Detection of Added L-Ascorbic Acid in Fruit Juices by Isotope Ratio Mass Spectrometry

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A method for the isotope effect free isolation of L-ascorbic acid from fruit juices has been developed. It consists of an anion exchange cleanup procedure and a subsequent liquid chromatography separation on a reversed phase column and implies a preceding reduction of L-dehydroascorbic acid. The recovery of L-ascorbic acid from model mixtures and spiked juices with this method was between 62 and 64%, but the $\delta^{13}C$ value difference between the applied and recovered test compound did not exceed 0.2‰. While commercial L-ascorbic acid has in general $\delta^{13}C$ values near −11.3‰, the mean $\delta^{13}C$ value of vitamin C from authentic orange juices is −20.7‰, and that of the corresponding sugar is −25.5‰. These differences are the basis for the determination of an addition of synthetic L-ascorbic acid to fruit juices, and it is shown with spiked samples that less than 20% of added commercial product can be identified.

INTRODUCTION

Vitamin C, L-ascorbic acid, has the highest reputation of the vitamins in the population. It is associated with health and vital force, and therefore food products are well accepted by the consumer when a high content of vitamin C is indicated. Natural fruit juices, especially citrus and black currant products, are generally assumed to be good vitamin C sources (Association of German Fruit Juice Industries, 1987).

L-Ascorbic acid is quite unstable, reacting very easily with oxygen, especially in the presence of heavy metal ions and light, forming L-dehydroascorbic acid and further degradation products (Bode et al., 1990; Angberg et al., 1993; Foerst, 1967). This instability is a serious problem in juice and nectar production, and inferior quality fruit material or the application of nonsuitable processing methods have for consequence losses of L-ascorbic acid. German food law demands minimum contents in juices, for example, for a natural citrus juice 200 mg/L, or even 300 mg/L, when a special high vitamin C content is advertised (Zipfel and Zipfel, 1988). The addition of industrially produced L-ascorbic acid is permitted but must be indicated.

A byproduct of biosynthetic vitamin C, produced from glucose by a combined chemical and microbial procedure (Christen, 1985), is D-isoascorbic acid; its occurrence in beer after addition of commercial vitamin C has been detected by HPLC (Seiffert et al., 1992). However, the application of this method to the analysis of fruit juices was not successful (W. Rieth, Association of Fruit Juice Producing Industries, personal communication, 1994).

A higher degree of security in adulteration detection would probably be attained by means of an isotope ratio determination, especially using the principle of intermolecular standardization (Bricout and Koziet, 1987; Parker, 1981; Schmidt et al., 1993; White and Winters, 1989). This is based on the experience that natural compounds from the same origin have not only metabolic but also isotopic correlations and that the observation of a difference from the normal δ value correlation of two compounds from the same origin is highly indicative for one of these compounds to originate from a foreign source. On the basis of this principle, the addition of commercially produced L-ascorbic acid to juices should be sensitively detectable, provided its $\delta^{13}C$ value is different from that of the original vitamin C in a given fruit juice.

The carbon isotope analysis of L-ascorbic acid presumes the micropreparative isolation of the compound. The most suitable principle for this would be high-performance liquid chromatography (HPLC) (Schwarzenbach, 1982), and as a matter of fact, HPLC on anion exchange (Hapette and Poulet, 1990), cation exchange (Bianco and Marucchi, 1991), amino phase (Romero-Rodriguez et al., 1992), and reversed phase materials (Racz et al., 1991; Lee and Coates, 1987) have successfully been used in context with the quantitative analysis of vitamin C. However, for isotope analysis, an absolutely isotope effect free micropreparation would be obligatory. We have recently reported that reversed phase chromatography meets this demand in the case of other organic acids (Gensler and Schmidt, 1994). Therefore, the corresponding material has also been used for the present task, and on this basis the isolation of L-ascorbic acid from fruit juices and its carbon isotope analysis, as well as the determination of the $\delta^{13}C$ values of commercial L-ascorbic acid and the compound from authentic, spiked, and commercial juices will be reported. Finally, the influence of partial vitamin C degradation on the carbon isotope ratio is investigated.

EXPERIMENTAL PROCEDURES

Materials. *Chemicals.* All applied chemicals (purchased from E. Merck and Aldrich Chemie GmbH & Co KG, Steinheim, Germany, respectively) were of analytical grade. Dithiothreitol was purchased from Aldrich Chemie. Water was purified using a Millipore system (Millipore Corp., Bedford, MA). All applied solutions were submitted for 5 min to an ultrasonic bath treatment and for 15 min to N_2 flushing.

