

Analytical, Nutritional and Clinical Methods

Determination of vitamin C in tropical fruits: A comparative evaluation of methods

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Abstract

Two analytical methods for extracting vitamin C (L-ascorbic and L-dehydroascorbic acids) in tropical fruits [banana, papaya, mango (at three maturity stages) and pineapple] were evaluated. These methods used ion-pair liquid chromatography (LC) for detecting ascorbic acid, but differed in the preparation of the sample (extraction with 3% metaphosphoric acid –8% acetic acid or 0.1% oxalic acid). Results were validated by comparison with ascorbic acid content obtained by the AOAC's official titrimetric method, by performing a recovery study and by the determination of within-day repeatability and inter-day reproducibility. There were differences in the efficiency of vitamin C extraction related to the fruit matrix and especially to the maturity stage in climacteric fruits. The LC-extraction method using 3% metaphosphoric acid –8% acetic acid shows high mean recoveries ($99 \pm 6\%$) for all matrices assayed, while the LC-extraction method with 0.1% oxalic acid proved to be unacceptable in some cases (unripe, half ripe and ripe banana and ripe mango) obtaining mean recoveries of $39.9 \pm 9.1\%$ and $72 \pm 13\%$ for banana and mango, respectively. The detection limit achieved with the metaphosphoric acid-acetic acid LC-extraction method for ascorbic acid (0.1 mg/l) allowed the determination of this vitamin in fruits analysed with good precision (5.94–12.8%), making its use as a routine analysis method perfectly valid. Recommendations about storage temperature, methods of thawing L-ascorbic acid extracts and the addition of antioxidants to extracts were made.

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1. Introduction

Vitamin C is the most important vitamin for human nutrition that is supplied by fruits and vegetables. L-Ascorbic acid (AA) is the main biologically active form of vitamin C. AA is reversibly oxidised to form L-dehydroascorbic acid (DHA), which also exhibits biological activity. Further oxidation generates diketogulonic acid (Fig. 1), which has no biological function (Davey et al., 2000; Deutsch, 2000). Since DHA can be easily converted into AA in the human

body it is important to measure both AA and DHA in fruits and vegetables to know vitamin C activity (Lee & Kader, 2000).

AA is widely distributed in plant cells where plays many crucial roles in growth and metabolism. As a potent antioxidant, AA has the capacity to eliminate several different reactive oxygen species, keeps the membrane-bound antioxidant α -tocopherol in the reduced state, acts as a cofactor maintaining the activity of a number of enzymes (by keeping metal ions in the reduced state), appears to be the substrate for oxalate and tartrate biosynthesis and has a role in stress resistance (Arrigoni & De Tullio, 2002; Davey et al., 2000; Klein & Kurilich, 2000).

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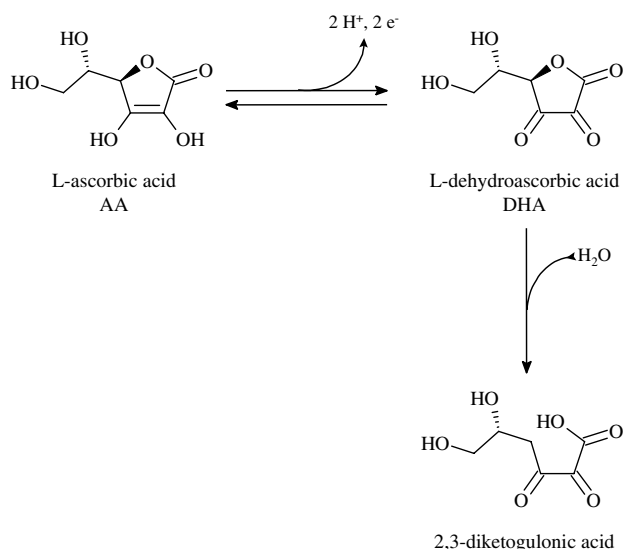


Fig. 1. Oxidation of L-ascorbic acid.

Since humans cannot synthesise ascorbate, their main source of the vitamin is dietary fruit and vegetables. Fruits (especially citrus and some tropical) are the best sources of this vitamin. An accurate and specific determination of the nutrients content of fruits is extremely important to understand the relationship of dietary intake and human health. Several analytical methods have been reported for the determination of vitamin C using titrimetry (AOAC, 1990; Kabasakalis, Siopidou, & Moshatou, 2000), spectrometry (Arya, Mahajan, & Jain, 1998) and amperometry (Arya, Mahajan, & Jain, 2000). Most of these methods may give overestimates due to the presence of oxidizable species other than AA and/or not to measure DHA. For example, the AOAC's official method (AOAC, 1990), based on the titration of AA with 2,6-dichloroindophenol in acidic solution, is not applicable in all the matrices. Substances naturally present in fruits such as tannins, betaninins, sulfhydryl compounds, Cu(II), Fe(II), Mn(II) and Co(II) are oxidised by the dye. Moreover, the method is applicable only when the concentration of DHA is low (Arya et al., 2000).

The preferred choice for AA determination are separation techniques: capillary electrophoresis (Versari, Mattioli, Parpinello, & Galassi, 2004), gas chromatography (Silva, 2005) and liquid chromatography (LC). LC avoids the problems of non-specific interference and ion-pair (Ke, El-Wazir, Cole, Mateos, & Kader, 1994), NH_2 bonded-phase (Silva, 2005; Zerdin, Rooney, & Vermuë, 2003) and reverse phase (Franke, Custer, Arakaki, & Murphy, 2004; Gökmen, Kahrman, Demir, & Acar, 2000) techniques have been reported. To increase the sensitivity for DHA, derivatisation prior to or after the chromatographic

separation is necessary. Usually, DHA is determined as the difference between the total AA after DHA reduction and AA content of the original sample. Various reducing agents, such as homocysteine, dithiothreitol (Gökmen et al., 2000; Silva, 2005) and L-cysteine (Zerdin et al., 2003) have been studied. Moreover, DHA can be determined after LC separation and detection by fluorimetry after a post-column derivatisation with *O*-phenyldiamine (Kall & Andersen, 1999).

