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# Ascorbic acid from lyophilized camu-camu fruit: stability and quality control of hard capsules

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## ABSTRACT

**Camu-camu, a fruit found in the Amazon Basin river banks and lake shores, is known for its high ascorbic acid content together with, other antioxidants. This feature shows high potential for being exploited in agribusiness industry and pharmaceutical processes. However, its high acidity, as well as peel bitterness associated with the phenolic substances content, has discouraged its consumption while still fresh. The development of alternative forms for consuming this fruit, while still preserving its ascorbic acid and polyphenol content, in addition to its great potential for maintaining human health, has become a major economic activity in coastal communities. The present study evaluated the ascorbic acid stability found in camu-camu capsules. Lyophilization was performed with the fruit pulp and peel. Both freeze-dried fruit and powder-filled capsules were stored at 5° C. Ascorbic acid stability was monitored for 90 days using HPLC assay technique. The encapsulation process of freeze-dried pulp was considered satisfactory in the ascorbic acid conservation, since there was only a loss of 10% of its initial concentration throughout the study period for 60 days.**

Keywords: Camu-camu. *Myrciaria dubia*, Lyophilization. Capsules. Ascorbic acid. Stability.

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## INTRODUCTION

Camu-camu (*Myrciaria dubia* (HBK) McVaugh) displays relevant nutritional content, characterized by high ascorbic acid concentration ranging from 1.72 to 2.90 g.100-1 (Silva *et al*., 2006a, 2006b, 2006c; Bardales *et al*., 2008), and the presence of other antioxidants such as total phenols especially ellagic acid and flavonoids, which rank amongst the most important components for the health of the population (de Sousa *et al*., 2015; Langley *et al*., 2015; Ribeiro *et al*., 2016).

The camu-camuzeiro is a shrub belonging to the Myrtaceae family. The fruit of which presents a round shape with a smooth, shiny dark red to dark purple-colored surface (Maeda *et al*., 2006).

Studies have demonstrated the ascorbic acid retention in lyophilized products to be significantly higher than that found in oven and sub dried ones (Sablani, 2006). And the degradative reactions are minimized, allowing the product to have longer shelf life due to the reduction of water activity (George & Datta, 2002).

The use of hard gelatin capsules presents several advantages to the consumer and manufacturer, such as: easy transport and oral administration, protection of the active ingredient preventing contamination, masking unpleasant taste, in addition to providing good availability of the active ingredient due to its rapid disintegration. The capsules are filled by surface leveling technique and, almost always, it is necessary to complete the capsule's volume with excipient (ANVISA, 2010; Allen Jr. *et al*., 2013). There are different sizes available for the hard capsules, and the volume the active ingredient occupies within them depends on its density and compressibility (Ferreira, 2011).

The chosen excipient must meet the following requirements: not interfering with the release and dissolution of the active ingredient, facilitating the process of filling the capsule with uniformity of weight and content, and ensuring the preparation stability throughout the established validity period (Villanova & Sá, 2009).

Microcrystalline cellulose (MC) is a single multifunctional excipient widely used as an additive for pharmaceuticals, foods and cosmetics. Among its functions, this study highlights its roles as pharmaceutical formulations homogenizer, stabilizer, adsorbent, non-adhesive agent and powders flow enhancer (Ramires *et al*., 2010).

Hence, its remarkable vitamin C and bioactive substances content, has raised an increasing need for developing novel alternative techniques for its human consumption. Despite the paucity of scientific production and impact studies addressing the encapsulated freeze-dried fruit, freeze drying shows to be a reliable preservation process, free from both preservatives and chemical agents for preserving the fruit, which enhances the concentration of ascorbic acid and other substances (Villanueva-Tiburcio *et al*., 2010).

The purpose of the present developed capsules containing camu-camu freeze-dried fruit and to assess its ascorbic acid content stability, for a period of 90 days at low temperatures.

