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Development and Validation of HPTLC-Densitometric Method Compared to Titrimetric Method for Determination of L-ascorbic Acid in Citrus Fruits

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Aims: The aim of this study is to develop and validate an efficient method for the specific determination of L-ascorbic acid in citrus fruits and to compare it with a usual method, method using 2,6-dichlorophenolindophenol
Methodology: The research for a specific method for determination of L-ascorbic acid has led to development and validation of High Performance Thin Layer Chromatography – Densitometric (HPTLC-D) method. The validation criteria evaluated are response function, determination reading wavelength, limit of detection, precision of the technique, limit of quantification and recovery rate.

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The validated method was applied to citrus juice samples for quantification of L-ascorbic acid. Then a 2,6-dichlorophenolindophenol titrimetric assay of L-ascorbic acid in the same sample was performed for comparison of method results.
Results: HPTLC-D method showed improved sensitivity on 360 nm scanner and a good linearity relationship between standard concentrations and absorbance responses. The regression coefficient obtained is R^2 = 0.99. The limit of detection (LOD) and the limit of quantification (LOQ) are respectively 3 ng and 9.5 ng per deposit. The method also exhibited good repeatability and precision, with intra-day (n = 3) and inter-day (n = 4) coefficients of variation (CV) of less than 6%.
Application of the HPTLC-Dmethod to citrus juices yielded a recovery rate ranging from 97% to 99%.
Conclusion: Comparison of results of the two methods shows that the contents obtained by titrimetry are greater than those obtained by HPTLC-D by 42.25%. This is explained by the presence of other redox compounds which are dosed at the same time as L-ascorbic acid. HPTLC-D makes it possible to specifically dose L-ascorbic acid.

Keywords: L-ascorbic acid; High Performance Thin Layer Chromatography – Densitometric method; 2,6-dichlorophenolindophenol, titrimetric method.

1. INTRODUCTION

L-ascorbic acid is one of nutrients that body is unable to synthesize and which is very essential for its functioning, growth and development. It must, therefore, be provided to it exogenously through food or as a food supplement. It exists both in natural form (in plants more particularly red fruits, citrus fruits and green vegetables [1] and in synthesized form [2,3].

Among citrus fruits, oranges are the most common in the diet in developing African countries. It is not rare to find that health professionals advise orange in diet to compensate for deficiency in L-ascorbic acid.

So, it is a necessity to have easy and reliable methods for:

-estimate L-ascorbic acid content, given the multiplicity of citrus varieties that currently exist,
- control foodstuffs quality,
-develop food guides and nutrition education.

L-ascorbic acid dosage in fruit juices is generally carried out by titrimetric and colorimetric methods based on its reducing properties [4]. These methods have the disadvantage that they are not specific, due to interference from other substances. The problem of effectiveness of these methods is in fact posed. It then becomes clear that there is a need for a technique that specifically doses L-ascorbic acid. Several separation techniques by chromatography exist today. Of all chromatographic methods for quantifying substances, High Performance Thin Layer Chromatography - Densitometric (HPTLC-D) with L-ascorbic acid derivatization of indeed allows identification and separation of difficult to detect compounds. It is also quickly sensitive [5].

The main objective of this study is to develop and validate High Performance Thin Layer Chromatography-Densitometric method for determination of L-ascorbic acid in citrus fruits and to compare it with titrimetric method in 2, 6-dichlorophenolindophenol.

2. MATERIALS AND METHODS

2.1 Plant Material

Grapefruits, limes, pomelo, oranges, tangors, tangelos harvested at HINVI, MASSI (Atlantic department); YOKO (Oueme department). Citrus fruits have been identificated at the National Herbarium of Benin of University of Abomey-Calavi [AA6323/HNB for *Citrus maxima* (grapefruit), AA6324/HNB for *Citrus medicaL.*, (Citrus), AA6325/HNB for *Citrus paradisi* M. (Pomelo), AA6326/HNB for *Citrus sinensis* (orange), AA6327/HNB for *Citrus sinensis* Osbeck x *Citrusreticulata* Blanco (Tangor), AA6328/HNB for *Citrus paradisi* x *Citrus reticulata* Blanco (Tangelo)].

