85% ortho-phosphoric acid (Merck) to 1 l with Milli-Q water. Then 100 ml of this stock solution was diluted to 1 l with Milli-Q water, then membrane-filtered and degassed by applying vacuum. Chromatographic peak in the samples was identified by comparing the retention time with that of a 4 μg/ml reference standard.

2.4. Calculations

Ascorbic acid quantitation was achieved by using the absolute calibration curve method of the data processor. The calculated analytical factor F1 was daily checked by injecting the work standard solution of ascorbic acid. The amount of ascorbic acid in mg/kg FW is calculated according to the equation:

$$\mathrm{AA(mg/kgFW)} = (A_{sa} \times \mathrm{F1}) \times 10\,000/\mathrm{wt} \times 100/\mathrm{rec}$$

where: A_{sa} is the area of ascorbic acid in the sample; F1 is the response factor for ascorbic acid and it is calculated by the ratio of the amount of standard to its area; wt is the sample weight (g); rec is the percentage of recovery of standard ascorbic acid added to the sample.

2.5. Sampling procedures from fresh fruits

Three sampling procedures were used: procedure S1, with which a pool of slices was prepared, followed the protocol by Lentheric et al. (1999); afterwards, samples were frozen in liquid nitrogen, put into polyethylene bags, sealed and stored at −20 °C for 1 month until extraction. Procedure S2 was similar to that described by Veltman et al. (2000), and each sample, made up of cylinders of pulp sampled using a cork borer, was frozen in liquid nitrogen in a little tulle bag and then stored either at −20 °C or at −80 °C for 1 month until extraction. Procedure S3 was exactly the same as procedure S2 except that each cylinder was divided axially into two parts, in order to have, for each sample, two ready-to-use portions for the parallel extraction of the sample without standard and of the same sample with standard addition for recovery evaluation.

2.6. Extraction procedures from frozen tissues

Three extraction procedures were compared. The first one (E1) was applied to S1 and S2 samples and it was according to Rizzolo, Forni, and Polesello (1984) and Brubaker et al. (1985); it was carried out on 5 g of thawed samples, extracting with 6% MPA, homogenizing, centrifuging and filtering through glass wool into a 50-ml volumetric flask, wrapped in aluminium foil. Procedure E2 was applied to 3–5 g of crushed frozen sample types S1 and S2 and was the same as E1, but all

the operations were performed under reduced light and on ice. The former condition was obtained by wrapping in black polyethylene film the centrifuge tubes and sealing them with a black cap; the temperature of +4 °C was maintained by putting on ice volumetric flasks and centrifuge tubes prior to weighing, during sample homogenization and moving tubes. In S2 at the moment of the extraction each replication was divided into two parts in order to determine the AA recovery by standard addition. Procedure E3 was applied to S2 and S3 samples and further improved the protection of samples from changes of temperatures during extraction. This was achieved by keeping all reagents at 2 °C, crushing both the sample portion and that for standard addition when just taken from −80 °C in a porcelain mortar with a porcelain pestle both pre-cooled at −20 °C prior to use, and carrying out homogenization and filtration through fluted paper into a 50-ml volumetric flask wrapped in aluminium foil in the dark in a cold room at 2 °C.

2.7. Storage of extracts

Before HPLC analysis the extracts were filtered through 0.45 m Nylon 66 membrane with a 1 μm glass wool pre-filter unit into 2-ml amber vials. When the HPLC separation was performed within 3–4 h from the end of the extraction procedure, the extracts were kept at 2 °C until HPLC analyses; otherwise, they were stored at −20 °C for a period ranging from 1 to 3 days until HPLC separation, and after thawing, they were kept at 2 °C until injection. In order to test the AA stability at 2 °C till the injection, either aluminium foil wrapped or amber vials containing MPA extract were kept over a 72-h period in cold room at +2 °C and were daily analysed for AA content.

2.8. Statistical analysis

Ascorbic acid results were analysed by one way analysis of variance and recovery results by two-way analysis of variance considering as factors the extraction procedure and the day of extraction. Statistically differences with *P*-values under 0.05 were considered significant and means were compared by 95% Tukey's HSD test, using STATGRAPH program, version 7.

3. Results and discussion

Using the aqueous orthophosphoric acid as mobile phase, according to Thompson and Trenerry (1995), on the ODS column AA had a retention time of 8.7±0.2 min with a room temperature between 18 and 22 °C, and a retention time of 8.725±0.008 min, when thermostated at 20 °C. This mobile phase presents the advantages of being cheap, easy and rapid to be prepared. Moreover, owing to the presence of a big unidentified peak which elutes at 30 min, two actions we had to made: (1) elapsing the analysis time to 35 min and (2) injecting a small volume of extract, i.e. 5 μl. The AA peak was well resolved from the other compounds which were co-extracted with MPA, such as malic acid which is present in such high amounts in pears, that it shows an absorbance value at 254 nm similar to that of the amount of ascorbic acid found in the samples of pears subjected to controlled atmosphere storage. Under these conditions the method was sensitive as the detection limit was ca. 0.1 ng per injection (5 μl) at a signal to noise ratio of 3. The limit of quantitation was 0.5 mg/kg FW and the calibration curve was linear in the concentration range of 0.01–400 μg/ml ($R^2 = 0.992$).

