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Assay of Ascorbic Acid by RP-HPLC in Samples Containing Also Citric Acid

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ABSTRACT

The ascorbic acid is widely used as antioxidant in food products. As this substance is a very unstable substance, its concentration needs an accurate monitorization. A RP-HPLC method was used for identification and quantification of ascorbic acid in mixtures used in sausage industry. Reliable results are obtained even the samples contain high quantities of citric acid. The regression coefficient of standard curve is 0.999.

KEY WORDS

ascorbic acid, citric acid, RP-HPLC, spectral ratio

INTRODUCTION

Ascorbic acid is a ubiquitously distributed in the vegatal and animal kingdom. Good sources of vitamin C are citrus fruits, hip berries, acerola, fresh tea leaves. Although initially it was isolated from adrenal cortex of ox, the first systematic studies were performed on ascorbic acid from lemon and paprika (1). Today the commercially available vitamin is produced by chemical synthesis (2)

Endogenous oxidative damage to proteins, lipids, and DNA is thought to be an important etiologic factor in aging and development of chronic

diseases such as cancer, atherosclerosis and cataract formation. The pathology associated with these diseases is likely to occur only after the production of reactive oxygen species has exceeded the body's or cell's capacity to protect itself and effectively repair oxidative damage (3). Vitamin C, vitamin E and beta-carotene, often referred to as "antioxidant vitamins," have been suggested to limit oxidative damage in humans, thereby lowering the risk of certain chronic diseases. Ascorbic acid was proven to be effective on improving myocardial energy metabolism and protecting against myocardial structural injury in hypoxic state (4). Being involved in biological oxidative processes ascorbic acid is the most used vitamin. Aside from its use as a vitamin, ascorbic acid or some derivatives (ascorbyl palmitate) are employed in complex pharmaceutical formulations (5), foodstuffs, to prevent rancidity, to prevent the browning of cut apples and other fruits, in meat curing, and so on (6).

Unfortunately, ascorbic acid is not very stable, being transformed relative quickly in compounds without biological activity (7). That is why its content has to be assayed in all commercial products those have to contain ascorbic acid. Although there is a large number of methods in use that claim to accurately quantify ascorbic acid, among them being spectrofotometric (5) and enzymatic (8) methods, only reliable ones are the chromatographic methods. Among them there is a great diversity of detection types, starting with relative simple methods that use UV and fluorescence (9) or electrochemical detection (10) till capillary electrophoresis methods (11). The method of detection has to be able to discriminate between vitamin C and other substances present as contaminants, because ascorbic acid is present in complex samples. One of the most frequent used compounds in food products simultaneously with ascorbic acid is citric acid. Therefore the assay has to distinguish between them and to be able to accurately quantify ascorbic acid in the presence of a high amount of citric acid. In this paper there is presented a HPLC method that efficiently measures the minute amount of ascorbic acid present as antioxidant in a mixture used in sausage industry, that contains also relatively large amount of citric acid.

MATERIAL AND METHODS

Chemicals. All chemicals, including the standards and the methanol for the mobile phase were of analytical grade from Merck (Darmstadt, Germany). Fresh glass double distillated water was used.

Sample preparation. A known amount of mixture used in sausage industry (Msi) was vortexed in 5 mL 40 % methanol with 0.1 M phosphoric acid for 10 minutes. The suspension was centrifuged at 5000 ***g*** for 10 minutes. Before injection in analytical column the sample was filtered on 0.2 μm Millipore filter.

Apparatus and HPLC conditions. The HPLC system (Beckman, USA) was equipped with Programmable Solvent Module 126 as HPLC pump, Programmable Detector Module 166, both connected with an IBM 586 personal computer and controlled by Gold® Software. The experiments were carried out on Lichrospher 100 RP-18 (5 μm) column (LichroCart 125 - 4 mm). The mobile phase was methanol in water, ranging from 40% to 5%, plus 0.1 M phosphoric acid. The solvent was filtered on 0.2 μm Millipore filter and degassed by ultrasonication before use. The elution was isocratic at a flow rate of 1 mL/min. The experiments were conducted at room temperature.