Standard L-ascorbic acid was of analytical grade and purchased from E. Merck GmbH, Darmstadt, Germany. Various commercial L-ascorbic acid samples from different origins were provided by the Association of Fruit Juice Producing Industries (SGF), Zornheim, Germany.

Column Material for Cleanup. An anion exchange resin AG1×8 (200–400 mesh/SO_4^{2-} form) from Bio-Rad GmbH, Munich, Germany, was employed. Anion exchange resin (60 g) was filled in a 2.5 cm i.d. glass column. After use, the anion
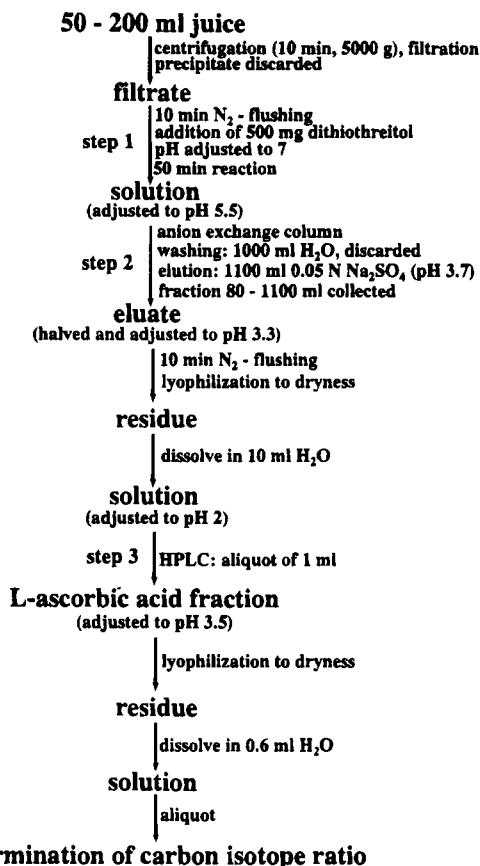
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50 - 200 ml juice

| centrifugation (10 min, 5000 g), filtration
| precipitate discarded

filtrate

step 1 | 10 min N₂ - flushing
| addition of 500 mg dithiothreitol
| pH adjusted to 7
| 50 min reaction

solution
(adjusted to pH 5.5)

step 2 | anion exchange column
| washing: 1000 ml H₂O, discarded
| elution: 1100 ml 0.05 N Na₂SO₄ (pH 3.7)
| fraction 80 - 1100 ml collected

eluate
(halved and adjusted to pH 3.3)

| 10 min N₂ - flushing
| lyophilization to dryness

residue

| dissolve in 10 ml H₂O

solution
(adjusted to pH 2)

step 3 | HPLC: aliquot of 1 ml

L-ascorbic acid fraction
(adjusted to pH 3.5)

| lyophilization to dryness

residue

| dissolve in 0.6 ml H₂O

solution

| aliquot

determination of carbon isotope ratio

Figure 1. Diagram for isolation of L-ascorbic acid from juices for carbon isotope ratio measurement.

exchange column was regenerated by washing with 800 mL of 0.6 N H_2SO_4 and 1000 mL of H_2O.

Fruit Juices. Authentic and commercial fruit juices and fruit juice concentrates, as well as fruit juices spiked with industrially produced L-ascorbic acid, were kindly provided by the Association of Fruit Juice Producing Industries (SGF), which also guaranteed the authenticity of origin and treatment. Applied juice aliquots were as follows: orange, 100 mL; black and red currant, 50 mL; pineapple, 200 mL; concentrates, dilution with H_2O 1:5 (pineapple 1:3).

A "test orange juice" for the control of the isotopic yield of the isolation procedure was prepared as follows: One hundred milliliters of an authentic orange juice was centrifuged (10 min, 5000g), the supernatant was adjusted to pH 8.5 (25% NH_3), and 18 mL of $CaCl_2$ solution (50 wt %) was added. Then the pH was again adjusted to 8.5, the solution was heated to 85 °C, and the acids, precipitated as Ca salts, were eliminated by centrifugation. The pH value of the supernatant was readjusted to 8.5, and then Na_2CO_3 solution (50 wt %) was added until complete precipitation of the excess of Ca^{2+}. After separation of the precipitated $CaCO_3$, the pH of the solution was adjusted to 3.5, and 25 mg of L-ascorbic acid, 300 mg of L-malic acid, and 1000 mg of citric acid of known $\delta^{13}C$ values were added.

