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Discrimination between Ascorbic Acid from Acerola and of Synthetic Origin by Measuring ^{13}C and ^{18}O Stable Isotope Ratios

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We examined the $^{13}C/^{12}C$ and $^{18}O/^{16}O$ stable isotope ratios ($\delta^{13}C$, $\delta^{18}O$) of ascorbic acid from acerola and of synthetic origin. Although the obtained $\delta^{13}C$ values were partially overlapping for the two species, $\delta^{18}O$ values were higher for ascorbic acid from acerola, than values obtained for synthetic ascorbic acid. From this study, it was demonstrated that carbon and oxygen isotopic composition analysis can discriminate ascorbic acid from acerola from that of synthetic origin.

Ascorbic acid plays an important role in the human body as a major antioxidant resource.[1] The antioxidant activity of ascorbic acid is largely a result of its ene-diol structure, leading to strong reducing ability. This characteristic ability of ascorbic acid has been demonstrated to be responsible for preventing a number of degenerative diseases.[2] In addition, ascorbic acid depletion induces scurvy, with symptoms such as dry skin, fatigue, and bleeding, due to defective collagen synthesis. Many animals can synthesize ascorbic acid in vivo; however, humans have lost the ability to produce ascorbic acid, due to mutations in the L-gulono-γ-lactone oxidase gene, which is essential for in vivo ascorbic acid synthesis.[3] As a result, humans must obtain ascorbic acid from dietary sources.

Fruit juices are generally assumed to be one of the primary ascorbic acid sources. Among these, acerola (*Malpighia emarginata* DC.) is well known to contain extremely high amounts of ascorbic acid. Acerola is a fruit originating from Central America to northern South America, that has become extremely popular in the daily lives of health-conscious persons, and is often used as a typical source of natural ascorbic acid due to its unique ascorbic acid content characteristics.

Recently, however, there have been many attempts to adulterate the ascorbic acid in acerola juices, with claims of totally natural ascorbic acid content, despite the addition of industrially synthesized ascorbic acid, in order to achieve the regulated ascorbic acid level at a lower cost. For this reason, an analytical method for detecting the addition of ascorbic acid in fruit juices with high precision is required.

Stable isotope analysis has widely been used to trace the origins of organic materials in various fields, including ecology, biochemistry, and food authenticity.[4,5] In the case of discrimination between substances of natural and biosynthetic origin, the technique of measuring the $^{13}C/^{12}C$ ratio is widely used.[6] This technique is based on the fact that C3 and C4 plants exhibit slightly different $^{13}C/^{12}C$ ratios; hence, it is possible to distinguish between natural ascorbic acid from acerola (a C3 plant) and synthesized ascorbic acid derived from C4 plants, as exemplified by maize or sugar cane.[7] However, this technique is limited, when applied to synthesized ascorbic acid derived from a C3 plant. As an alternative technique, it has been reported that a measurement of the positional $^{13}C/^{12}C$ ratios in the ascorbic acid molecule enables a validation of the origins of ascorbic acid from different sources, via ^{13}C NMR.[8] This method, however, requires measurements which are quite time-consuming, in order to obtain a precision which is adequate for quantification. Accordingly, in this study, we aimed to establish a novel method for discriminating between acerola and synthetic ascorbic acid, by means of simultaneous measurements of the $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios.

Table 1. $\delta^{13}C$ and $\delta^{18}O$ values of different sources of ascorbic acid

	Number of samples	$\delta^{13}C$ /‰	$\delta^{18}O$ /‰
Acerola	50	-21.6 ± 1.2	34.2 ± 2.5
Brazil	27	-21.9 ± 1.3	35.7 ± 2.2
Vietnam	23	-21.2 ± 0.9	32.5 ± 1.6
Synthetic	51	-12.8 ± 4.3	24.6 ± 2.6

(means $\pm$ SD)