2.2 Methods

2.2.1 Development method of High Performance Thin Layer Chromatography-Densitometric (HPTLC-D)

Camag Application notes instrumental Thin-Layer Chromatography for Quantitative determination of vitamin C in fruit juices. method [6] isused to develop.

2.2.1.1 Preparation of stationary phase

A TLC plate was placed in a TLC tank containing 100 mL isopropanol for 1 hour. The plate is then removed and dried at 120 °C for 30 minutes.

2.2.1.2 Derivatization of standard ascorbic acid solution

A mixture composed of2 mL of sample (citrus juice orreference solution), 25 mL oxalic acidaqueoussolution (4%), 2 mL 2,6-dichlorophenolindophenolaqueous solution (0.5%) was incubated for 5 minutes forcomplete oxidation of L-ascorbic acid. Then 10 mg thioureawere added to mixture to neutralize excess oxidant, followed by adding 100 mL of distilled water.

An amount of20 mL mixture obtained were added to 4 mL 2,4-dinitrophenylhydrazine solution (2%) in sulfuric acid (70%).Then 6 mL ethyl acetate-acetic acid (98:2) were added three times to extract hydrazone. All hydrazoneswere combined and then added to 20 mL ethyl acetate-acetic acid mixture to form the solution to be analyzed by HPTLC-D.

2.2.1.3 Quantification by High Performance Thin Layer Chromatography-Densitometric method developed in the CAMAG laboratories

1 mL of the last mixture obtained, as well as the standard solution is introduced into vials of automatic depositor which deposited them on a stationary phase (HPTLC Silica gel 60 F254S 20 x 10 plates (Merck®) on a glass support). The volumes deposited were 0,5 ; 1 ; 1,5 ; 2 ; 2,5 µL for standard and1ou 2 µL for sample. The deposits were strips 6 mm long spaced 11,2 mm apart.

After the deposits, plate is removed, air dried for 10 minutes and developed in a chromatographic tank containing mobile phase (ethyl acetate - chloroform1:1, v/v). Saturation time of plate is 20 minutes. Then plate is removed, dried, observed with UV light and placed in the Camag TLC Scanner III densitometer, read at 360 nm, and then data are acquired and processed by the winCATS 1.2.4 software. .

2.2.2 Validation of High Performance Thin Layer Chromatography-Densitometric method

The validation was made according to the Strategy of French Society of Pharmaceutical Sciences and Technics [7, 8, 9].

2.2.2.1 Determination of the limit of detection (LOD)

It consists in determining the concentration whose response is equivalent to the amplitude of the background noise multiplied by three [10].

2.2.2.2 Determination of the limit of quantification(LOQ)

It is determined by multiplying the lower limit of the dosing interval by ten times the background noise.

2.2.2.3 Determination of recovery rate

This is the extraction yield. It is calculated as r = [(A- B) / C] x100, where A = the amount of analyte extracted from citrus juice with the addition of ethyl acetate - acetic acid; B = the amount of analyte in the juice without addition, C = the amount of ascorbic acid added.

2.2.2.4 Precision of the method

It consists to verify the repeatability and the intra and inter day precision for the technic. The inter-day fidelity is carried out over 4 days and calculated from 3 series per day which are used for calculation of intra-day fidelity.

2.2.2.5 Establishment of response function

It consists in determining the proportionality between the chromatographic response (y) expressed in arbitrary surface unit and the analyte content (x). When the regression is linear: $Y = ax + b$.
The response function is established from a range of calibrations, each point in the range being repeated 3 times. The calibration interval is chosen taking into account the limits of detection and quantification.

2.2.3 Titrimetric method: 2,6-dichlorophenolindophenol method

L-ascorbic acid quantification was carried out according to 2,6-dichlorophenolindophenol method [11]

2.2.3.1 Indophenol solution standardization

2 mL of L-ascorbicacid solution (1 mg/mL) are mixed with 5 mL of a solution obtained by dissolving 15 g of metaphosphoric acid in a mixture composed of 40 mLof acetic acid and 200 mL of distilled water, which mixture after

complete dissolution of metaphosphoric acid is brought to 500 mL with distilled water. The mixture obtained is rapidly titrated with a solution of indophenol [50 mg 2,6-dichlorophenolindophenol, 50 mL aqueous solution (42 mg of sodium carbonate, 200 mL of distilled water)]. A blank is also titrated.