For a "test cherry juice", 25 mL of an authentic cherry juice (no original L-ascorbic acid was detectable) was spiked with 40 mg of L-ascorbic acid and 1500 mg of citric acid of known $\delta^{13}C$ values.

Methods. *Isolation of "Total L-Ascorbic Acid".* The procedure developed for the isolation of total L-ascorbic acid (L-ascorbic acid + L-dehydroascorbic acid) from centrifuged juices (Figure 1) comprised a reduction step (step 1), one chromatographic cleanup step (step 2), and a subsequent high-performance liquid chromatographic (HPLC) separation (step 3).

In the first step, L-dehydroascorbic acid is reduced to L-ascorbic acid. The cleanup step has for aim the elimination of the nonpolar ingredients, the sugars, amino acids, and excess of oxidized and reduced dithiothreitol, as well as the main part of the L-malic and citric acid. In the anion fraction,

L-ascorbic acid is separated from residual L-malic acid by reversed phase HPLC.

The efficiency of the procedure was controlled by its application to a corresponding test solution and to test juices. Detailed conditions, also in correlation with the evaluation, are given below.

(*a*) All juices are centrifuged (10 min, 5000g) and filtered.

(*b*) An aliquot of the supernatant (50–200 mL, depending on juice species) is flushed with nitrogen for 10 min and then 500 mg of dithiothreitol is added and the pH is adjusted to 5.5 (step 1, Figure 1).

(*c*) The solution from (b) is applied to the anion exchange column, followed by a washing step with 1000 mL of water and an elution with 1100 mL of 0.05 N Na_2SO_4 (pH 3.7). The elution fraction from 80 to 1100 mL is collected (step 2, Figure 1). A 50% aliquot is adjusted to pH 3.3, and after 10 min of nitrogen flushing, is brought to dryness by lyophilization. The residue is dissolved in 10 mL of water, and the pH of the solution is adjusted to 2.0.

(*d*) One milliliter of the solution from (c) is injected into a 50 × 2 cm i.d. stainless steel column packed with octadecyl silica, Lichrospher 100 RP 18, E. Merck, of 5 μm particle size of a high-pressure liquid chromatograph (S 1000, Sykam, Gilching, Germany), equipped with a manual injection valve (Rheodyne, Berkeley, CA) and a 1 mL sample loop. Detection of L-ascorbic acid is performed by an UV detector (S3310, Sykam) at 210 nm. The mobile phase consists of 0.02 M H_3PO_4 (pH 2.0); the flow rate is set to 7 mL/min (step 3, Figure 1). The L-ascorbic acid fraction is collected, adjusted to pH 3.5 (5 N NaOH), and lyophilized to dryness. The residue is dissolved in 0.6 mL of water.

(*e*) From an aliquot of the dissolved residue (d) after lyophilization the carbon isotope ratio is determined by IRMS.

Evaluation of the Method. (1) Control of the Complete Reduction of L-Dehydroascorbic Acid in the Juice Aliquot. To 100 mL of two authentic and one commercial orange juice, after centrifugation and filtration, portions of 50 mg of dithiothreitol were added, and the pH was adjusted to 7. After 50 min of reaction at 20 °C, the yield of reduction was monitored by means of the area of the L-ascorbic acid peak in an analytical HPLC separation. The chromatographic system was equipped in this case with a manual Rheodyne injection valve, a 20 μL sample loop, and a 25 × 0.4 cm i.d. stainless steel column, packed with octadecyl silica (Hypersil ODS, Grom, Herrenberg, Germany, 5 μm particle size). The mobile phase consisted of 0.02 M H_3PO_4; the flow rate was set to 1 mL/min, and the temperature to 22 °C. The pH of the sample was adjusted to 2.0 (50% H_3PO_4). Detection was performed by an UV detector (S3310, Sykam) at 210 and 242 nm. A calibration of the chromatographic system was obtained by standard mixtures (pH 2) in water containing 0.1, 0.25, 0.5, 0.75, and 1.0 g/L (measurement at 210 nm) or 0.02, 0.05, 0.1, 0.15, and 0.2 g/L (measurement at 242 nm) of L-ascorbic acid, respectively.

Storage and processing of data from the detector were carried out on a personal computer by the use of chromatographic software (Axxiom Chromatography Software, Calabasas, CA).