We collected more than 50 samples of both acerola and synthetic ascorbic acid, as shown in Table 1. The acerola samples were collected from two major growing regions (Brazil and Vietnam), since the $^{18}O/^{16}O$ ratio is often reported to be affected by geographical origin.[9] Prior to the measurements, we isolated the ascorbic acid from acerola according to the procedure of Gensler et al.[10] On the other hand, synthetic ascorbic acid was purchased from several chemical suppliers, and used directly for the measurements. For carbon, 1.0 mg of samples were weighed into a tin capsule (5×9 mm). Each sample was then analyzed via elemental analyzer (EA)–isotope ratio mass spectrometry (IRMS) using a Finnigan Delta V ADVANTAGE (Thermo Electron Corporation) interfaced with a Flash EA 1112 (Thermo Electron Corporation) to determine the carbon isotope ratios. For oxygen, each 1.5 mg of sample was weighed into a silver capsule (3.3×5 mm). Oxygen isotope analysis was then carried out via thermocombustion EA (TCEA)/IRMS using a Finnigan Delta V ADVANTAGE interfaced with a Finnigan TCEA (Thermo Electron Corporation). The isotopic composition was reported using the δ notation:

$$\delta\ (‰) = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000 \quad (1)$$

where R_{sample} is the isotope ratio (i.e., $^{13}C/^{12}C$, $^{18}O/^{16}O$) of the sample, and R_{standard} is the isotope ratio of the international standards (Peedee Belemnite (PDB) for carbon; Standard Mean Ocean Water (SMOW) for oxygen). Each isotope value is expressed in permil (‰). C and O isotopic compositions can normally be measured with an analytical uncertainty of ± 0.1 to 0.3‰.

A two-dimensional plot of the $\delta^{13}C$ and $\delta^{18}O$ values obtained for ascorbic acid of differering origins is presented in Figure 1, and the mean values for the respective origins are summarized in Table 1. The $\delta^{13}C$ values obtained for ascorbic acid from acerola ranged from -25.1 to -20.0‰, and were concentrated within a range of roughly 5‰. On the other hand, the $\delta^{13}C$ values obtained for synthetic ascorbic acid could be divided into three groups: a first group ranging from -12.6 to -10.3‰, a second group from -17.1 to -15.6‰, and a third group from -25.7 to -23.0‰ (as confirmed by p-values systematically lower than 0.01, in a Tukey multiple comparisons test). Taking the previously reported knowledge into consideration, these three groups might be attributed as ascorbic acid derived from C4 plants for the first group, from a mixture of C3 and C4 plants for the second group, and from C3 plants for the third group.[7] Although the first and second groups of the synthetic ascorbic acids were distinguishable from the acerola group, the third group partially overlapped with the acerola group, when considering the $\delta^{13}C$ parameter, alone. However, a clear-cut discrimination is evident from the vertical axis in Figure 1. The $\delta^{18}O$ values obtained for ascorbic acid from acerola were significantly higher than those obtained for synthetic ascorbic acid (as confirmed by p-values systematically lower than 0.01 in t-tests). The oxygen atoms in ascorbic acid are thought to originate primarily from locally falling rainwater. Plant water is known to be rich in ^{18}O (and deuterium), compared to groundwater due to repeated evaporation from leaves.[11] Therefore, the low $\delta^{18}O$ values for the synthetic ascorbic acids obtained in this study are probably due to the uptake of the oxygen from groundwater or purified water used for factory ascorbic acid production. In addition, $\delta^{18}O$ values are also well known to vary with geographical factors.[9] In this study, we examined whether $\delta^{18}O$ values exhibit any differences, between acerola obtained from Brazil and Vietnam (Table 1). The ascorbic acid $\delta^{18}O$ values were 35.7 ± 2.2‰ and 32.5 ± 1.6‰ for Brazil and Vietnam acerola, respectively, showing a significantly lower value for acerola from Vietnam (as confirmed by p-values systematically lower than 0.01 in t-tests). Based on this result, the $\delta^{18}O$ values were clearly affected by regional factors; however, this deviation was not at a level adequate for affecting discrimination between ascorbic acids from acerola and of synthetic origin.

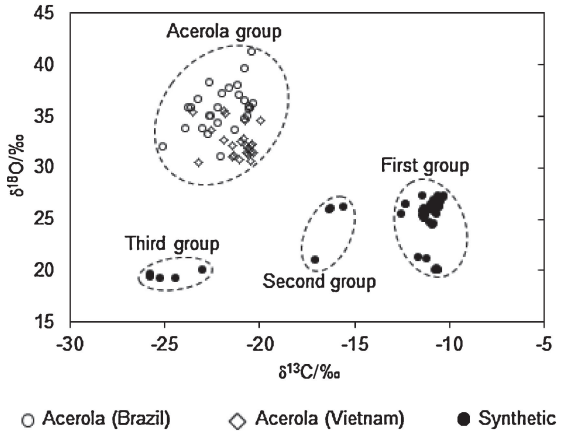

Figure 1. Two-dimensional plot of the $\delta^{13}C$ and $\delta^{18}O$ values obtained for different sources of ascorbic acid.