To ensure that the subsequent LC analysis is effective, it is very important to optimise sample extraction when analysing vitamin C in complex samples such as fruits. It is essential to inactivate degradative enzymes which can destroy AA during the extraction and to fix the AA/DHA redox equilibrium. Extraction methods may also differ between different fruits or maturity stages (in climacteric fruits) because of their different matrices. Moreover, fruits contain large amounts of potentially interfering compounds. For these reasons great caution should be exercised in the employment of methods that have been developed for the analysis of specific plant tissue types (Davey et al., 2000). AA is readily oxidised under alkaline conditions, so the use of a high ionic strength, acidic extraction solvent is required to suppress metabolic activity upon disruption of the cell and to precipitate proteins. Metaphosphoric acid may provide efficient AA extraction by preventing oxidation (Cano et al., 1997; Franke et al., 2004) compared to other acids. On the other hand, it may cause serious analytical interactions with silica-based column materials, (RP- C_{18} or NH_2 bonded-phases) which can result in drifts in the baseline and retention time (Kall & Andersen, 1999). Oxalic acid (Kabasakalis et al., 2000; Ke et al., 1994) has also been reported as an AA extractant, moreover, at the concentrations usually employed, it is cheaper and less toxic than metaphosphoric acid. However, sometimes it does not recover the total AA present in the sample and the extracts are less stable than in metaphosphoric acid. A metal chelator such as EDTA is also usually required.

The main objective of the present study was to optimise a simple and rapid LC method for the determination of AA and DHA in tropical non-climacteric (pineapple) and climacteric (banana, papaya and mango, at three maturity stages) fruits and to compare two different methods of fruit extraction with metaphosphoric-acetic acid or oxalic acid. Taking into account that there is no appropriate reference material containing AA in fruit samples analysed, in order to validate the method the results were tested with (i) the AOAC's official titrimetric method, (ii) a recovery study and (iii) the determination of within-day repeatability and inter-day reproducibility.

2. Materials and methods

2.1. Chemicals and reagents

L-Ascorbic (AA), metaphosphoric (MPA), oxalic and citric acids, dithiothreitol (DTT), 2,6-di-tert-butylhydroxytoluene (BHT), ethylenediaminetetraacetic acid disodium salt (EDTA), 2,6-dichloroindophenol (DCIP), thymol blue and methylene blue were all supplied by Sigma (Madrid, Spain). Indigo carmine, *tert*-butylhydroquinone (TBHQ) and Darco granular activated carbon were purchased from Aldrich (Madrid, Spain) and sodium hydroxide solution (0.1 N) from Merck (Darmstadt, Germany). All other reagents (acetic acid, orthophosphoric acid and sodium bicarbonate) were obtained from Panreac (Madrid, Spain). Ethrel[®] 48 (48% ethephon) was given by ETISA (Spain). For chromatographic analysis, deionised water of 18 M Ω /cm resistivity, purified with a Milli-Q system (Millipore, Bedford, USA) was used. Stock standard solution containing 10 mg/ml of AA was prepared in water and stored in a glass-stoppered bottle at 4 °C in the dark. Solutions of variable concentrations were prepared by diluting the stock standard solution in water or 3% MPA–8% acetic acid. As L-dehydroascorbic acid (DHA) standard was not commercially available, it was obtained through the oxidation of AA stock solution by activated carbon (Cano et al., 1997; Gökmen et al., 2000).

2.2. Plant material

Banana (*Musa acuminata* AAA, cv. “Gran Enana”), papaya (*Carica papaya* L., cv. “Baixinho do Santa Amalia”), mango (*Mangifera indica* L., cv. “Keitt”) and orange (*Citrus sinensis*, cv. “Navelino”) were obtained from the Instituto Canario de Investigaciones Agrarias lands in Tenerife (Canary Islands, Spain) in October of 2002. Pineapple (*Ananas comosus*, cv. “Roja Española”) was provided from El Hierro (Canary Islands, Spain). Climacteric fruits, banana, papaya and mango, were harvested at physiological maturity stage (mature-green). Banana fingers were dipped in a 1 ml/l solution of Ethrel 48 for 1 min to accelerate ripeness. Climacteric fruits were allowed to ripen at 18 °C. Non-climacteric fruits, pineapple and orange (chosen for comparative purposes of vitamin C content), were collected at full-ripeness.

2.3. Methods for characterising ripeness stages

Ripeness stage of fruits was characterised and classified by colour, firmness and taste as unripe (physiological maturity stage), half-ripe and full-ripe (consumption stage). Nine uniform units of each fruit were analysed at each maturity stage and assessed for weight, length and

maximum fruit diameter. Peel and pulp colour were measured with a Minolta Chroma Meter model CR-300 (Wheeling, USA) colour difference meter, using attributes lightness (L), Hue and chromaticity (Chroma). Peel and pulp firmness were measured as penetration force using a Chatillon Mod. DPP-5 Kg with a 1 cm² tip (New York, USA). Total soluble solids (TSS) were determined using a hand refractometer Atago ATC-1 (Tokyo, Japan) and pH was measured by a WTW pHmeter (St Woburn, USA). After determination of pH, titratable acidity was measured with 0.1 N sodium hydroxide standard solution up to pH 8.1, and the results were expressed as mg main acid/100 g fruit (main acid: citric acid for orange, papaya, mango and pineapple, malic acid for banana). To determine water content (%) thin slices of the fruit were heated in an oven (65 °C, 24–72 h) until a constant weight was obtained and the weight loss was used to calculate the water content in fruit.

2.4. Extraction of vitamin C

For vitamin C determination, fruits were sliced, frozen into liquid nitrogen and stored at –80 °C until the analyses were carried out. Frozen pulverised fruit samples were weighed (0.5 g for orange, 1.5 g for banana, 0.25 g for papaya, mango and pineapple) and mixed with 2.5 ml of the extractant solution (3% MPA and 8% acetic acid for MPA–acetic acid extraction and 0.1% oxalic acid for oxalic acid extraction). The mixture was homogenised in a Politron PT 6000 (Kinematica AG, Switzerland) high-speed blender at 18000 g (in ice and darkness) for 1 min and then centrifuged at 9000 g (refrigerated at 4 °C) for 20 min. This procedure was repeated twice and the two resulting supernatants were mixed together. All extractions were carried out in quintuplicate. Several precautions were taken in order to perform all the operations under reduced light and at 4 °C temperature. Moreover, in order to stabilise vitamin C in the extractant solutions, adding 1 mM TBHQ is recommended.