(2) Determination of the Optimum Fraction Size for L-Ascorbic Acid Isolation from the Anion Exchange Column. One hundred milliliters of test mixture (25 mg of L-ascorbic acid, 300 mg of L-malic acid, and 1000 mg of citric acid) or 100 mL of supernatant of reduced authentic orange juices or 50 mL of black currant juice, respectively, was applied to the anion exchange column. After a washing step with 1000 mL of water, an elution with 50 mL portions of 0.05 N Na_2SO_4 solution was performed. The presence of L-ascorbic acid in the washing water (after concentration by lyophilization) and the elution fractions was controlled by analytical HPLC [HPLC apparatus and conditions see (1)]. L-ascorbic acid was found in elution fractions 4–16 (150–800 mL); routinely the eluate between 80 and 1100 mL was collected.

Analysis of Carbon Isotope Ratios. Carbon isotope ratios of isolated and pure compounds were determined, as described by Winkler and Schmidt (1980), two times in sequence. The carbon isotope ratio of the sugar fractions was determined, as described by Rossmann et al. (1990) and Koziet et al. (1993).

Table 1. Recovery and Isotope Yield of L-Ascorbic Acid from the Isolation Method, Analyzed on a Test Mixture and Test Juices[a]

	recovery (%) from absorption at		$\delta^{13}C$ values (‰)PDB	
	210 nm	242 nm	found[b]	shift from original value
test solution				
test mixture	63	61	−18.9	+0.1
test orange juice	62	61	−18.8	+0.2
test cherry juice	65	63	−18.8	+0.2

[a] $\delta^{13}C$ value of applied standard L-ascorbic acid: −19.0‰. [b] Mean value of three independent determinations (standard deviation always <0.1‰).

All $\delta^{13}C$-values were related to the PDB standard.

$$\delta^{13}C \text{ value} = \frac{(R_{sample}) - (R_{standard})}{(R_{standard})} \times 1000(‰)$$

$$R = [^{13}C]/[^{12}C], \text{ for PDB} = 0.011237$$

The isotope ratio mass spectrometer used was a VG Micromass 903 (VG Isogas, Middlewich, U.K.).

RESULTS

Evaluation and Reliability of the Method. To determine the *yield and isotopic recovery* of the L-ascorbic acid isolation, a test mixture of 25 mg of isotopically analyzed L-ascorbic acid, 300 mg of L-malic acid, and 1000 mg of citric acid in 100 mL of H_2O, 100 mL of a test orange juice, or 25 mL of a test cherry juice was fractionated according to Figure 1. The recovery of L-ascorbic acid, as determined from the mean of the absorption at 242 and 210 nm on the analytical HPLC (see Control of the Complete Reduction of L-Dehydroascrobic Acid in the Juice Aliquot), was 62% (Table 1). This is in the range of comparable isolation procedures (Gensler and Schmidt, 1994). The shift of the analyzed $\delta^{13}C$ values was within the standard error of carbon isotope ratio measurements, 0.1−0.2‰. Results obtained with the test juices confirmed those from the test mixture.

For the control of the *reproducibility*, one orange juice and one black currant juice were analyzed four times in repeat. The maximum difference of $\delta^{13}C$ values found for each juice was 0.2‰ (standard deviation <0.1‰).

By the addition of dithiothreitol in aliquots it could be shown that the *reduction* of any *dehydroascorbic acid* present in normal juices was complete after addition of 200 mg of dithiothreitol/100 mL of juice. Nevertheless, in the routine procedure 500 mg of dithiothreitol was added to the juice aliquot.

In this context the question arose whether a partial loss of L-ascorbic acid by oxidation in the course of aging of a juice had an effect on the isotope ratio of the analyte. Two samples of orange juice products were fractionated according to Figure 1; in addition, a second aliquot of each, with omission of the reduction step, was fractionated. In all samples, the amount and $\delta^{13}C$ value of ascorbic acid were determined. In both cases, the $\delta^{13}C$-value of the original L-ascorbic acid was more negative than that of the total L-ascorbic acid after reduction (Table 2). We do not know whether this indicates an isotope effect on the oxidation, and we do not have an idea why the effect is greater in one sample; however, it is quite clear from this result that in the case of practical analysis of unknown samples this treatment at the beginning of the isolation is obligatory.