Although the discrimination between ascorbic acid from acerola and of synthetic origin could be achieved via a combination of $\delta^{13}C$ and $\delta^{18}O$ values, as shown in Figure 1, it was uncertain as to whether or not this discrimination could be regarded as statistical. Accordingly, to ascertain the statistical discrimination of the different ascorbic acid sources used in this study, a hierarchical cluster analysis was also performed using the $\delta^{13}C$ and $\delta^{18}O$ isotopic standardized variables. Figure 2 represents a dendrogram, which visualizes the similarity of the ascorbic acids. From this analysis, it is evident that the ascorbic acids used in this study are primarily divided into acerola and synthetic groups. Furthermore, the synthetic group is divided into a third group (presumably derived from C3 plants) and other

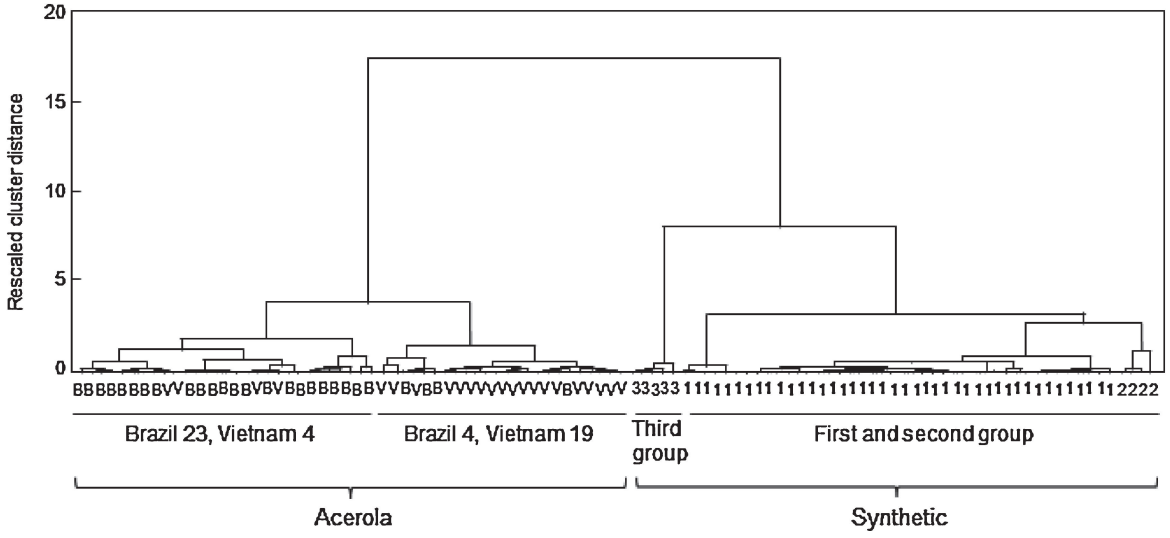

Figure 2. Dendrogram showing the clustering of the ascorbic acid based on the $\delta^{13}C$ and $\delta^{18}O$ parameters, using the Euclidean distance via the Ward method; (B): Brazil acerola, (V): Vietnam acerola, (1): synthetic (first group), (2): synthetic (second group), (3): synthetic (third group).

groups. Regarding the acerola group, it is further grouped into two clusters, which are mainly from Brazil (23 for Brazil, 4 for Vietnam) and from Vietnam (4 for Brazil, 19 for Vietnam), respectively. Thus, based on this cluster analysis, it was statistically demonstrated that the present method of combining $\delta^{13}C$ and $\delta^{18}O$ parameters surely discriminates ascorbic acid from acerola and of synthetic origin. Moreover, additional information could also be obtained, including the origin of the C3 or C4 plants, as well as the geographical origin, using this novel method.

In conclusion, this study strongly demonstrates that simultaneous carbon and oxygen isotopic composition analyses can discriminate ascorbic acid from acerola and of synthetic origin. This is the first report which investigates the oxygen isotopic composition for the purpose of assessing the natural and synthetic origins of ascorbic acid. Further investigations are now in progress to determine whether the amount of synthetic ascorbic acid, added as a mixture into acerola juice can also be determined using this method.

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