RESULTS AND DISCUSSION

In sausage industry frequently are used compounds in order to improve and preserve the quality and appearance of sausages. Among them are binders, emulsifying agents, dyes, artificial flavors, preservatives, antioxidants, organic acids and so on. The most used antioxidant is ascorbic acid, and (perhaps because it is cheep) the most used organic acid is citric acid. Taking into account that the ascorbic acid is not very stable it is necessary to determine its content in raw materials because the stability of the final product is directly related with its concentration.

High Performance Liquid Chromatography (HPLC) is a very sound, rapid and reliable technique extensively used in modern analytical laboratories. The eluted compounds are normally identified by comparing the retention time of the substance of interest with the retention time of the

standard eluted in the same chromatographic conditions. Although this comparison is necessarily it is not sufficient for a positive identification

Although the two eluted substances (from the sample and the standard) have the same retention time they are not necessary the same substance, because many compounds can elute at the same time even they belong to different classes. It is necessary to introduce at least a second criterion to positive matching both substances (from the sample and the standard). These criteria fall dawn in several categories: to modify the chromatographic elution, to change the column, to detect the eluted compound at least at two specific wavelengths, to compare the spectra of the standard and that of the analyte, to use specific detectors (like fluorimetric, electrochemical, MS), and so on. Of course, the easiest way is to change the elution conditions, *i.e.* to modify the composition of mobile phase.

In figure 1 there are presented the spectra of ascorbic acid (analyte of interest) and citric acid (here considered as contaminant). Fortunately these two substances have maximal absorbance at different wavelengths: ascorbic acid at λ_{max} 245 nm and citric acid at λ_{max} 205 nm.

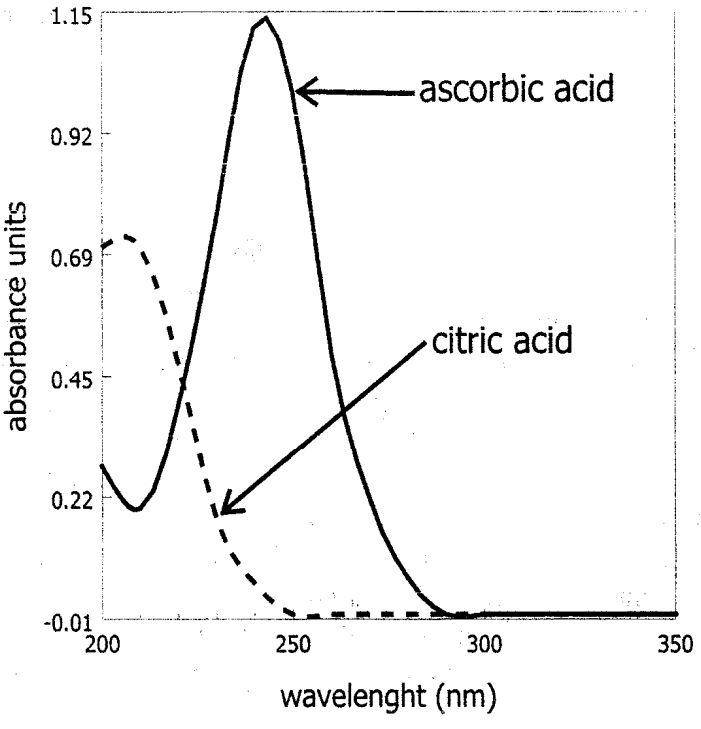


Fig. 1.
The absorption spectra of ascorbic acid and citric acid in methanol 40%, 0,1 M phosphoric acid.

Figure 2 presents some trials to improve the separation of the two substances, ascorbic acid and citric acid. Figure 2a shows that when it is injected a mixture of two standards, at 40% methanol concentration in mobile phase, the chromatogram presents a single peak, meaning that the separation has not taken place. Decreasing the methanol concentration till 10% in mobile phase there are eluted two peaks, although not totally separated (figures 2b and 2c). A second criterion of discrimination was also introduced, based on the fact that where ascorbic acid has a minimal absorbance citric acid has a maxim (at 205 nm). The trials presented in figure 2b and 2c indicate that at high wavelengths the peak of ascorbic acid is higher than that of citric acid and at low wavelengths the highest peaks belongs to citric acid.