2.5. Chromatographic determination of vitamin C

The liquid chromatographic method used for the determination of AA consisted of an isocratic elution procedure with UV-visible detection. The analyses were carried out on a Shimadzu modular chromatographic system (Kyoto, Japan) equipped with a LC-10 AD pump, a SPD-10AV UV-visible detector and controlled with a Class LC-10 data acquisition software (also from Shimadzu). The injection valve was a Rheodyne (Cotati, USA) Model 7725i with an injection loop of 20 μ L. The chromatographic system was equipped with a Shodex RSpak KC-811 column (5 μ m particle size, 250 \times 4.6 mm I.D.), using an isocratic 0.2% orthophosphoric acid mobile phase at a

flow rate of 1.2 ml/min. The temperature of the analytical column was kept at 25 °C using a Shimadzu CTO-10 A chromatography column oven. Detection wavelength for the UV-visible detector was set at 245 nm. The standard solutions and extracts were filtered through a 0.45 µm Nylon membrane before their injection in the chromatograph. AA peak was identified by comparing its UV-visible spectral characteristics and retention time with a commercial standard of AA. The spectrum (detection wavelengths from 200 to 700 nm) was recorded for the peak identified as AA by retention time, using a Shimadzu SPD-M6A UV-visible diode array detector. For each sample type the efficiency of peak separation was checked by the peak purity test carried out at maximum absorbance.

Each fruit extract was diluted six times with deionised water and injected twice ($n = 10$). To determine DHA, DTT was added to the fruit extract to obtain a concentration in the final extract of 5 mM DTT and then the mixture was kept in the dark at 30 °C for 15 min to convert any DHA to AA. After the conversion was complete, the sample was analysed for its total AA content using LC (sample injected in duplicate). The DHA content of the sample was calculated by subtracting the initial AA content from the total AA content after conversion. The reduction kinetics of DHA to AA were calculated by reacting a 10 mM DHA solution in water with a DTT solution at concentrations of 1, 5 and 20 mM (in the dark at 30 °C) until complete conversion was achieved.

To prevent the loss of AA, it was necessary to protect the standard solutions and the samples from light by using amber flasks.

2.6. The AOAC method to determine ascorbic acid

Results were validated comparing AA content obtained by LC (for the two extraction methods) with those obtained by the AOAC's official titrimetric method (AOAC, 1990). To summarise, 2 ml of the 3% MPA–8% acetic acid extracts (see Section 2.4) were titrated with indophenol solution (25% DCIP and 21% NaHCO₃ in water) until a light but distinct rose pink colour appears and persists for more than 5 seconds. The indophenol solution was standardised daily with AA solution. All determinations were repeated ten times. This titration method only determines AA and not DHA.

2.7. Stabilising ascorbic acid by adding antioxidants

The effect that antioxidants had on AA stability was tested by adding antioxidants EDTA, TBHQ or BHT at a concentration of 1 mM to the extracts. In the test, they were added to a standard solution of 50 mg/l AA in 3% MPA–8% acetic acid and to fruit

extracts spiked with 50 mg/l AA. Controls of 50 mg/l AA in both water and 3% MPA–8% acetic acid (without the addition of antioxidants) were used. In all cases, standard solutions or extracts were kept at room temperature, in contact with oxygen and light to favour AA oxidation. An aliquot of each of the samples was analysed at 0, 30, 60, 90, 120, 180 and 240 min by AA content and all determinations were done in triplicate. Neither of the antioxidant peaks co-eluted with the AA peak.

2.8. Statistical analysis

Data analysis was carried out with Systat statistical program version 10 (SPSS Inc., USA). Analysis of variance was used to evaluate physical and physicochemical characteristics of climacteric fruits (at three maturity stages), vitamin C content obtained from the chromatographic method and the AOAC's official titrimetric method and the effect of storage, thawing and addition of antioxidants to the extractant on AA stability in extracts. Fisher's Least-Significant-Difference test (LSD) was applied to experimental results to assess intra-pair significant differences ($p < 0.05$). Simple linear correlation analysis was used to measure the correlation between the results obtained for AA content from the chromatographic methods and the AOAC method.

3. Results and discussion

3.1. Chromatographic conditions

Initially, the chromatographic conditions described by Ke et al. (1994) were followed to analyse AA. Chemical, hydrodynamic, and physical variables were optimised to reduce the analysis time while keeping a good resolution between the peaks of AA and other co-extracted compounds in the samples. Good results were obtained using a mixture of water and orthophosphoric acid as the mobile phase. The concentration of orthophosphoric acid was studied over the range 0–0.5% and a concentration of 0.2% was found to be optimal. The flow-rate significantly influenced AA retention time; the best flow value was 1.2 ml/min (optimised between 0.4 and 1.6 ml/min) due to the better retention time (5.80 ± 0.06 min) and resolution for AA and other compounds. The oven temperature was studied over the range 25–80 °C and 25 °C was considered optimum because at higher temperatures there were AA losses.

Calibration equation for AA was constructed by plotting the UV response against the AA concentration at seven concentration levels (analysed in triplicate). UV response (y) of AA over a concentration (x) range of 0.5 to 50 mg/l was linear ($y = 6.07 \cdot x - 0.18$) with a regression coefficient (r^2) of 0.999. Detection limit,

defined as the minimum concentration capable of giving a chromatographic signal three times higher than background noise, was 0.1 mg/l. Sensitivity obtained with this method was similar to that obtained by Franke et al. (2004) and Gökmen et al. (2000) when an UV-visible detector was used, but lower (six times) than sensitivity obtained using an electrochemical detector (Franke et al., 2004). The RSD values for repeatability (11 consecutive injections of a standard solution containing 25 mg/l of AA) and inter-day reproducibility (five parallel determinations carried out for five consecutive days) were 0.06–1.05% for retention times and 1.97–10.9% for peak areas.

The reduction of DHA to AA was catalysed by DTT, but the DTT amount required to complete the reaction depends on how much DHA is present in the sample and this compound also affects the rate of reaction (Gökmen et al., 2000). The DTT concentration was optimised by reaction of a 10 mM DHA solution with a DTT solution at variable concentrations (1, 5 and 20 mM) in the dark at 30 °C. The complete conversion was achieved within 30, 10 and 2.5 min for 1, 5 and 20 mM DTT, respectively (Fig. 2). Gökmen et al. (2000) found that DHA could be completely converted to AA within 90–120 min at room temperature for $6 \cdot 10^{-4}$ – $6 \cdot 10^{-3}$ mM DTT, respectively. Relating the total AA concentration to reaction time, the pattern of DHA reduction gave a better mathematical fit when a zero order kinetics model was used, in agreement with the results obtained by Gökmen et al. (2000). The estimated rate constants were 0.267 ± 0.018 ($r^2 = 0.920$), 0.530 ± 0.018 ($r^2 = 0.969$) and 1.944 ± 0.087 ($r^2 = 0.941$) mg/l min for 1, 5 and 20 mM DTT, respectively. Because it is important to reduce the consumption of expensive chemicals for routine analysis, a concentration of 5 mM DTT (which gave a