## MATERIAL AND METHODS

### Sample collection and Lyophilizing preparation

Camu-camu fruits were collected on tropical Brazilian Amazon non-flooded soil in the Municipality of Manaquiri (AM), at km 8, located 150 km from the city of Manaus (03º 25' 41" S e 60º 27' 34" W), in November 2014. About 3,400 kg of the fruit were harvested and transported in the same day to the National Research Institute of Amazonia (INPA), packed in black plastic bags and kept in icepack-filled cooling boxes. The fruits were selected manually in the Nutrition Biochemistry Laboratory/INPA, using ripeness, color, texture, and lack of blemishes and cuts as selecting criteria. Then, fruit batches were randomly mixed, by performing the following steps: pre-washing in running water, followed by sanitization where the fruits were kept immersed in 50 ppm chlorine solution for 10 minutes (Cenci, 2006). After being selected and washed, the fruits were cut into halves with the aid of a sanitized stainless steel knife, for removing the seeds. The pulp with pods was frozen at -18º C. For lyophilizing the samples, we used the benchtop Lyophilizer SP Plus Scientific Model ES-53 with 3 stainless steel, 208/230 V, 10 amps, 60 Hz, trays with initial pressure of 100 mTorr.

Lyophilization is a drying process consisting of three steps: freezing, primary and secondary drying. This process occurred through the - 50° C frozen fruit dehydration by sublimation, followed by desorption using low, drying temperatures at reduced pressure (Tattini *et al*., 2006).

### Preparation of capsules containing freeze dried camu-camu powder.

Capsules were filled out in the Laboratory of Innovation and Development in Pharmaceutical Technology (LIDETEF) at Federal University of Amazonas (FUA). Freeze dried camu-camu was powdered on o Osterizer Blender Food Processor at II per 1 minute velocity to turn the lyophilized fruit into powdered material. The microcrystalline cellulose increased by 20% relative to the mass of the lyophilized fruit, as diluent and stabilizer of the ascorbic acid present in the pericarp. All components were mixed in a V-Mixer S1-147 Solab for 20 minutes at 22 rpm and sieved in a 20 mesh stainless steel sieve.

The assay to determine the bulk density ($\rho_B$) of this mixture was conducted as described by Santos & Pessim (2007), who have calculated $\rho_B$ as the relation between the powder mass which fills the volumetric capacity ($V_c$) of the chosen capsule, according to Equation 1. The mass powder was calculated by difference between the full ($m_F$) and empty ($m_E$) capsule weigths. Assays were performed in triplicates at capsules n. 0 (volumetric capacity of 0.68 mL).

$$\rho_B = \frac{(m_F - m_E)}{V_c}$$

Equation 1

Capsules were prepared in manual capsule filling machine Multilabor, model MO-60. Three 60-unit batches were produced to be submitted to quality control tests.

### Quality control tests of capsules containing camu-camu freeze dried powder

For capsules obtained through masterful process in small batches, the National Formulary of the Brazilian Pharmacopoeia (ANVISA, 2012) recommends calculating the average weight (by a non-destructive method), the coefficient of variation and the percentage of the theoretical content variation, to evaluate the quality of capsules.

The average weight ($\bar{X}$) was calculated as the arithmetic mean of the individual weight of 10 units of capsules randomly selected within the manipulated batch. Afterward, the standard deviation from the mean (SD) was measured to determine the coefficient of variation (*CV*), according to Equation 2. In order to evaluate the theoretical content variation, the lightest ($X_L$) and the heaviest ($X_H$) capsules were selected among the 10 units sampled from each batch for calculating the Minimum Theoretical Amount ($Q_{theor.min.}$) and the Maximum Theoretical Amount ($Q_{theor.max}$), respectively, as described by Equations 3 and 4. The Theoretical Weight of Capsules containing freeze dried camu-camu ($X_T$) was estimated by the sum of the weights of the empty capsule and the powder content (Equation 5). Due to small but known weight variation of empty capsules, its mass ($m_E$ ) was calculated as the arithmetic mean of the individual weights of 20 empty units. Since density is an intensive quantity which relates mass by its volume, the powder content was estimated by multiplying $\rho_B$ by $V_c$.