The absolute $\delta^{13}C$ value of the L-ascorbic acid in the commercial sample is by 3.4‰ more positive than that

Table 2. Proportion of L-Ascorbic Acid on Total L-Ascorbic Acid (Sum of L-Ascorbic Acid + L-Dehydroascorbic Acid) in Two Orange Juices and Carbon Isotope Ratios of Both Fractions[a]

		$\delta^{13}C$ values (‰)PDB		
orange juice	unchanged L-ascorbic acid (% of total)	total L-ascorbic acid	original L-ascorbic acid	diff
authentic concentrate, 1991	52	−20.5	−21.7	+1.2
commercial juice, 1993	73	−16.5	−18.3	+1.8

[a] All values mean of two determinations.

Table 3. Carbon Isotope Abundance of L-Ascorbic Acid and of the Sugar Fraction from Authentic and Commercial Juices[a]

		$\delta^{13}C$-values [‰]PDB		
juice sample	origin, year	L-ascorbic acid	sugar	diff, L-ascorbic acid − sugar
orange 1	Italy, 1993	−19.8	−24.5	+4.7
orange 2	Italy, 1993	−20.1	−24.6	+4.5
orange 3	Italy, 1993	−20.1	−24.9	+4.8
orange 4	Brazil, 1993	−20.7	−24.7	+4.0
orange 5	Brazil, 1993	−20.4	−25.5	+5.1
orange 6	Brazil, 1993	−19.9	−25.8	+5.9
orange 7	Brazil, 1991	−20.5	−25.0	+4.5
orange 8	Argentina, 1992	−21.5	−26.5	+5.0
orange 9	Argentina, 1992	−21.6	−26.8	+5.2
orange 10	Florida, 1993	−21.3	−26.0	+4.7
orange 11	commercial, 1993	−16.5	−26.0	+9.5
black currant 1	Germany, 1992	−20.5	−25.0	+4.5
black currant 2	France, 1993	−21.0	−25.8	+4.8
red currant 1	Germany, 1994	−21.2	n.d.	
pineapple 1	Thailand, 1992	−9.7	−12.1	+2.4
mean of authentic C-3-plant samples		−20.7	−25.5	+4.7

[a] All juices were authentic with the exception of orange 11. Mean values of two determinations, differences <0.2‰. Sugar = sum of mono- and disaccharides.

of the authentic sample; as will be shown later, this suggests the addition of a biosynthetic product.

Carbon Isotope Ratios of L-Ascorbic Acid and Sugar in Authentic and Commercial Fruit Juices. Authentic and commercial fruit juices were submitted to the fractionation scheme depicted in Figure 1, and the carbon isotope ratio of the isolated L-ascorbic acid was determined. The isotope ratio of the sugar fraction from the same sample was obtained according to Rossmann et al. (1990), and Koziet et al. (1993). Analyses of 10 authentic orange juice products (9 juices and 1 concentrate) of controlled harvest and treatment from different regions (Brazil, Argentina, Florida, and Italy), of one commercial product, of 2 authentic black currant juices (from Germany and France) and 1 red currant juice (from Germany), and of 1 authentic pineapple juice concentrate from Thailand were performed (Table 3).

In spite of the fact that the $\delta^{13}C$ values of L-ascorbic acid and sugar were relatively characteristic for their origin (more positive for juices from Italy as compared to juices from Brazil, and the latter showed a higher ^{13}C content than those from Argentina and Florida), the difference of the $\delta^{13}C$ values of sugar from L-ascorbic acid were, with two exceptions, between +4.5 and +5.2‰. The same value was found for an authentic orange juice concentrate, produced in 1991 and stored for over 3 years (storage conditions: −20 °C, dark, O_2-free). This is in line with the biosynthetic origin of L-ascorbic acid from glucose and the existence of an isotope effect implied in it. The $\delta^{13}C$ value of the sugar from the commercial orange juice was in the range of authentic juices, while the $\delta^{13}C$ value of the L-ascorbic

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Table 4. Carbon Isotope Abundance of Commercially Available L-Ascorbic Acid

sample origin	year	$\delta^{13}C$ value [‰]$_{PDB}$
Germany	1990	−11.2
Germany 1	1991	−11.3
Germany 2	1991	−11.5
Germany 3	1991	−14.5
Germany	1994	−10.0
Bulgaria	1993	−10.1
China 1	1993	−19.7
China 2	1993	−17.8
Russia	1993	−9.9
Colombia	1994	−12.4
Venezuela	1993	−10.5
mean[a]		−11.3

[a] Except samples from China.

acid, as well as its difference from that of the sugar, was considerably more positive. As will be shown in the following section, this is probably due to the addition of biosynthetically produced L-ascorbic acid.