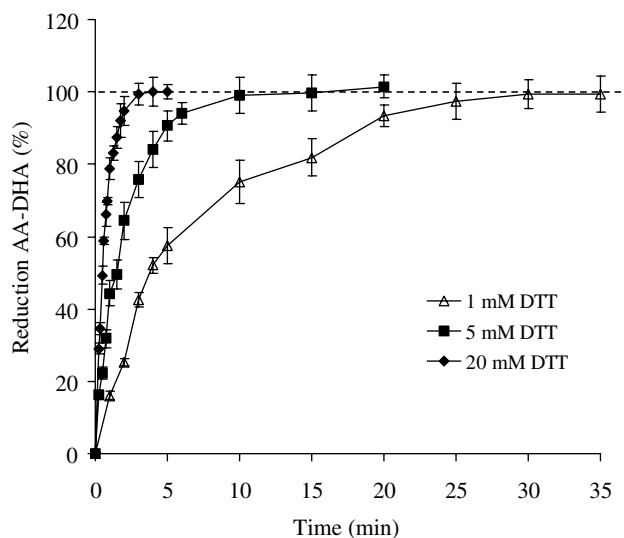


Fig. 2. Conversion (%) of L-dehydroascorbic acid in L-ascorbic acid at different dithiothreitol (DTT) concentrations.

reaction time sufficiently short) was selected as optimal. Since the amount of DTT required depends on how much DHA is present, the amount of DHA used to optimise the concentration of DTT was higher than the expected in fruit extracts. Nevertheless fruit extracts (orange, banana, papaya, mango, and pineapple) in 3% MPA–8% acetic acid and 0.1% oxalic acid were made to react with a 5 mM DTT solution in the dark at 30 °C and in each case the reaction was complete at 15 min. AA formed by DTT was stable for 2 h in the dark and 4 °C.

3.2. Optimisation of the ascorbic acid extraction

The AA extraction method may be influenced by the fruit type and/or the maturity stage (in climacteric fruits) due to the differences in the composition of their matrices. Since one of the objectives of this study was to develop a method to extract vitamin C from banana, papaya and mango (climacteric fruits) at three maturity stages, samples of these fruits at different stages of ripeness were taken (unripe, half-ripe and ripe) and characterised by colour, firmness and taste prior to the analysis of AA. The data regarding peel and pulp colour (L, Hue, Chroma), peel and pulp firmness, TSS, pH, titratable acidity and water content are reported in Table 1. Fruits experienced changes in colour, texture and composition during their ripening. There were changes in colour due to chlorophyll degradation and the appearance or synthesis of yellow and red pigments as the decrease in peel (banana and papaya) and pulp (banana, papaya and mango) hue shows. The degradation of pectins, compounds that contribute to fruit structure, produces losses in peel and pulp firmness. A starch transformation in sugars also takes place, increasing fruit sweetness (TSS) and also influencing texture.

Two different methods for preparing the sample prior to chromatographic analysis were evaluated: extraction with a mixture of MPA and acetic acid (AOAC, 1990; Cano et al., 1997) and with oxalic acid (Ke et al., 1994). The extraction conditions described in Section 2.4 were selected after performing various recovery tests of AA. Since AA extraction is usually carried out in acidic medium the efficiency of extraction was compared using a 0.1% oxalic (pH 2.8) and 0.1% citric acid (pH 2.2) solutions and water (containing an antioxidant, 0.01% BHT) adjusted at pH 2 with 1 N HCl. There were no significant differences in the extraction efficiency for papaya and mango obtained with the three extractants, but this extraction efficiency diminished 40% for water with 0.01% BHT for banana and pineapple and between 10 (pineapple) and 23% (banana) with 0.1% citric acid compared with 0.1% oxalic acid (selected as optimum extractant). The number of extractions necessary to obtain the maximum extraction efficiency was optimised with both extractants and it was fixed at 2. The efficiency of the first extraction was higher when 3% MPA–8%

Table 1
Physical and physicochemical characteristics of non-climacteric and climacteric fruits (at three maturity stages)

Characteristics	Orange	Banana			Papaya			Mango			Pineapple
	Ripe	Unripe	Half-ripe	Ripe	Unripe	Half-ripe	Ripe	Unripe	Half-ripe	Ripe	Ripe
<i>Fruit peel</i>											
L	76 ± 3	54 ± 2 c	66 ± 1 b	75 ± 2 a	41.7 ± 2.3 b	60 ± 4 a	66 ± 2 a	44.8 ± 2.8 a	43.0 ± 3.0 a	45.5 ± 3.4 a	49.5 ± 1.5
Hue	90 ± 3	122 ± 1 a	109 ± 4 b	97 ± 1 c	126 ± 3 a	100 ± 7 b	81 ± 4 c	107 ± 16 a	108 ± 16 a	113 ± 11 a	72 ± 4
Chroma	71 ± 3	40.7 ± 1.5 b	51 ± 3 a	53 ± 5 a	29.2 ± 3.2 c	50 ± 6 b	60 ± 2 a	28.0 ± 5.7 a	22.9 ± 3.9 a	20.7 ± 3.5 a	34.0 ± 1.2
Firmness (N)	41.9 ± 4.7	40.8 ± 3.5 a	18.6 ± 1.4 b	14.7 ± 1.3 c	>49.1	22.2 ± 6.3 a	7.68 ± 1.57 b	>49.1	>49.1	37.5 ± 5.8 a	35.6 ± 5.5
<i>Fruit pulp</i>											
L	47.6 ± 1.1	84 ± 1 a	74 ± 2 b	75 ± 4 b	67 ± 2 a	62 ± 3 ab	59 ± 3 b	79 ± 2 a	79 ± 3 a	73 ± 2 b	67 ± 2
Hue	101 ± 1	95 ± 1 a	90 ± 1 b	92 ± 1 b	71 ± 4 a	68 ± 3 ab	64 ± 2 b	103 ± 4 a	98 ± 3 ab	91 ± 5 b	102 ± 1
Chroma	27.1 ± 1.8	33.3 ± 1.0 b	41.8 ± 2.3 a	39.3 ± 3.0 a	41.9 ± 3.0 b	45.9 ± 2.0 ab	49.0 ± 2.2 a	46.8 ± 9.0 b	55 ± 5 ab	64 ± 4 a	16.4 ± 1.3
Firmness (N)	1.47 ± 0.35	27.3 ± 2.6 a	4.54 ± 0.47 b	3.01 ± 0.19 c	46.5 ± 1.9 a	3.19 ± 0.85 b	0.74 ± 0.27 c	47.9 ± 2.9 a	12.6 ± 2.7 b	3.36 ± 0.56 c	15.0 ± 1.4
Total soluble solids (°Brix)	12.2 ± 1.1	2.47 ± 0.20 c	17.3 ± 2.3 b	22.8 ± 1.2 a	8.60 ± 0.45 b	10.0 ± 1.0 ab	12.1 ± 1.1 a	3.73 ± 0.40 c	8.28 ± 1.30 b	12.2 ± 2.1 a	14.2 ± 0.4
pH	3.25 ± 0.14	5.36 ± 0.03 a	4.56 ± 0.05 b	4.69 ± 0.10 b	5.62 ± 0.08 b	5.41 ± 0.06 c	5.79 ± 0.07 a	3.33 ± 0.06 b	3.31 ± 0.11 b	3.69 ± 0.20 a	3.34 ± 0.13
Titrateable acidity (mg acid/100 g fruit)	139 ± 6	304 ± 49 b	573 ± 48 a	453 ± 82 a	63 ± 8 a	66 ± 11 a	72 ± 8 a	453 ± 88 a	453 ± 35 a	369 ± 77 a	1044 ± 99
Moisture content (%)	83 ± 2	73 ± 1 a	72 ± 1 a	74 ± 1 a	88 ± 4 a	87 ± 1 a	90 ± 3 a	84 ± 2 a	82 ± 2 a	80 ± 3 a	85 ± 1