There was a great influence of the sampling procedure from fresh fruit. In fact, using the sampling S1 coupled with either the extraction E1 (Brubaker et al., 1985; Lentheric et al., 1999; Rizzolo et al., 1984) or E2 we did not find any detectable amount of ascorbic acid in pear extracts, whatever the type of fruit sampled. In addition, after dehydroascorbic acid reduction with dl-homocysteine, according to Chiari, Nesi, Carrea, and Righetti (1993), in E1 and E2 extracts, AA was detected at very low levels, close to the quantitation limit of the method, and only in four cases out of twenty (data not shown). We concluded that the extensive slicing prior to freezing into liquid nitrogen used in sampling S1 is not suitable for AA determination in pears, as it likely favoures the action of ascorbate peroxidase, inducing not only the AA oxidation, but also the degradation of dehydroascorbic acid to 2,3-diketo-gulonic acid, a reaction probably irreversible (Washko, Welsh, Dhariwal, Wang, & Lewie, 1992). These drawbacks were overcome preparing aliquotes of samples ready-to-use at the moment of the extraction (sampling S2). However, there was a slight thawing of little pieces of sample during the weighing operation of the second portion of sample in spite of working on ice. So the final sampling procedure S3, during which sample thawing did not occur, was set up.

As for the storage temperature of samples prior to extraction, the temperature of −20 °C was not low enough to prevent enzyme-induced oxidations in pear tissue; in fact, S2 samples, when stored at −20 °C, showed a slight browning of the tissue when they were just taken from storage and some tissue thawing when kept at room temperature for the extraction. So, it is advisable to lower the storage temperature of samples till the extraction to −80 °C.

On samples S2, at first AA was extracted using the extraction procedure E1; results obtained from just sampled outer and inner part of pulp, and pulp frozen in liquid nitrogen and stored for one month at −80 °C showed that there was no significant difference between

Table 1
Ascorbic acid (AA) content (mg/kg FW) in fresh and frozen pear tissues stored at −80 °C of different pear samples obtained with the three extraction procedures and literature data of similar samples

Samples	Portion	Extraction	Fresh	Frozen	Literature data
Pear stored in air at −0.5 °C for 15 days	Inner	E1	15.6	15.87	25[a]
	Outer	E1	12.47	11.96	
A pear stored in 2% O_2 + 0.7% CO_2 at −0.5 °C for 6 months	Inner	E2	1.86	1.88	–
Pear at harvest	Inner	E3	42.8	42.4	45[b]

Each value is the mean of three determinations.
[a] Veltman et al. (2000).
[b] Lentheric et al. (1999).

fresh and frozen tissue stored at −80 °C (Table 1); however, the AA amounts found were lower than those found in literature for similar samples of Conference pears. In addition E1 extraction showed the lower average recovery with the higher standard error (Table 2), and no significative influence of the day of analysis. So, in developing the extraction procedure, several precautions were taken in order to perform all the operations under reduced light and at a temperature of +4 °C (Section 2) and a further temperature control was achieved in procedure E3 by skipping centrifuging and immediately filtering the homogenate through fluted paper into the volumetric flask putting it in a cold room at 4 °C. Results obtained both on fresh and frozen tissue samples obtained with E2 and E3 procedures are shown in Table 1 and are compared with available literature data for similar Conference pear samples. Also for the extractions E2 and E3 there was no significant difference between fresh and frozen tissue stored at −80 °C and for E3 the AA amount found was similar to that of literature. Extraction E3 is preferable as it showes a significantly higher percentage AA recovery (Table 2), with a lower day to day variability (Table 4). Using extraction E2 percentage AA recovery was higher than E1, and when standard addition was performed by only measuring the volume, the standard error was comparable with that found for method E1 (Table 2). Furthermore extraction E2 had a higher day-to-day variability than extraction E3 (Table 3) having statisti-

Table 2
Percentage recovery of ascorbic acid (AA) from Conference pear tissue sampled with procedures S2 or S3 and extracted with procedures E1, E2 and E3[a]

Extraction method	Recovery (%)	S.E	Tukey's test $P<0.05$
E1[b]	60.41	2.60	c
E2[b]	73.43	2.38	b
E2[c]	75.48	1.76	b
E3[c]	82.84	0.60	a

[a] Means with different letters at Tukey's test are significantly different ($P<0.05$).
[b] Standard addition measured by volume.
[c] Standard addition measured both by volume and weight.