Similar $\delta^{13}C$ values and differences were obtained for L-ascorbic acid from black and red currant juices and, as expected for a product of a CAM (Crassulacean acid metabolism) plant, the $\delta^{13}C$ value of L-ascorbic acid from the pineapple juice was −9.7‰ and the difference from the sugar $\delta^{13}C$ value was +2.4‰.

$\delta^{13}C$ Values of Commercial L-Ascorbic Acid and L-Ascorbic Acid from Juices Spiked with the Commercial Product. L-Ascorbic acid is produced from glucose by means of a combined chemical and microbiological procedure (Christen, 1985). As the acid is a main product in this process, it is to be expected that its $\delta^{13}C$ value is largely influenced by that of the starting material. The $\delta^{13}C$ values of samples of commercially available L-ascorbic acid from different origin and years (Table 4) showed that they had obviously—with the exception of the samples from China—been produced from C4 sugars or possibly from C4/C3 sugar mixtures. This supports the finding on the commercial juice samples mentioned previously (Tables 2 and 3) and could be used as a basis for detection of commercial vitamin C addition.

To verify this expectation, original orange and black currant juices were spiked in a double-blind experiment with commercial L-ascorbic acid from two different sources. Neither the original nor the total amount of L-ascorbic acid in these samples nor the amount and

the $\delta^{13}C$ value of the added product were given. The $\delta^{13}C$-values of the L-ascorbic acid and the sugar fraction of the samples were determined (Table 5). Assuming a $\delta^{13}C$ value of −20.7‰ for "standard" authentic juice L-ascorbic acid (mean value, Table 3) and a $\delta^{13}C$ value of −11.3‰ for biosynthetic L-ascorbic acid (mean value, Table 4), the fraction f of added commercial product will result from the value δ_{AM}, measured for L-ascorbic acid of the sample:

$$f(-11.3) + (1 - f)(-20.7) = \delta_{AM} \qquad (1)$$

$$f = \frac{\delta_{AM} + 20.7}{20.7 - 11.3} \qquad (2)$$

On the other hand, the individual $\delta^{13}C$ value of L-ascorbic acid δ_{AA} in a given sample will be available on the basis of its correlation (in the range of natural variations) to that of the corresponding sugar, $\delta_{AA} = \delta_S + \Delta\delta^{13}C$ (in the present case corresponding differences from Table 3 have been taken, namely +5.2‰ for orange and +4.8‰ for black currant juice), permitting us to replace the standard $\delta^{13}C$ value for authentic L-ascorbic acid by an individual one (e.g. for orange juice):

$$f = \frac{\delta_{AM} - \delta_{AA}}{-\delta_{AA} - 11.3} = \frac{\delta_{AM} - \delta_S - 5.2}{-\delta_S - 16.5} \qquad (3)$$

The corresponding results are compiled in Table 5; the fraction f is expressed in percent.

In Table 5, the L-ascorbic acid from orange juices a, e, and i and from black currant juice b had $\delta^{13}C$ values that were more negative than the supposed natural standard value; furthermore, the $\delta^{13}C$ value difference L-ascorbic acid/sugar did not exceed the indicated limit of +5.2‰ (+4.8‰). Therefore, these samples must be regarded as authentic. According to both criteria, to all other juice samples an addition of commercial L-ascorbic acid must be assumed. For the spiked samples, only the amount and the $\delta^{13}C$ value of the added product were later available. Therefore, its relative part was estimated on the basis of RSK-values (mean L-ascorbic acid concentrations in ordinary commercial juices; Association of German Fruit Juice Industries, 1987). The agreement between the two estimations is excellent, and orange samples b−d and h and black currant samples a and c are all in the range as expected from RSK parameters; even two of the samples adulterated with the product $\delta^{13}C$ value of −17.8 (orange f and g) would