2.2.3.2 Determination of ascorbic acid content in juices

2 mL of filtered juice of each citrus fruit is determined using the same method as for standard ascorbic acid.

3. RESULTS AND DISCUSSION

3.1 Results of Validation of High Performance Thin Layer Chromatography-Densitometric Method

3.1.1 Spectrodensitometry

The UV spectrum of standard obtained by HPTLC-Dscanner (Fig. 1.) clearly showed an maximum absorption between 254 and 360 nm. Wavelength of 360 nm has better sensitivity on the scanner, it was chosen.

3.1.2 Response function

The response /concentration values obtained during 4 different days with 3 daily repetitions for the reference solutions (n = 3), made it possible to calculate correlation coefficient of regression lines obtained (Fig. 2). It showed that there was a good linearity relationship between concentrations of the standard and absorbance responses: the regression coefficient R^2 was 0.99 with an equation: Y = 87.09x - 440.6. The linear regression model was chosen for validation of the method given the value of R^2.

3.1.3 The detection limit

The detection limit was 3 ng per spot (concentration giving a peak height equal to three times the background noise)

3.1.4 Fidelity of the method (intra-day and inter-day coefficient of variation)

Table 1 showed the coefficients of variations (CV) intra-day or repeatability (n = 3) and inter-days or intermediate precision (n = 4) obtained. They were less than 6% for entire series of concentrations analyzed. These values express good repeatability and precision of the method according to the Strategy of French Society of Pharmaceutical Sciences and Technics.