Values are the mean ± standard deviation of $n = 5-9$ determinations.

Within a row (a-c), different letters denote significant differences ($P < 0.05$) between maturity stages in each climacteric fruit.

acetic acid was used as extractant for all fruits. The percentage of extraction in first extraction ranged between 83.0% (banana) and 98.3% (mango) and in second extraction between 1.7% (mango) and 17.2% (banana), respectively. Banana extracts in 0.1% oxalic acid were less stable, showing light brown colour, than in 3% MPA–8% acetic acid. Moreover, papaya and mango extracts in 3% MPA–8% acetic acid were cleaner than those in 0.1% oxalic acid.

3.3. Analysis of ascorbic acid

Table 2 shows AA, DHA and total vitamin C content for the different fruit samples [banana, papaya and mango (at three maturity stages), pineapple and orange], obtained with the two extraction methods evaluated. AA and DHA contents in the different fruits varied greatly. AA content were comprised of between 6.00 ± 0.88 (ripe banana) and 149 ± 16 (ripe papaya) mg/100 g fresh weight (FW) for the MPA–acetic acid extraction method, whereas for the oxalic acid extraction method concentrations were between 1.57 ± 0.08 (ripe banana) and 147 ± 10 (ripe papaya) mg/100 g FW. DHA content ranged between 0.36 ± 0.09 (pineapple) and 7.33 ± 1.12 (half-ripe mango) mg/100 g FW for the MPA–acetic acid extraction method, whereas for the oxalic acid extraction method concentrations were between zero (banana at the three maturity stages analysed and pineapple) and 5.08 ± 0.75 (half-ripe mango) mg/100 g FW. There were statistically significant differences in AA content obtained by the MPA–acetic acid and oxalic acid

extraction methods for orange, banana (at three maturity stages) and ripe mango. For this reason AA content obtained by the LC methods were validated with those obtained using the AOAC (1990) official titrimetric method (Table 2). Because the AOAC method may overestimate the AA content all extracts were tested for interferences such as basic substances (using pH indicator thymol blue) and reducing ions Fe(II), Sn(II) and Cu(II) (using indicators methylene blue and indigo carmine) before AA determination. None of the extracts contained interfering substances so titrimetric method could be applied to determine AA in all fruits examined. Simple linear correlation analysis was used to measure the relationship between the results obtained for AA using the chromatographic method and those of the AOAC method, obtaining a relatively strong significant correlation ($r^2 = 0.980$, $slope = 1.075$ for MPA–acetic acid extractant; $r^2 = 0.965$, $slope = 1.083$ for oxalic acid extractant). For each of the matrices analysed, there were no significant differences in the values obtained by the chromatographic (with two extraction methods) and the titrimetric method for orange, unripe, half-ripe and ripe papaya, unripe and half-ripe mango and pineapple. However, there were statistically significant differences in the AA content obtained by the oxalic acid LC-extraction and the AOAC method for unripe, half-ripe and ripe banana and ripe mango (the results were identical for the MPA–acetic acid LC-extraction and the AOAC method). Fig. 3 shows the chromatograms obtained for unripe banana, half-ripe banana and ripe mango obtained by both of the procedures.

Table 2

Quantification of vitamin C in non-climacteric and climacteric (at three maturity stages) fruits determined by the AOAC's titrimetric method and liquid chromatography (LC) after 3% MPA–8% acetic acid (E1) or 0.1% oxalic acid (E2) extraction

Fruit	AA ^a			DHA ^a		Total vitamin C ^a	
	E1–Titrimetry	E1–LC	E2–LC	E1–LC	E2–LC	E1–LC	E2–LC
Orange							
Ripe	68 ± 6 ab	75 ± 3 a	64 ± 2 b	2.32 ± 0.17 a	2.19 ± 0.17 a	77 ± 3 a	66 ± 2 b
Banana							
Unripe	13.8 ± 2.0 a/A	15.1 ± 0.9 a/A	6.87 ± 0.62 b/A	0.38 ± 0.05 a/A	– b/A	15.5 ± 1.0 a/A	6.87 ± 0.62 b/A
Half-ripe	13.2 ± 2.1 a/A	14.6 ± 1.3 a/A	4.66 ± 0.32 b/B	0.48 ± 0.08 a/A	– b/A	15.1 ± 1.4 a/A	4.66 ± 0.32 b/B
Ripe	7.15 ± 1.17 a/B	6.00 ± 0.88 a/B	1.57 ± 0.08 b/C	0.61 ± 0.18 a/A	– b/A	6.61 ± 1.06 a/B	1.57 ± 0.08 b/C
Papaya							
Unripe	81 ± 14 a/B	79 ± 10 a/B	82 ± 9 a/C	6.69 ± 1.76 a/A	1.02 ± 0.23 b/C	86 ± 12 a/B	83 ± 9 a/C
Half-ripe	80 ± 13 a/B	92 ± 6 a/B	93 ± 4 a/B	5.59 ± 0.69 a/A	5.01 ± 0.46 a/A	98 ± 7 a/B	98 ± 4 a/B
Ripe	151 ± 13 a/A	149 ± 16 a/A	147 ± 10 a/A	5.32 ± 1.15 a/A	1.88 ± 0.25 b/B	154 ± 17 a/A	149 ± 10 a/A
Mango							
Unripe	80 ± 8 a/A	76 ± 4 a/A	84 ± 9 a/A	5.42 ± 0.53 a/A	4.63 ± 0.96 a/A	81 ± 5 a/A	89 ± 10 a/A
Half-ripe	59 ± 11 a/B	61 ± 5 a/B	58 ± 4 a/B	7.33 ± 1.12 a/A	5.08 ± 0.75 b/A	68 ± 6 a/B	63 ± 5 a/B
Ripe	55 ± 8 a/B	54 ± 5 a/B	38.7 ± 1.4 b/C	5.73 ± 1.16 a/A	1.01 ± 0.73 b/B	60 ± 6 a/B	39.7 ± 2.1 b/C
Pineapple							
Ripe	26.9 ± 2.7 a	26.2 ± 3.2 a	26.5 ± 3.3 a	0.36 ± 0.09 a	– b	26.6 ± 3.3 a	26.5 ± 3.3 a