Table 5. $\delta^{13}C$ Values of L-Ascorbic Acid and the Sugar Fraction from Fruit Juices, Spiked with Commercial L-Ascorbic Acid, and Calculated Relative Amount of This Addition[a]

juice samples	$\delta^{13}C$ values (‰)$_{PDB}$			added L-ascorbic acid (% of total)			
				estimated on			
	L-ascorbic acid (δ_{AM})	sugar (δ_S)	diff ($\delta_{AM} - \delta_S$)	measd $\delta^{13}C$ values	$\delta^{13}C$ value diff	expd from addition	L-ascorbic acid actually added (mg/L)
orange a	−21.7	−26.1	+4.3				
orange b	−19.3	−25.7	+6.4	14.9	13.0	22	100
orange c	−17.8	−25.6	+7.8	30.9	28.6	36	200
orange d	−17.5	−25.6	+8.1	34.0	31.9	42	250
orange e	−20.8	−25.6	+4.8			22	100*
orange f	−20.3	−26.0	+5.7	4.3	5.3	36	200*
orange g	−19.7	−25.7	+6.0	10.6	8.7	42	250*
orange h	−17.5	−25.7	+8.2	34.0	32.6	36	200
orange i	−21.4	−25.7	+4.3				
black currant a	−20.6	−25.7	+5.1	1.1	3.1	13	200
black currant b	−21.3	−25.6	+4.3				
black currant c	−19.6	−25.6	+6.0	11.7	12.6	23	400

[a] The calculations were made by assuming a standard authentic $\delta^{13}C$ value for fruit L-ascorbic acid of −20.7‰ and for commercial L-ascorbic acid of −11.3‰ (eq 2) and by assuming the latter value and a standard difference for the $\delta^{13}C$ values of L-ascorbic acid and sugar of 5.2‰ (orange) and 4.8‰ (black currant), respectively (eq 3). The actually added products had $\delta^{13}C$ values of −10.0 and −17.8‰ (values with *). On the basis of the amounts added, the theoretical percentage was calculated, using RSK values.

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have qualitatively been detected. Their values, calculated on the real $\delta^{13}C$ value ($-17.8\%_{oPDB}$) of the added L-ascorbic acid, would have been 14 and 35%, respectively.

Certainly, the detection limit for ascorbic acid addition by this method cannot exactly be fixed, because it will depend on the $\delta^{13}C$ value of the added product which is normally not known. However, one could indicate that for the most probable cases [$\delta^{13}C$ value of the added biosynthetic L-ascorbic acid from -10 to $-12\%_{oPDB}$ (Table 4)] and ascorbic acid concentrations between 200 and 350 mg/L, a detection limit of 20% or better will be attained.

DISCUSSION

L-Ascorbic acid is a minor but quality and price determining component of fruit juices, and its quantification and origin assignment are of great importance for fruit juice quality characterization. Our results show that on the basis of the ^{13}C analysis of vitamin C the addition of less than 20% of the biosynthetic product to C3 plant juices will be detectable, provided this product has been prepared from C4 plant sources. From a practical point of view, it is important, that the total L-ascorbic acid is analyzed and that an isotope effect free isolation is possible. It is worthwhile to mention that vitamin C adulteration in other fruit juice beverages can be detected according to the same principle; however, one must take into account that in these cases sugar addition would be allowed.

It should be mentioned that the ^{13}C enrichment of $5\%_o$ of vitamin C relative to that of glucose is quite large and unexpected because L-ascorbic acid is a direct descendant of this carbohydrate. Probably, we have come upon another example of an "isotopic balance" after a metabolic branching, leading to depleted products on one side and enriched ones on the other, a fact that will mainly become evident for minor components, as has already been found by us for glycerol (Weber et al., 1995). Ascorbic acid is certainly a minor product from glucose, compensating for depletions in main products such as cellulose, secondary products, or CO_2 produced by the energy metabolism. The ^{13}C enrichment of L-ascorbic acid meets our finding that also L-tartaric acid in grape juice is ^{13}C-enriched relative to cellulose; the biosynthetic pathway of L-tartaric acid passes L-ascorbic acid as an intermediate (Loewus and Loewus, 1987). Thus, the biochemical background of our results reported here is quite reasonable.

Finally, the principle of the intermolecular isotopic standardization as a general tool for authenticity evaluation has another time demonstrated its value. An objection to its application in the present case could be that its validity could be questionable in the future, because the industrial production of vitamin C could easily be shifted to a C3 plant base. This is true; however, we believe that this could also be detected, e.g. by determinations of $\delta^{18}O$ and δ^2H values or through isotopic patterns of vitamin C.

LITERATURE CITED

Angberg, M.; Nyström, C.; Castenson, S. Evaluation of heat-conduction microcalorimetry in pharmaceutical stability studies VII. Oxidation of ascorbic acid in aqueous solution. Int. J. Pharm. **1993**, 90, 19–33.

Association of German Fruit Juice Industries (VdF). RSK Values. In RSK Values, The Complete Edition; Flüssiges Obst: Schönborn, Germany, 1987.