$$CV = \frac{SD}{\bar{X}}.100$$

Equation 2

$$Q_{theor.min.} = \frac{X_L}{X_T}.100$$

Equation 3

$$Q_{theor.max.} = \frac{X_H}{X_T}.100$$

Equation 4

$$X_T = \overline{m_E} + (V_c - \rho_B)$$

Equation 5

**Analysis of ascorbic acid (AA)**

The determination of ascorbic acid content of the lyophilized and encapsulated samples was performed using the quantification method of analysis by high-performance liquid chromatography (HPLC), which consists of extracting the AA from the lyophilized product (100 mg) using 10 mL of extracting solution comprising water, 8% acetic acid (v / v), EDTA 1 mM, 0.3N sulfuric acid and m-phosphoric acid (MPA) 3% (v / v) in ice bath, following the procedures described by Valente *et al*. (2011). AA was titrated in duplicate, in samples at time zero (right after encapsulation), 30, 60 and 90 days. In the intervals between sample takings, the material was kept under refrigerating temperature at 5º C. The equipment used was the Thermo® chromatograph with automatic injector and quaternary pump, diodes array detector (HPLC-DAD) computer equipped with XCalibur® software for obtaining spectrometric data. Analytical column Phenomenex, Synergi Hydro-RP ™ (150 × 4.6 mm ID, 4.0 mM) from Phenomenex (Torrance, California, USA) with its pre-AQ C18 column (40 × 2.0 mm ID, 5 µm) from Phenomenex (Torrance, California, USA). The analyses were performed in isocratic mode with a flow rate of 0.6 mL.min$^{-1}$. The column temperature was maintained at 25° C and the shelf containing the samples and self-gun at 4° C. Analytical curve was made from L - (+) - ascorbic acid standard solution injection, in duplicate at six different concentrations (1, 20, 40, 60, 80, 100 µg.mL$^{-1}$). The L - (+) - ascorbic acid standard was solubilized in aqueous solution MPA at 1% (v / v) (Valente *et al*., 2011).

The mobile phase consisted of a mixture of water buffered with ($NH_4$) $2HPO_4$ 20 mM MPA 0.015% (pH 3.5 stabilized with formic acid). Elution was performed in isocratic mode for 8 minutes.

The limit of detection was determined by the lowest chromatographic concentration, the AA solution chromatogram peak level of which stood out at three times that of the average noise fluctuations in the chromatographic baseline (ICH, 2005). Thus, the detection limit was obtained from duplicate analysis of AA analytical solutions, at concentrations of 1 µg.mL$^{-1}$. LD is the lowest concentration able to produce a perfectly detectable peak (three times the average of the oscillations produced at baseline) (Snyder *et al*., 2010), according to Equation 6, where SD corresponds to the standard deviation of the average of the obtained areas, and b to the linear coefficient of the straight line obtained from the standard AA calibration slope.

$$LD = \frac{(3xDP)}{b}$$

Equation 6

**Microbiological analysis**

The microbiological analyses were performed on the capsules contents, freeze-dried fruit, for the quantification of total coliform, fecal coliform bacteria and fungi, in triplicate, meeting the recommendations of the American Public Health Association (APHA) (Sveum *et al*., 1992) and according to the current legislation (ANVISA, 2001; ICMSF, 2006).

Ten milliliters, 1 mL aliquot lauryl sulfate tryptose broth was the culture medium used for growth of coliforms. EC broth was used for fecal coliforms (*E. coli*) 8 mL, one heave and the agar with potato dextrose isolation of 1 mL aliquot at concentrations of $10^{-1}$, $10^{-2}$ to $10^{-3}$ was used for the growth of the fungi.

## RESULTS

The calculated $\rho_b$ was 0.21 g.mL$^{-1}$, that is, a n. 0 capsule, which had the volumetric capacity of 0.68 mL, held a mass of 0.1428 g of the resulting lyophilized powder of the mixture of crushed pulp and camu-camu plus 20% (w/w) of microcrystalline cellulose. Since the weight of the empty capsule n. 0 should to be 0.1009 g (determined by the average of 20 units), the expected XT from each capsule was 0.2437 g.