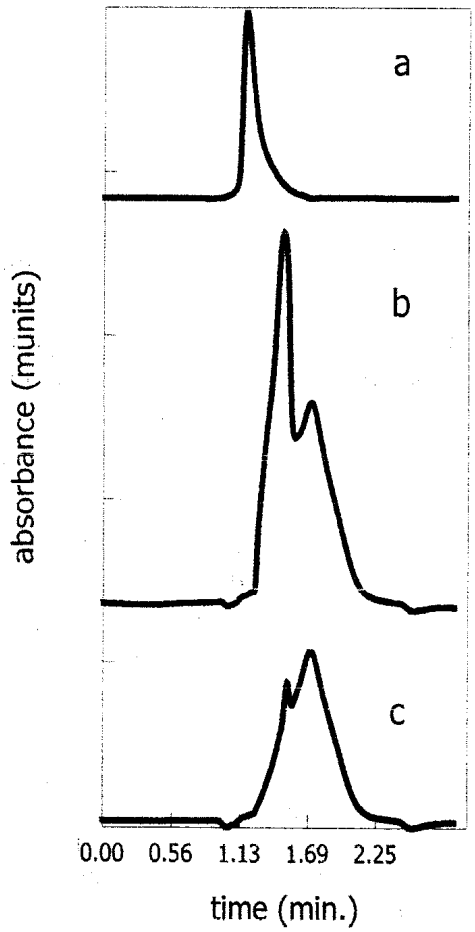


Fig. 2. Separation of a mixture of ascorbic acid and citric acid. **a)** mobile phase 40% methanol with 0.1 M phosphoric acid, detection at 245 nm; **b)** mobile phase 10% methanol with 0.1 M phosphoric acid, detection at 245 nm; **c)** mobile phase 10% methanol with 0.1 M phosphoric acid, detection at 205 nm.

The option to decrease the concentration of organic modifier in the mobile phase in order to separate the two substance of interest has been proven to be effective. Unfortunately, the concentration of the organic

modifier can not be decreased below 5% when a silicagel-based column is used. As it is shown in figure 3 this decrease of methanol concentration till 5% was sufficient to separate adequately ascorbic acid from citric acid. In order to increase the accuracy of the method, the second criteria of identification was also used, i.e. the identification of the elution peak in the sample with the eluted peak in standard is made by both comparisons: of the elution time and spectral ratio. This last criterion is obtained dividing the absorbance value from the chromatogram taken at 205 nm at the absorbance value from the chromatogram taken at 245 nm, for both substances of interest. For example, the spectral ratio of ascorbic acid was obtained dividing the absorbance value of peak eluted at 1.55 min. from the chromatogram detected at 205 nm (fig. 3a) to the absorbance value taken at the same elution time but from the chromatogram detected at 245 nm (fig. 3b). The spectral ratios for ascorbic acid (in our chromatographic conditions) were 1,44 for ascorbic acid and 16 for citric acid.

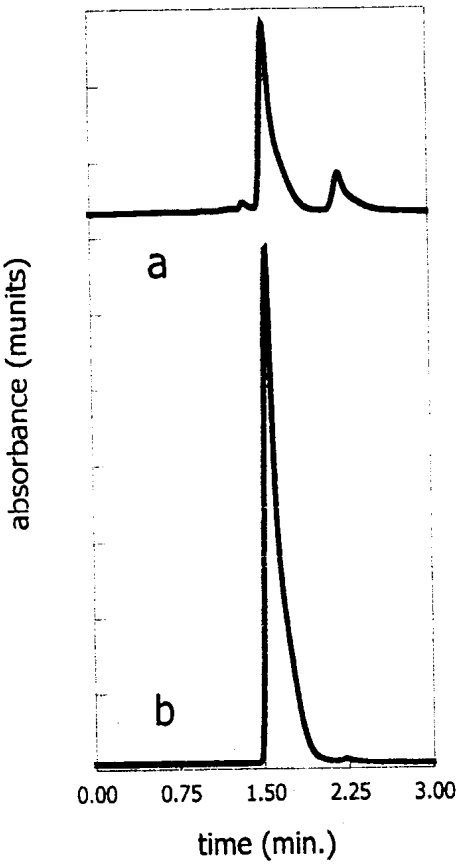
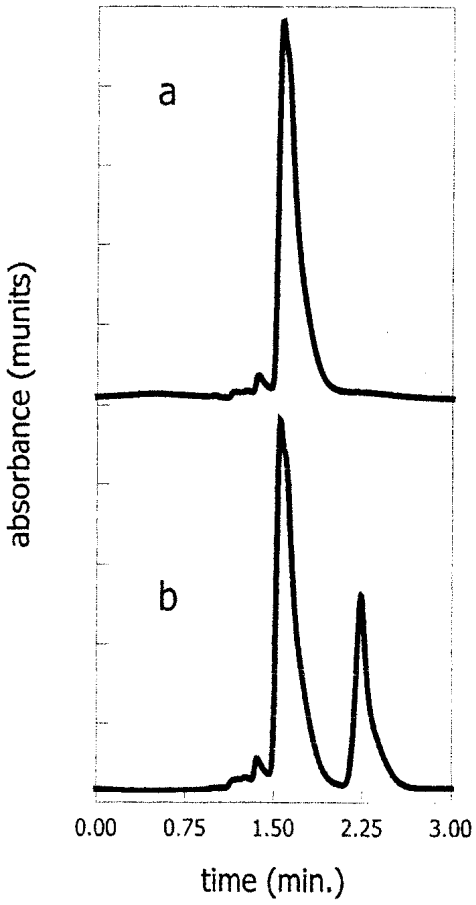