Within a row (a–b) or a column (A–C), different letters denote significant differences ($p < 0.05$) between analytical methods evaluated or between AA and DHA content among maturity stages in each climacteric fruit, respectively.

^a mg/100 g fresh weight (mean ± standard deviation, $n = 10$).

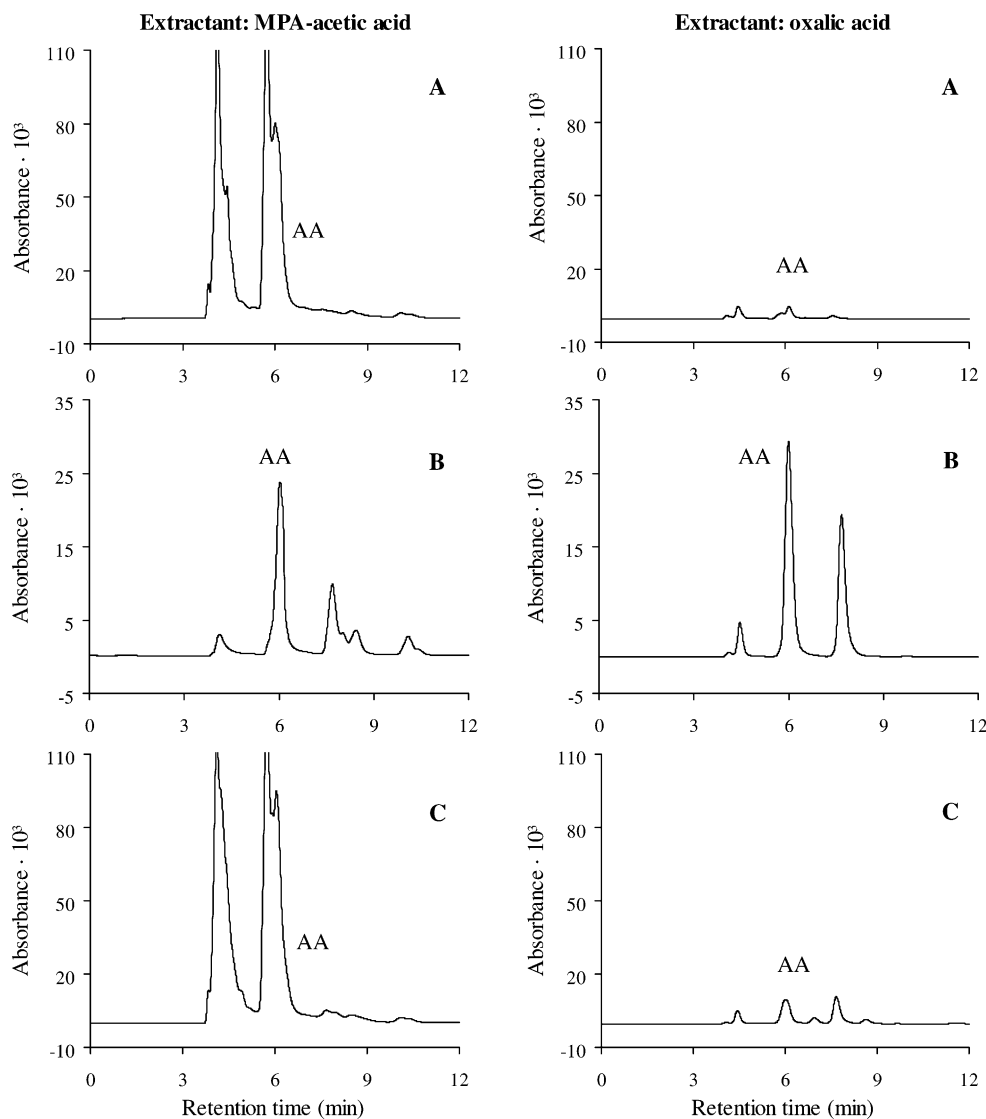


Fig. 3. Chromatograms obtained at 245 nm for unripe banana (A), for half-ripe papaya (B) and for ripe mango (C) extracted with 3% metaphosphoric acid (MPA)–8% acetic acid or 0.1% oxalic acid. AA, L-ascorbic acid.

AA and DHA amounts found for orange cv. “Navelino” in this study were similar to those found by Vanderslice, Higgs, Hayes, and Block (1990) for orange cv. “Florida” (54.7 and 8.3 mg/100 g for AA and DHA, respectively). However, Gökmen et al. (2000) established a lower content of AA (43.5 mg/100 g) and a higher content of DHA (3.5 mg/100 g). Unripe bananas contain twice as much vitamin C as ripe bananas. When the results obtained (analysing banana with the MPA–acetic acid LC-extraction method) were compared with those described by other authors, differences were found. For ripe banana cv. “Gran Enana”, Forster, Rodríguez-Rodríguez, Darias-Martín, and Díaz-Romero (2003) reported an average AA quantity of 11.5 ± 3.3 mg/100 g, while Cano et al. (1997) found higher amounts: 33.2 ± 0.6 mg/100 g. On the other hand, Leong and Shui (2002) described an AA content

of 2.1 ± 0.8 mg/100 g for ripe banana, although in this study the banana cultivar was not specified. For papaya, AA increased during ripening. In this study the AA amount found for ripe papaya was higher than that obtained by other authors: 74 ± 7 mg/100 g (Franke et al., 2004) and 68 ± 13 mg/100 g (Leong & Shui, 2002). The mango’s AA content decreased significantly during ripening. Franke et al. (2004) and Leong and Shui, 2002 established AA content for ripe mango (13.2 ± 5.1 and 19.7 ± 9.1 mg/100 g, respectively) and pineapple (5.00 and 54.0 ± 7.9 mg/100 g, respectively) which also differed from the results we found. These differences could be attributed to the variation in AA content among cultivars, which either were not referenced or were different from the ones that we analysed, and to pre-harvest factors (Lee & Kader, 2000). DHA did not account for more than 10% of total vitamin C in any of the analysed

fruits as has been described by Lee and Kader, (2000). It has been noted that when reporting vitamin C levels, many researchers have not taken into account DHA.