Bianco, L.; Marucchi, M. Determination of acids in flours, bread and improvers by HPLC. Ind. Aliment. **1991**, 30, 625–634.

Bode, A. M.; Cunningham, L.; Rose, R. C. Spontaneous decay of oxidized ascorbic acid (dehydro-L-ascorbic acid) evaluated by high-pressure liquid chromatography. Clin. Chem. **1990**, 36, 1807–1809.

Bricout, J.; Koziet, J. Control of the authenticity of orange juice by isotopic analysis. J. Agric. Food Chem. **1987**, 35, 758–760.

Christen, H. R. Carbohydrates. In Principles of Organic Chemistry; Otto Salle Verlag: Frankfurt a.M., Germany, 1985; p 952.

Foerst, W. Ascorbic Acid. In Ullmann's Encyclopedia of Technical Chemistry; Urban & Schwarzenberg: Munich, Germany, 1967; Vol. 18, p 220.

Gensler, M.; Schmidt, H.-L. Isolation of the main organic acids from fruit juices and nectars for carbon isotope ratio measurements. Anal. Chim. Acta **1994**, 299, 231–237.

Hapette, A. M.; Poulet, S. A. Application of high-performance liquid chromatography to the determination of ascorbic acid in marine plankton. J. Liq. Chromatogr. **1990**, 13, 357–370.

Koziet, J.; Rossmann, A.; Martin, G. J.; Ashurst, P. R. Determination of carbon-13 content of sugars of fruit and vegetable juices. Anal. Chim. Acta **1993**, 271, 31–38.

Lee, H. S.; Coates, G. A. Liquid chromatographic determination of vitamin C in commercial Florida citrus juices. J. Micronutr. Anal. **1987**, 3, 199–209.

Loewus, F. A.; Loewus, M. W. Biosynthesis and metabolism of ascorbic acid in plants. CRC Crit. Rev. Plant Sci. **1987**, 5, 101–119.

Parker, P. L. Application of the $^{13}C/^{12}C$-ratio for detection of sugar addition to fruit juice concentrates. Fluess. Obst **1982**, 12, 692–694.

Racz, E.; Parlagh-Huszar, K.; Kecskes, T. HPLC method for determination of ascorbic acid in fruits and vegetables. Period. Polytech., Chem. Eng. **1991**, 35, 23–30.

Romero-Rodriguez, A.; Vazquez Oderiz, L.; Lopez Hernandez, J.; Simal Gandera, J. Comparison of two HPLC methods for the determination of ascorbic acid in Carica pentagona. Sci. Aliments **1992**, 12, 593–600.

Rossmann, A.; Rieth, W.; Schmidt, H.-L. Possibilities and results of the combination of stable hydrogen- and carbon isotope ratio measurements with results of conventional analyses (RSK values) for detection of sugar addition in fruit juices. Z. Lebensm. Unters. Forsch. **1990**, 191, 259–264.

Schmidt, H.-L.; Butzenlechner, M.; Rossmann, A.; Schwarz, S.; Kexel, H.; Kempe, K. Inter- and intramolecular isotope correlations in organic compounds as a criterion for authenticity identification and origin assignment. Z. Lebensm. Unters. Forsch. **1993**, 196, 105–110.

Schwarzenbach, R. High-performance liquid chromatography of carboxylic acids. J. Chromatogr. **1982**, 251, 339–358.

Seiffert, B.; Swaczyna, H.; Schaefer, J. Simultaneous determination of L-ascorbic acid and D-isoascorbic acid by HPLC in wine and beer. Dtsch. Lebensmittel. Rundsch. **1992**, 88, 38–40.

Weber, D.; Kexel, H.; Schmidt, H.-L. ^{13}C-pattern of natural glycerol, an isotopic antipode of its precursor glucose. **1995**, to be submitted for publication.

White, J. W.; Winters, K. Honey protein as internal standard for stable isotope ratio detection of adulteration of honey. J. Assoc. Off. Anal. Chem. **1989**, 72, 907–911.

Winkler, F. J.; Schmidt, H.-L. Scope of the application of ^{13}C isotope mass spectrometry in food analysis. Z. Lebensm. Unters. Forsch. **1980**, 171, 85–94.

Zipfel, W.; Zipfel, G. Guidelines for fruit juices. In German Food Law; C. H. Beck: Munich, Germany, 1988; Vol. 2, Chapter 31.