TABLE 1. Calculated parameters for the quality control of the manipulated capsules.

| Lot | $\bar{X}$ (g) | SD (±) | *CV* (%) | Light Capsule | | Heavy Capsule | |
|---|---|---|---|---|---|---|---|
| | | | | m (g) | $Q_{theor.min}$ (%) | m (g) | $Q_{theor.max}$ (%) |
| 1 | 0.2496 | 0.0010 | 0.3783 | 0.2328 | 95.53 | 0.2597 | 106.57 |
| 2 | 0.2427 | 0.0056 | 2.3306 | 0.2316 | 95.03 | 0.2476 | 101.60 |
| 3 | 0.2503 | 0.0069 | 2.7594 | 0.2422 | 99.38 | 0.2601 | 106.73 |

Table 1 notes calculated values of $\bar{X}$, *CV* and $Q_{theor.min.}$ and $Q_{theor.max}$. for the 3 handled batches. All *CV* values are very close to the $X_T$. Since the filled capsules weigh less than 300 mg, batches where all individual capsule values have remained within the ± 10% *CV* range should

be approved. As no unit presented individual mass value higher or lower than the limits calculated for each lot, all were considered approved in this regard. The lots were also considered approved with respect to the coefficient of variation or *CV*, since the law stipulates that the calculated values should not exceed 4% (ANVISA, 2010).

$Q_{theor.min}$ and $Q_{theor.max}$ are calculated to enable estimating the acceptable range of the weight of the capsules, since by the non-destructive method, the capsules were not opened for experimentally determining their contents homogeneity. Thus, assuming the encapsulated powder mass to be perfectly homogeneous; the acceptable content should be within the range of 90 to 110%. Since the lowest and maximum determined levels showed to be 95.03% and 106.73%, respectively, all lots were considered definitely approved.

When analyzed under the same chromatographic conditions as those of the standard AA, the camu-camu pulp presented a chromatographic peak with a similar 4.41 ± 0.02 minutes retention time (Figure 1). The calibration slope was obtained from the average of the areas of standard AA chromatographic peaks for six different concentrations.

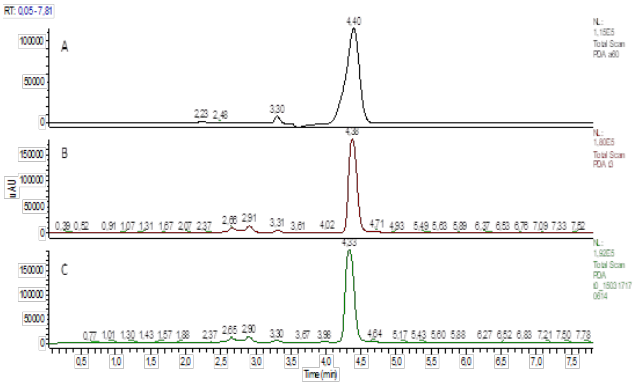


FIGURE 1. Gas chromatograms obtained by HPLC-DAD: A) AA standard; B) AA content in camu-camu fruit at time zero; C) AA content in camu-camu fruit following 90 days (time 3).

The linear correlation coefficient ($r^2$) for the calibration slope was 0.9960 (Figure 2). The method shows good linearity in the concentration range tested (1-100 $\mu g.mL^{-1}$). From interpolation of the mean value of the area for the AA signal obtained on camu-camu fruits, by employing the Straight Line equation obtained on the AA standard calibration slope, enabling to determine the fruit's AA content at the beginning ($T_0$) and three months later ($T_3$).

The results from AAs analysis from contents set at respective times (Table 2).