To evaluate the effect of the sample matrix on the accuracy of the analysis, taking into account that there is no appropriate reference material containing AA in fruit samples analysed, a recovery test was carried out. AA was added to fruit samples at two different concentration levels (100 and 250 µg of AA) and analysed in triplicate using the extraction methods evaluated in this study. The results of the recovery experiments are shown in Table 3. The mean recovery value for AA with MPA–acetic acid extraction method was $99 \pm 6\%$. The RSD for AA ranged from 1.49% to 9.65%. The oxalic acid extraction method was unacceptable for analysing AA in unripe, half-ripe and ripe banana and ripe mango. This was mainly because of the low percentage recoveries (mean recovery $39.9 \pm 9.1\%$ and $72 \pm 13\%$ for banana and mango, respectively). In these samples some difficulty was encountered also in obtaining reproducible determinations (RSD > 10%). In summary, from the two methods evaluated, MPA–acetic acid extraction method seemed to be the most suitable for giving a satisfactory quantitative analysis of AA in the fruits analysed [banana, papaya and mango (at three maturity stages) and pineapple] and in the whole range of concentrations tested.

Franke et al. (2004) described that extraction with MPA led to an extraction solution that was incompatible with the mobile phase of an ion-pair system by showing double peaks for AA. For this reason the sensitivity (slope of the calibration graph), linear range and limit of detection for AA in 3% MPA–8% acetic acid were ob-

tained. There were not significant differences in the values obtained for AA in water (data shown in Section 3.1) and in the selected extractant. Moreover, no double peaks were observed; this was confirmed by recording the spectra of the different peaks in the chromatograms. The repeatability of the fruit extracts ranged between 5.94% (pineapple) and 11.9% (banana and mango), and the RSD values for reproducibility between 8.90% (papaya) and 12.8% (mango). No significant differences were found between the within-day and inter-day precision indicating that the method has good reproducibility and that the LC-extraction procedure is stable and reliable.

3.4. Storage and thawing of fruit extracts to determine L-ascorbic acid

Definitive procedures for storage and thawing of AA extracts have not been established and procedures remain controversial. With this in mind, the effect of storage temperature (4 and -80°C) on the AA stability was studied in two standard solutions (50 mg/l AA in water and 3% MPA–8% acetic acid, $n = 3$) and a ripe banana extract spiked with 50 mg/l AA ($n = 3$), which was selected as the extract model because it had the highest complexity among the extracts. After 24 h, the stability of AA kept at 4°C was $95 \pm 5\%$ from the initial AA content; after 4 days it was $75 \pm 4\%$ and after 8 days $51 \pm 3\%$. These results are similar to those obtained by Kall and Andersen (1999) who found that 1% MPA–0.5% oxalic acid, adjusted to pH 2, provides excellent extraction and stabilisation of AA and DHA in extracts from broccoli, tomato, plum and cabbage, kept at 4°C

Table 3
L-Ascorbic acid recovery test

Fruit	E1-LC ^a						E2-LC ^a					
	100 µg AA added ^b			250 µg AA added ^b			100 µg AA added ^b			250 µg AA added ^b		
	R (%)	RSD (%)	H (%)	R (%)	RSD (%)	H (%)	R (%)	RSD (%)	H (%)	R (%)	RSD (%)	H (%)
Banana												
Unripe	100 ± 5	5.04	8.50	104 ± 2	2.07	7.41	47.3 ± 6.6	13.9	8.50	52 ± 6	12.2	7.41
Half-ripe	104 ± 4	3.71		103 ± 8	7.33		33.5 ± 5.7	14.0		39.8 ± 5.3	13.4	
Ripe	100 ± 4	4.01		102 ± 2	1.49		31.9 ± 6.8	21.3		34.6 ± 4.8	13.8	
Papaya												
Unripe	98 ± 4	4.41	6.49	96 ± 4	4.19	5.66	96 ± 2	2.36	6.49	100 ± 1	1.16	5.66
Half-ripe	95 ± 5	5.15		104 ± 5	4.81		102 ± 6	6.04		99 ± 5	5.26	
Ripe	99 ± 6	6.41		99 ± 4	3.76		106 ± 6	6.02		103 ± 3	2.54	
Mango												
Unripe	99 ± 5	5.11	6.49	94 ± 2	2.05	5.66	101 ± 2	1.53	6.49	98 ± 2	2.46	5.66
Half-ripe	99 ± 4	4.16		93 ± 1	0.77		101 ± 2	2.13		101 ± 5	5.04	
Ripe	96 ± 1	0.73		94 ± 3	2.97		67 ± 10	14.4		76 ± 17	22.2	
Pineapple												
Ripe	102 ± 2	2.17	6.49	97 ± 3	3.52	5.66	93 ± 1	0.74	6.49	99 ± 4	4.50	5.66

^a E1-LC, 3% MPA–8% acetic acid extraction–liquid chromatography; E2-LC, 0.1% oxalic acid extraction–liquid chromatography.

^b R, recovery (mean ± standard deviation, $n = 3$); RSD, relative standard deviation; H, Horwitz value ($H = \%RSD_R = 2 \cdot (1 - 0.5^{\log C})$, where C is the concentration of AA added in g/g fresh weight).