Table 3
Percentage of ascorbic acid (AA) recovery with extraction E2: day-to-day variability[a]

Day	No. of extractions	Recovery (%)	SE	Tukey's test $P<0.05$
1	6[b]	76.99	1.011	a
2	9[b]	91.51	2.566	b
3	9[c]	84.09	0.750	ab
4	9[c]	91.06	2.448	b
5	9[c]	84.50	0.983	ab
6	9[c]	79.57	2.039	a

[a] Means with different letters at Tukey's test are significantly different ($P<0.05$).
[b] Standard addition measured by volume.
[c] Standard addition measured both by volume and weight.

Table 4
Percentage ascorbic acid (AA) recovery with extraction E3: day-to-day variability[a]

Day	No. of extractions	Recovery (%)	SE	Tukey's test $P<0.05$
1	9	85.94	1.336	bde
2	8	82.08	1.624	bcde
3	8	72.58	3.256	a
4	5	85.20	0.982	bcde
5	10	88.41	1.353	e
6	6	81.25	4.296	abcde
7	8	78.79	1.764	abcd
8	6	81.32	1.035	abcde
9	6	83.04	1.120	bcde
10	6	82.95	1.902	bcde
11	6	76.80	1.792	ab
12	6	77.41	1.916	abcd
13	10	77.40	1.817	abc
14	10	85.25	1.330	bcde
15	6	83.74	0.895	bcde

[a] Means with different letters at Tukey's test are significantly different ($P<0.05$).

Table 5
Ascorbic acid (AA) content (mg/kg FW) in stored pears after 7 months in $2\%O_2+0.7\%\ CO_2$ (2+0.7) or in $2\%\ O_2+5\%\ CO_2$(2+5)[a]

Experiment		Storage atmosphere		
		2+0.7	2+5	SE
Influence of the position on the tree	Low	3.65	1.88	
	High	9.50	2.36	1.81
Influence of pulp position	Outer	6.25	1.02	
	Inner	3.51	2.05	0.79
Influence of harvest	Harvest 1	3.36	0.69	
	Harvest 2	4.59	1.31	0.97
	Harvest 3	6.71	2.59	

[a] All data were obtained with sampling S3, storage of sample till extraction at −80 °C, and extraction E3.

cally different percentage recoveries in 4 days out of 6 versus 12 days out of 15 not statistically different of extraction E3 (Table 4).

AA stability data of extracts kept at 2 °C till the injection showed that AA was stable over the first 24 h, having an average AA retention of 102±6% (average of 10 assays). After 72 h, AA retention was on average 88±6% (average of 10 assays).

Table 5 shows some examples of results for Conference pears which had been stored for seven months in two types of controlled atmosphere at −0.5 °C obtained with the set up procedure, i.e. sampling S3, storage of sample till the extraction at −80 °C and extraction E3.

4. Conclusions

The sampling procedure from fresh fruits and the storage temperature of frozen samples prior to extraction are key factors in ascorbic acid determination for pears. Only by avoiding extensive cutting, enzymes such as ascorbate oxidase, polyphenol oxidase, cytochrome oxidase and peroxidase, responsible for ascorbic acid oxidation (Ashoor & Monte, 1984; Wright & Kader, 1997) are not induced, as the mixing of enzymes and substrates that would be compartmentalized in undamaged tissues is prevented. In addition this study demonstrates that even small variations in temperature and light exposure during extraction significantly affect ascorbic acid recovery. So it is advisable, for fruits like pears, to couple each sample with its recovery test.

So the recommended method for determining AA in pear tissue comprises the following steps: sampling by taking pulp cylinders, deviding them axially into two parts, in order to have, for each sample, two ready-to-use portions for the parallel extraction of the sample without standard and of the same sample with standard addition and freezing each sample in liquid nitrogen in a little tulle bag; storing samples before extraction at −80 °C ; extracting AA keeping all the reagents at 2 °C; crushing as quick as possible sample when just taken from −80 °C in a porcelain mortar with a porcelain pestle both pre-cooled at −20 °C prior to use; weighing sample into a 90-ml black polyethylene film wrapped centrifuge tube in the presence, for the sample without standard, of 30-ml of 6% MPA on ice and, for the sample with standard addition, of 25 ml of MPA on ice plus 5-ml of working standard solution at 0.008% of ascorbic acid on ice previously weighed; and, in a cold room at 2 °C, homogenizing both samples with an Ultra-Turrax mixer, filtering through fluted paper into a 50-ml volumetric flask wrapped in aluminium foil and bringing the volume to the mark with 6% MPA; filtering the extracts through 0.45 μm Nylon 66 membrane with a 1 μm glass wool pre-filter unit into amber vials and keeping the extracts at 2 °C until HPLC analysis.

This procedure, besides giving the highest AA recoveries, is easier to perform and has high extraction rates: in 1 day two sets of six samples with their recovery tests can be carried out. The overnight HPLC separation further speeds up the work.

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