TABLE 2. Lyophilized camu-camu ascorbic acid contents (g.100 $g^{-1}$) stored at 5° C for 3 months as determined by high-performance liquid chromatography.

| Storage time (months) | AA |
|---|---|
| 0 | 3.04+0.04 |
| 3 | 2.60+0.30 |

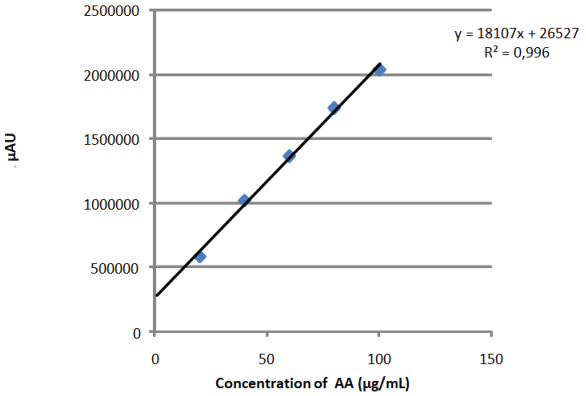


FIGURE 2. Analytical curve obtained from standard AA analyses in six different concentrations by employing HPLC-DAD.

During the study, we conducted two analyses of vitamin C in the lyophilized fruits of camu-camu. At baseline (t = 0) vitamin C estimate was 3.04 g.100 $g^{-1}$ and in the third month (t = 3) it was 2.60 g.100 $g^{-1}$. Whereas at 45-days it showed to be the same as at one month t = 1.5, and the difference between T = 3 and T = 0 was 3 months, and the difference of vitamin C between t = 0 and t = 3 equals 0.44 g.100 g -1 (14.47%). Through simple rule 3 the difference between t = 0 and t = 1.5 calculates a decrease of 0.22 g.100 $g^{-1}$ (7.24%). Thus, T = 1.5 is tantamount to 2.82 g.100 $g^{-1}$.

The average weight of the capsule content No 0 was 0.1455 ± 0.02 g, while at t = 0 it was equivalent to 442.3 mg of vitamin C and at t = 1.5 to 410.3 mg of vitamin C.

The microbiological analyzes showed acceptable results within the limits established by ANVISA (2001) for total coliforms, fecal coliforms and fungi (Table 3).

TABLE 3. Microbial Count in Lyophilized camu-camu.

| | Total coliform | Fecal coliform | Fungus |
|---|---|---|---|
| Temperature (º C) | 37 | 45 | 35 |
| Incubation time | 24-48 h | 24-48 h | 5 dias |
| MPN/g* | 0,0 | 0,0 | - |
| FUC/g** | - | - | <10 |

* MPN- Most Probable Number

** CFU – Colony Forming Unit

Ascorbic acid in addition to other major, antioxidant activity-bearing components content, brought about a demand in the market for camu-camu fruit, yet, its consumption is still low on account of the fresh fruit being both highly acid to consume and highly perishable to be kept at air temperature. These factors have confirmed the need for the development of technologies, which would enable a longer shelf life period and the consumption of the fruit in a processed form.

The lyophilization process benefits transportation and storage due to its compactness combined with the food's microbiological and chemical stability beneficial effects,

thereby providing a product, which will remain viable for a long time gap (Sagar & Suresh, 2010).

However, the shelf life of a dehydrated food depends on extrinsic factors, such as packaging size and properties, environmental storage conditions (humidity, oxygen concentration, light, time and temperature), shipping and handling, as well as intrinsic factors such as chemical composition of food, type and concentration of additives (Kowalska & Lenart, 2005; Maeda *et al*., 2007).

As shown by the results, the method used with the hard gelatin capsules provided the lyophilized camu-camu with, among other inherent properties, a light barrier, protecting it against oxidation and moisture absorption. These factors contribute to reduce the degradation of the processed fruit, ensuring shelf life lengthening, as well as the stabilization of the antioxidant components associated with the refrigeration temperature.

## DISCUSSION

Due to the higher bark, ascorbic acid concentration, its incorporation into the pulp becomes a positive contribution, which was also observed in studies by Maeda *et al*. (2006).