Table 4
Effect of thawing method on the recovery (%), $n = 3$) of L-ascorbic acid

Thawing method	50 mg/l AA, standard solution		Ripe banana extract, spiked with 50 mg/l AA
	water	3% MPA–8% acetic acid	3% MPA–8% acetic acid
No frozen	100 ± 2 a/A	100 ± 5 a/A	100 ± 6 a/A
Room temperature	89 ± 6 ab/B	88 ± 3 a/B	76 ± 5 b/B
Refrigerator	98 ± 5 a/AB	93 ± 5 a/AB	81 ± 1 b/B
Microwave oven	98 ± 5 a/AB	99 ± 6 a/A	93 ± 3 a/A

Within a row (a–b) or a column (A–B), different letters denote significant differences ($p < 0.05$) between standard solution and sample evaluated or between thawing method, respectively.

and protected against daylight. On the other hand, a concentration of 3% MPA–8% acetic acid (standard solution or fruit extract) stabilises AA during at least one month of storage at -80°C , while the losses of AA in water were $80 \pm 3\%$ at 19 days and $40 \pm 4\%$ at 22 days. The influence of the thawing method was also tested with the two standard solutions (50 mg/l AA in water and 3% MPA–8% acetic acid) and a ripe banana extract spiked with 50 mg/l AA, which were immediately frozen at -80°C . The standard solutions and extracts were thawed in a refrigerator (4°C , 50 min), at room temperature (21°C , 10–15 min) or in a microwave oven (0.5–2 min). All analyses were carried out in triplicate. Variability (%) in AA recovery (Table 4), relative to the content before freezing, differed depending on the thawing method ($84 \pm 7\%$, $91 \pm 9\%$ and $97 \pm 6\%$ when thawing at room temperature, in the refrigerator and in the microwave oven, respectively). There were no significant differences in the initial content and the values measured after microwave thawing. Thawing at room temperature resulted (for both standard solutions and extract) in lower AA content than thawing in the microwave oven. Microwave thawing was chosen on the basis of these results and because it was the most practical method for routine analysis. The effect that adding antioxidants (EDTA, TBHQ or BHT) to the extractant had on the AA stability was analysed (Fig. 4) under forced oxidation conditions (room temperature, contact with oxygen and light). Antioxidants were added to a ripe banana extract, which was selected as the extract model to determine the effect of adding antioxidants because it was the most complex fruit extract. In addition, antioxidants were added to a standard solution of 50 mg/l AA in 3% MPA–8% acetic acid. A control (without the addition of antioxidants) of 50 mg/l AA in water and in 3% MPA–8% acetic acid were used. It was found that AA recovery in water was inferior to 90% after 1 h, and only $78 \pm 2\%$ at 4 h. In 3% MPA–8% acetic acid, AA recovery decreased to $91 \pm 4\%$ in 30 min, but this recovery remained stable for 1.5 h, which indicates that the acidic pH of the extractant prevents the oxidation of AA (if the results are compared with AA standard in water). In the described conditions of forced oxidation, AA loss for the ripe banana extract (without the addition of antioxidants) increased, relative to that obtained for the

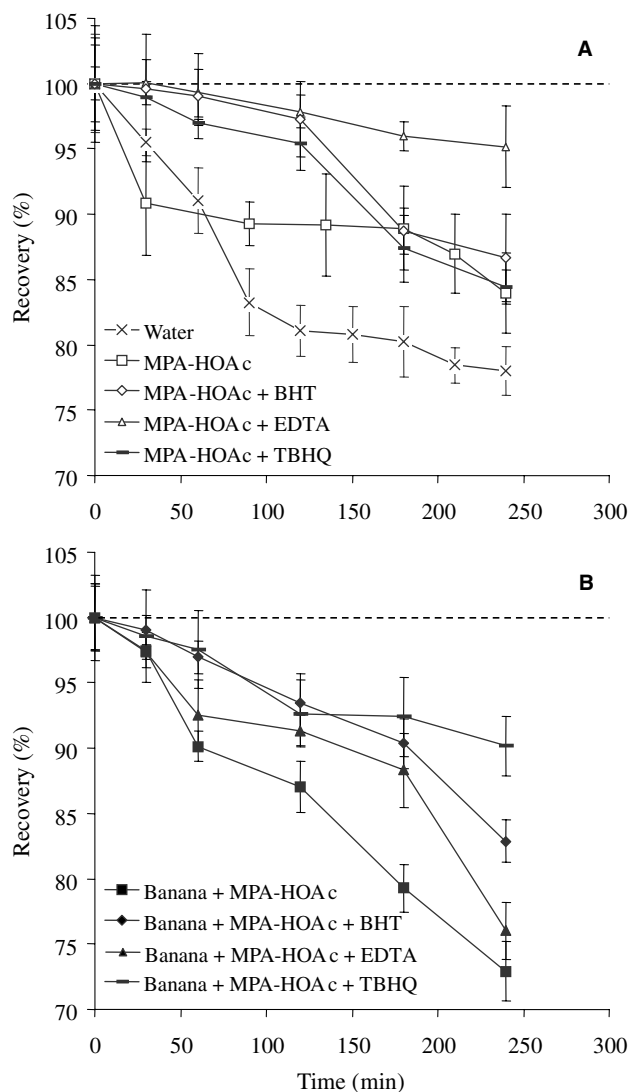


Fig. 4. Changes of L-ascorbic acid (AA) content in standard solutions (A) and ripe banana (B) extracts in 3% metaphosphoric acid (MPA)–8% acetic acid added with different antioxidants (concentration, 1 mM) at room temperature, in contact with oxygen and light. Both standard solutions and extracts were spiked with 50 mg/l AA.

standard solution in 3% MPA–8% acetic acid, being $13 \pm 2\%$ at 2 h and $27 \pm 2\%$ at 4 h, which indicates that in the banana extract some compounds are co-extracted that are capable of oxidising AA. All antioxidants assayed diminished AA loss providing recoveries higher

than 90% at 2 h in standard solutions and in banana extracts. 1 mM TBHQ was the most effective antioxidant in stabilising AA in the banana extract, because loss (under conditions of forced oxidation) was less than 10% at 4 h. For this reason the addition of 1 mM TBHQ to 3% MPA–8% acetic acid is recommended.

4. Conclusions

The 3% MPA–8% acetic acid LC-extraction method affords enough sensitivity and selectivity in L-ascorbic acid determination in tropical fruits [banana, papaya and mango (at three maturity stages) and pineapple] and the method is also free of interferences from other concomitants or the solvent peak. The method delivers results within 5.8 min after fruit extraction. The proposed method can be used by control laboratories to identify and quantify vitamin C in a wide variety of fruits. Finally, fruit extracts were stable during 24 h at 4 °C and during at least one month stored at –80 °C. Moreover, the most suitable method to thaw extracts is in a microwave oven. The use of an antioxidant (1 mM TBHQ) in the extractant solution is recommended.

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