Fig. 3. The chromatograms of a mixture of ascorbic acid and citric acid detected at 205 nm (**a**) and 245 nm (**b**). Isocratic elution at 1 mL/min with 5 % methanol and 0,1 M phosphoric acid.

Although for routine analyses using two criteria of matching of analyte in the sample with the standard is usually accepted, often additional matching criterion are applied. In figure 4 there are presented the chromatogram of a sample of Msi (fig. 4a) and the chromatogram of the



same sample spiked with a known amount of citric acid (fig. 4b). Both chromatograms were taken at 205 nm, where the citric acid has maximum absorbance (fig. 1). Although it was expected that the Msi would contain citric acid it was not detected neither at 205 nm or 245 nm (data not shown). For this reason the Msi sample was spiked with citric acid to demonstrate that the other components presented in Msi do not interfere with the separation and detection of citric acid.

Fig. 4. The chromatograms of a sample of mixture used in sausage industry recorded at 205 nm (**a**) and of the same sample spiked with citric acid (**b**). Isocratic elution at 1 mL/min with 5 % methanol and 0,1 M phosphoric acid.

Besides the positive identification of the presence of ascorbic acid in Msi, the HPLC method was used for quantitative assessment of this substance. Figure 5 presents the calibration curve for ascorbic acid obtained when an isocratic elution with 1 mL/min of 40% methanol and 0,1 mM phosphoric acid was performed. The detection was done at 245 nm. The accuracy of the method was illustrated by the regression coefficient r = 0.999895.

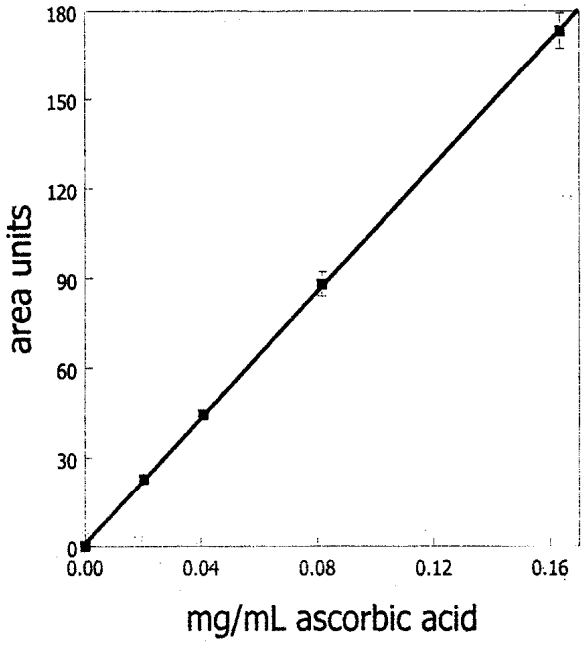


Fig. 5. Calibration curve for quantitative determination of ascorbic acid. Chromatographic conditions: Isocratic elution at 1 mL/min with 5 % methanol and 0,1 M phosphoric acid. Detection at 245 nm.

In conclusion, the HPLC method we presented here proved to be a sound, accurate and rapid method for identification and quantification of ascorbic acid in mixtures used in sausage industry. The method can be equally used for other types of samples that contain ascorbic acid (like soft drinks, pharmaceutical formulations, and so on).

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