This Amazonian fruit with its remarkable vitamin C content is an important food for diet. The recommended daily intake of vitamin C for adults was estimated to be 75 mg for women and 90 mg for men (NRC, 2000a, 2000b).

In this study the camu-camu provides six and fivefold the vitamin C daily recommendations for women and men, respectively.

Regarding stability of vitamin C in the pulp of camu-camu in storage at - 18° C, Justi *et al*. (2000) found a 23% decrease in its concentration up to the twenty-eighth day. By the hundredth day the decrease had come to 26%, remaining constant up to the 335th.

As to the loss of ascorbic acid in the nectars Maeda *et al*. (2007) found there to be higher losses in those stored at the temperature of 26º C, reaching 20% at the end of the storage period (120 days), while those stored under refrigeration (5º C) had from 12 to 14% losses.

As to the packaging, studies conducted by Juliano *et al*. (2014) on lyophilized camu-camu pulp, kept at 25° C for five months in aluminum layer-coated polyethylene containers, presented satisfactory storing characteristics for 150 days, maintaining the product stable due to the intake of less moisture and the prevention of pigments oxidation and coloring change.

The authors noted that, from 90 days onwards, the freeze-dried pulps packed in polyethylene and nylon bags, no longer had quantifiable anthocyanin contents. And from day 60 on, it was not possible to quantify the ascorbic acid content.

Maeda *et al*. (2007) also assessed the reduction of anthocyanins and ascorbic acid during the storage time, and room temperature, proving these pigments to be heat-sensitive, photosensitive, and to tend to decrease with storing time. However, for nectars stored at refrigeration temperature and protected from light, they found there to be no reduction on their contents.

As to microbiological analyses showing acceptable findings within the limits establishes by Anvisa they demonstrating the used sanitizer to having been effective when it came to eliminating pathogenic and spoilage microorganisms throughout all processing steps (pulping, freezing and packaging), assuring hygienic and sanitary safety.

In conclusion, we determined the stability of ascorbic acid in the freeze-dried and encapsulated camu-camu in accordance with the rules of the Brazilian Pharmacopoeia. During 90 days, the loss was considered acceptable, once ascorbic acid is readily oxidized when exposed to oxygen, light, air temperature, long shelf life, packaging and others. By 60 days can be considered the term of validity, defined as the time taken by the active ingredient to diminish its initial concentration by 10%. Besides, it was verified that the controlled, encapsulating process stages, and conservation under refrigeration temperature were paramount to warrant the physical-chemical and microbiological quality of the fruit.

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## RESUMO

*Ácido ascórbico do fruto de camu-camu liofilizado: estabilidade e controle de qualidade de cápsulas duras*

**Camu-camu, um fruto encontrado nas margens dos rios e lagos da Bacia Amazônica, é conhecida por seu alto teor de ácido ascórbico, juntamente com outros antioxidantes. Esta característica apresenta grande potencial para ser explorado na agroindústria e em processos farmacêuticos. Entretanto, sua elevada acidez, assim como o amargor da casca relacionado ao teor de compostos fenólicos, desencoraja o seu consumo in natura. O desenvolvimento de formas alternativas de consumo desse fruto, que preservem o conteúdo em ácido ascórbico e polifenóis, além do seu grande potencial na manutenção da saúde humana, é importante para a atividade econômica de populações ribeirinhas. Este estudo avaliou a estabilidade do ácido ascórbico do camu-camu em cápsulas. A liofilização do fruto foi realizada com a polpa e a casca. Tanto o fruto liofilizado quanto as cápsulas manipuladas foram armazenadas em refrigerador a 5º C. A estabilidade do ácido ascórbico foi monitorada por 90 dias, empregando CLAE como técnica de doseamento. O processo de encapsulamento da polpa liofilizada foi considerado satisfatório na conservação do ácido ascórbico, uma vez que houve perda de 10% de sua concentração inicial, no período de estudado por 60 dias.**
Palavras-chave: Camu-camu. *Myrciaria dubia*. Liofilização. Cápsulas. Ácido ascórbico. Estabilidade.

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