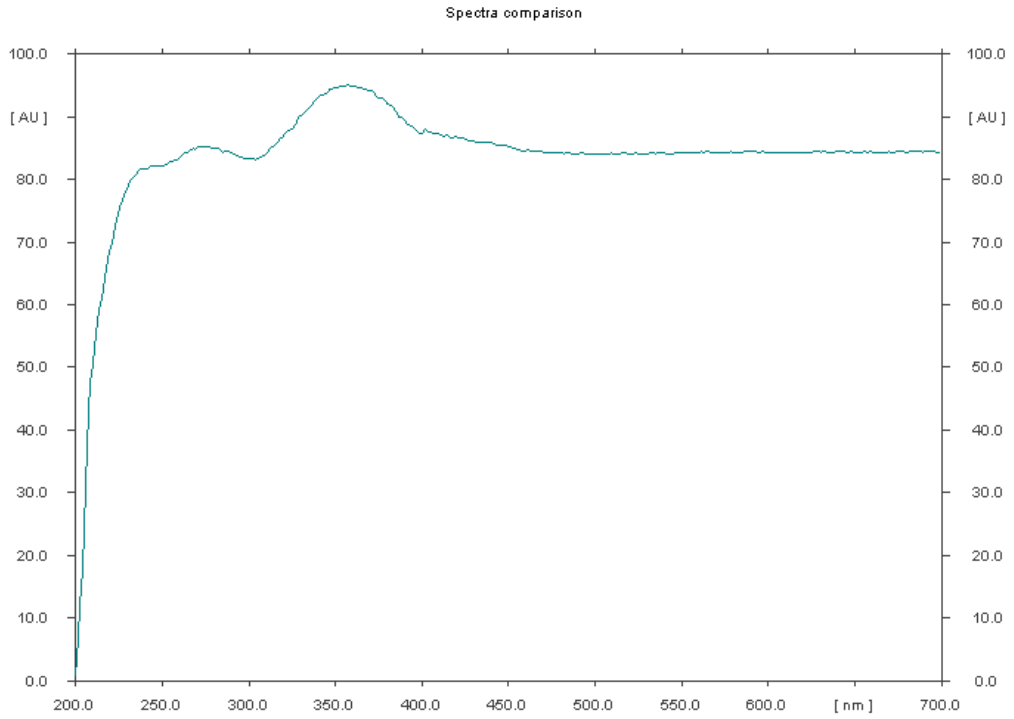

Fig. 1. UV spectrum ofascorbic acid taken by the scanner between 200 and 700 nm

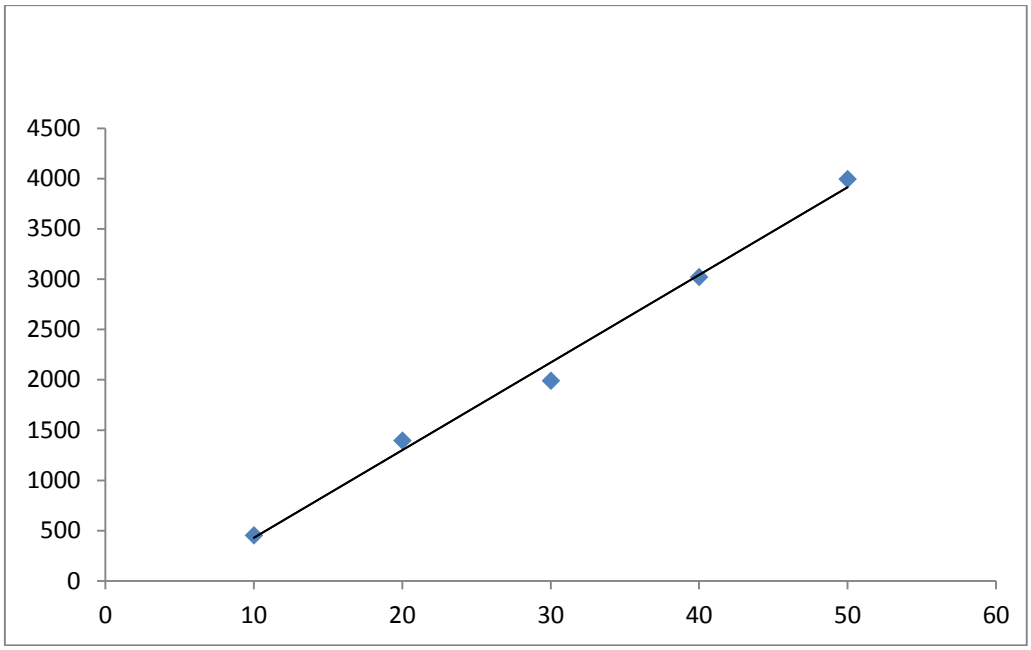

Fig. 2. Regression models tested for calibration

Table 1. Coefficients of variations of intra-day and inter-day analyzes

Standard	*Quantity (ng)*	*CV (%)*	
		Intra-day (n = 3)	*Inter-days (n = 4)*
	10	5.54	5.05
	20	4.83	3.86
	30	2.71	0.80
	40	4.71	3.39
	50	5.56	1.36

3.1.5 Limit of quantification

The limit of quantification was 9.5 ng per spot (quantity giving a peak height equal to 10 times the background noise).

3.2 Application to Citrus Juices and Determination of L-ascorbic Acid Content

3.2.1 Identification of L-ascorbic acid in citrus juices

L-ascorbic acid was identified in citrus juices analyzed by comparison of UV spectra of the standard spots and that at the same Rf in the juice sample (Fig. 3). This Fig. 3 showed the peaks corresponding to L-ascorbic acid in the standard and in two samples respectively.

The homogeneity of the three spectra clearly showed that the spot separated and analyzed in the sample was that of L-ascorbic acid. For analysis, the peak of L-ascorbic acid was identified as follows (Fig. 4) in the samples.

3.2.2 Recovery rate

The recovery rates (extraction yields) after L-ascorbic acid derivatization of three varieties of citrus juice taken at random were between 97% and 99% (Table 2), which was excellent considering the complexity of juice fruit.

3.2.3 L-ascorbic acid content in citrus juices (titrimetry)

The results of titrimetry method (Fig. 5) showed that all citrus juices studied contain L-ascorbic acid but with varying levels. The results showed that of all citrus fruits studied; orange was the richest in L-ascorbic acid.

3.3 L-ascorbic Acid Content of Citrus Fruits Obtained by HPTLC-D

The method developed and then validated was used to determine L-ascorbic acid content in juices. Resultswererepresented by Fig. 6.

3.4 Comparison of Results of the Two Analytical Methods

The comparison of contents obtained by titrimetry and by HPTLC-D analysis of these results revealed that contents obtained by titrimetric method are higher than those obtained by HPTLC-D. This variation can reach about 60% in some cases and may explain the specificity of HPTLC-D compared to titrimetry.

In fact, only L-ascorbic acidhydrazones of each juice obtained after derivatization are dosed after separation by HPTLC-Dand this compared to the standard.

The high titrimetric contents could be explained by the presence of tannin and other reducing, oxidizing or ketone-functional compounds which react with 2,6-dichlorophenolindophenol at the same time with L-ascorbic acid. This distorts the true content of L-ascorbic acid. It appears that HPTLC-D method was more specific, therefore more reliable than titrimetry for L-ascorbic acid in fruits quantification.

HPTLC-D method is also a fast, simple and reliable method.

Used to determine vitamin C content in other matrices, this method is qualified as simple, accurate, reproducible, sensitive, selective, precise [12,13,14].

Table 2. Estimated recovery of L-ascorbic acid

Sample	Quantity of standard (ng)	Recovery rate (%)
C. paradisi	10	98.69
	50	97.98
C.sinensis	10	98.03
	50	98.78
C. medica	10	98.13
	50	97.88

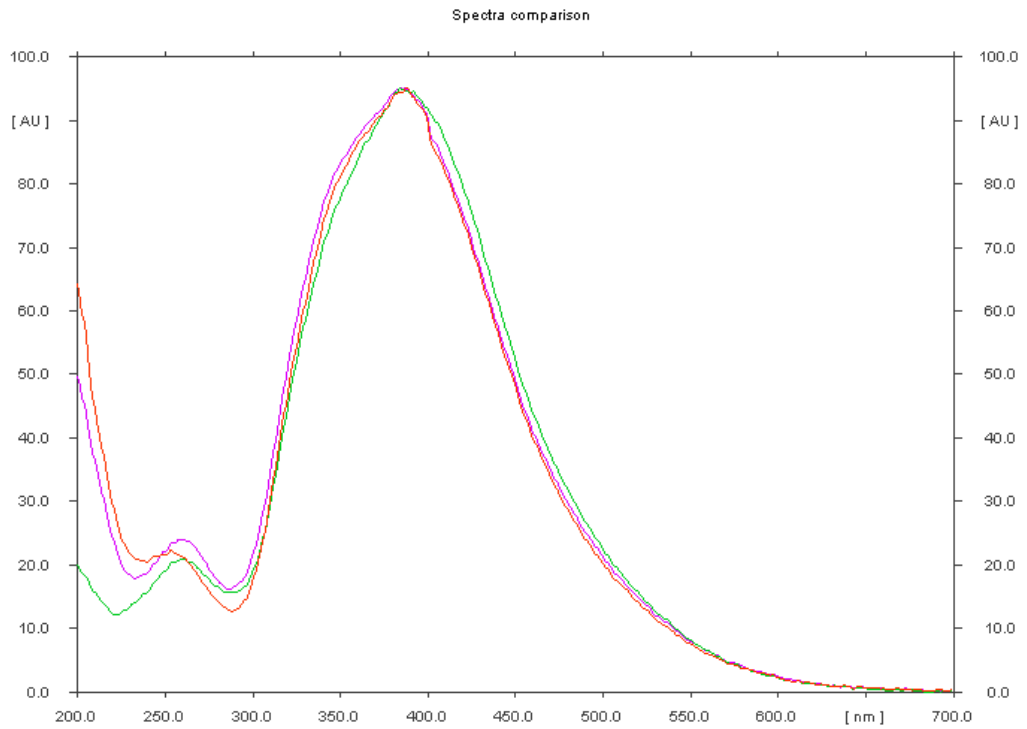

Fig. 3. Comparative UV spectrum of standard of L-ascorbic acid and ascorbic acid of two samples:

Standard's spectrum

C. medica'sspectrum

C. paradisi x C. reticulate'sspectrum

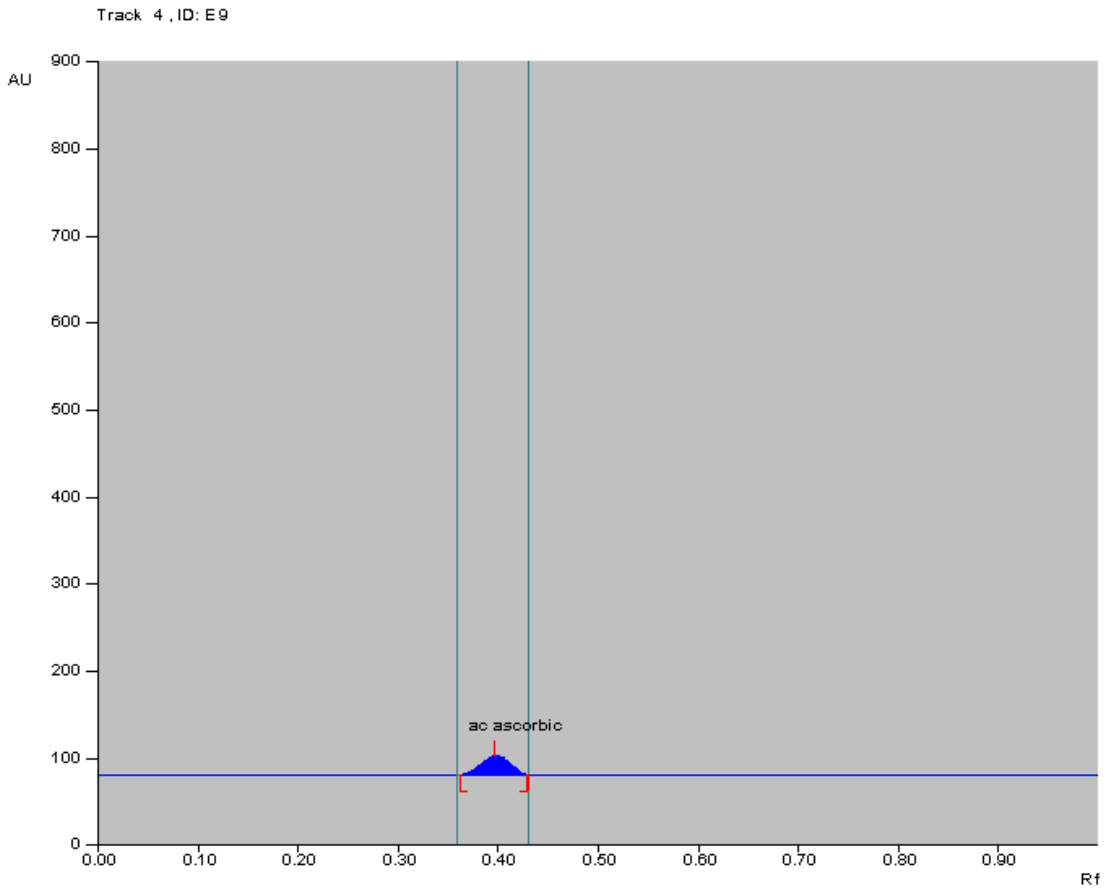

Fig. 4. Chromatogram of L-ascorbic acid in a sample juice analyzed

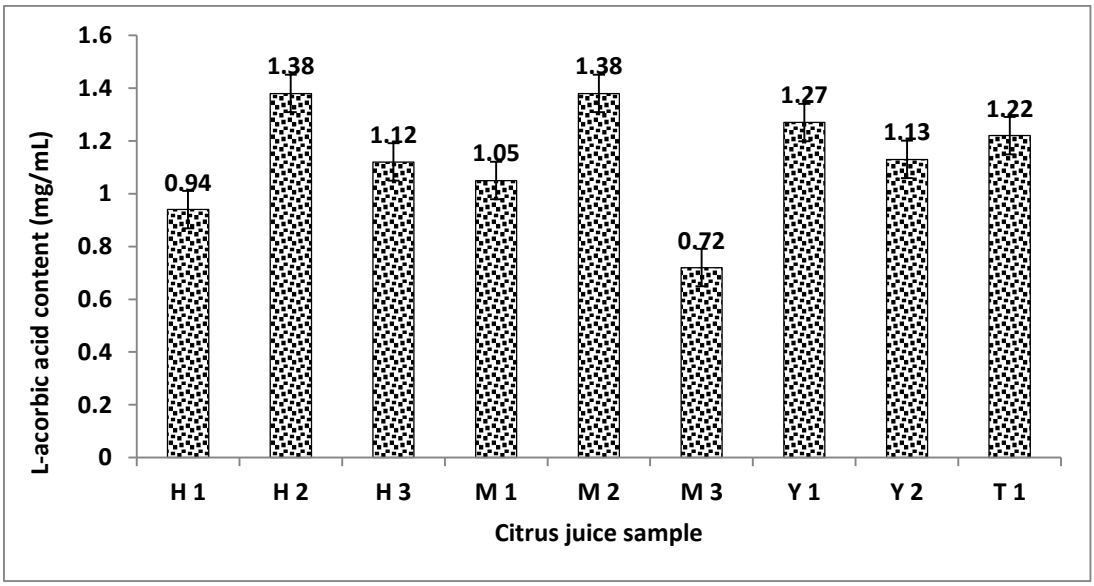

Fig. 5. L-ascorbic acid content of some citrus fruits obtained by titrimetry

H1= C. paradisi, H2 = C.sinensis, H3 = C. medica
M1 = C. sinensis x C. reticulate blanco, M2 = C. sinensis, M3 = C. paradisi x C. reticulata
Y1 = C. sinensis, Y2 = C. maxima
T1 = C. sinensis

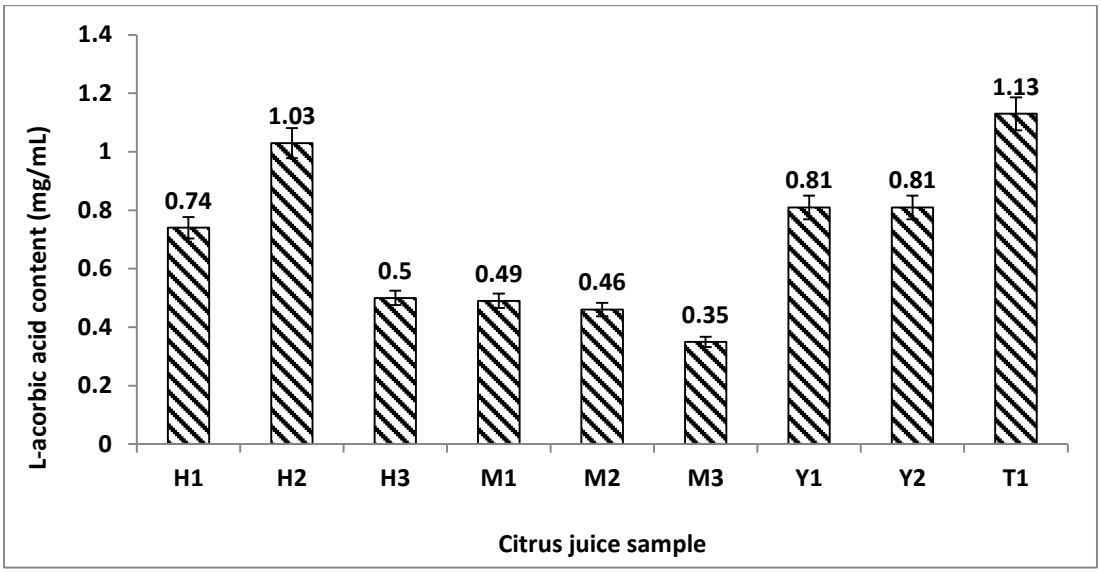

Fig. 6. L-ascorbic acid content of citrus fruits obtained by HPTLC-D
H1= C. paradisi, H2 = C.sinensis, H3 = C. medica
M1 = C. sinensis x C. reticulate blanco, M2 = C. sinensis, M3 = C. paradisi x C. reticulata
Y1 = C. sinensis, Y2 = C. maxima
T1 = C. sinensis

4. CONCLUSION

The HPTLC-Dmethod developed using a linear model, exhibits good repeatability and precision for the range of concentrations analyzed. The intra-day (n = 3) and inter-day (n = 4) coefficients of variation (CV) are less than 6% for the entire series of concentrations analyzed. The recovery rate of L-ascorbic acid in juices of different citrus varieties by the method is over 97%.

This method developed and valided was used to measure L-ascorbic acid in citrus juices. It was also quantified by titrimetry method with 2,6-dichlorophenolindophenol.

A comparison of results of the two methods reveals that the contents obtained by titrimetry are on average higher than those obtained by densitometric -high performance thin layer chromatography by 42.25%. This is explained by the presence of other redox compounds which are dosed at the same time along as L-ascorbic acid. Indeed, 2,6-dichlorophenolindophenol titrimetric assay of L-ascorbic acid is an oxidation-reduction reaction between 2,6-dichlophenolindophenol and L-ascorbic acid HPTLC-D makes it possible to dose L-ascorbic acid in a specific way.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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