

ADULTERATION OF FRUIT JUICE BEVERAGES

edited by

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Application of Natural Variations in $^{13}\text{C}/^{12}\text{C}$ Ratios to Detect Adulteration of Orange, Lemon, and Apple Juices

I. INTRODUCTION

Among the most abundant elements in living tissues are hydrogen, carbon, nitrogen, oxygen, and sulfur. Each possesses one major and one or more minor stable (nonradioactive) isotopes. Important biological elements and the relative proportions of their major and minor stable isotopes are listed in Table 1. The atomic weight of an element is determined by its isotopic composition, and the tabulated value for carbon is 12.01115. There are, however, variations in isotope ratios such as $^{13}\text{C}/^{12}\text{C}$ among inorganic and organic reservoirs of the carbon cycle. These variations are small, but much greater than the precision with which they can be measured by stable isotope ratio mass spectrometry. Isotope effects are responsible for the dependence of the value of a given isotope ratio on what is being analyzed.

For nearly 50 years it has been known (Nier and Gulbransen, 1939) that carbon dioxide, the source of carbon for all living things, possesses a slightly higher $^{13}\text{C}/^{12}\text{C}$ ratio than does the carbon of organisms. A landmark survey of ratios in hundreds of samples confirmed these findings (Craig, 1953). Many years passed before such observations could be explained. Now the basic physicochemical

Table 1 Stable Isotope Abundances
of Biological Elements

Isotope	Natural abundance (atom %)
^1H	99.983
^2H	0.014
^{12}C	98.916
^{13}C	1.084
^{14}N	99.634
^{15}N	0.366
^{16}O	99.758
^{17}O	0.038
^{18}O	0.204
^{32}S	95.018
^{33}S	0.750
^{34}S	4.215
^{36}S	0.017

and biochemical processes responsible are quite well understood, and the reasons for variations in isotope ratios among classes of compounds from a given natural source are being investigated.

Some of the important processes that are responsible for natural variations in $^{13}\text{C}/^{12}\text{C}$ ratios will be briefly described. To appreciate studies of the purity of foods by stable carbon isotope ratio analysis, and to envisage future applications, some understanding of such variations is important. Most important are the initial enzymes that fix carbon dioxide in C_3 and C_4 plants (ribulose biphosphate carboxylase and phosphoenolpyruvate carboxylase, respectively), which are largely responsible for variations in $^{13}\text{C}/^{12}\text{C}$ ratios among foods derived from these plants. A comprehensive review on carbon isotope fractionation in plants, with an extensive bibliography, has been published (O'Leary, 1981). Application of these variations to detecting adulteration of the juices from orange, lemon, and apple will be described in some detail.

II. CARBON ISOTOPE FRACTIONATION BY PLANTS

Stable isotope ratios of carbon (and other elements) are reported as values of δ , which are differences, in parts per thousand (per mil) between the isotopic composition of a sample and a standard. The standard most often used for carbon ratios is the calcium carbonate PeeDee Belemnite (PDB). Organic material is quantitatively combusted to carbon dioxide and the relevant ion beam (masses 45 and 44) ratios are compared to those generated from the PDB standard. The values of δ are then determined from the formula.

$$\delta^{13}\text{C} = \left[\frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{standard}}} - 1 \right] 10^3$$

The reproducibility of these mass spectrometric measurements is ± 0.2 per mil or better. A negative value for ^{13}C indicates that the sample is depleted in the less common isotope with respect to the standard. Most organic carbon does give a negative value, and it is the magnitude of this value that has proved to be extremely useful in attempts to detect adulteration in foods. The discussion that follows shows that the value of $\delta^{13}\text{C}$ varies extensively among plants and their derived products. These variations can be largely accounted for by considering the plant photosynthetic categories C_3 , C_4 , and crassulacean acid metabolism (CAM). Foods that have been the major targets for adulteration use the C_3 type of photosynthesis, while the major adulterants have been derived from the C_4 sources, sugarcane and corn.

A. C_3 and C_4 Plants

For several years after its elucidation, the Calvin cycle (also known as the C_3 or photosynthetic carbon reduction cycle) of photosynthesis was thought to be the common pathway for assimilation of carbon dioxide by all photosynthetic organisms. The C_3 cycle is ultimately operative in all plants, but two other modes of initial carbon dioxide fixation are now known, the C_4 (Hatch and Slack, 1970) and CAM cycles. The C_3 cycle begins with the addition of carbon dioxide to ribulose biphosphate, a reaction catalyzed by ribulose biphosphate carboxylase ($\text{RuBP}_{\text{case}}$). The product of this reaction is 3-phosphoglyceric acid, a C-3 acid. Plants that possess the C_3 cycle have $\delta^{13}\text{C}$ values ranging from about -22 to -33 per mil.

The C_4 cycle begins with the addition of carbon dioxide to phosphoenolpyruvate, catalyzed by phosphoenolpyruvate carboxylase (PEP_{case}).

The product is a C-4 compound, oxaloacetic acid, which is converted to either malic or aspartic acid. Table 2 summarizes plant photosynthesis and the three main carbon dioxide fixation pathways. Plants that possess the C₄ cycle have values of δ¹³C ranging from about -9 to -20 per mil, with most being nearer the upper end of the range. The value for carbon dioxide is quite uniform throughout the atmosphere, -7 per mil, and in recent years reasons we have learned why plant carbon, especially from C₃ plants, is depleted in ¹³C relative to its source carbon dioxide.

Detailed in vitro studies have established that the carbon isotope fractionation associated with carboxylation of ribulose biphosphate is 29 per mil (Roeske and O'Leary, 1984). This accounts for most of the deviation in values from source carbon dioxide, and the further variability in δ¹³C values among C₃ plants will be discussed in a later section. Little or no fractionation is associated with the carboxylation of phosphoenolpyruvate in C₄ plants. Since the transfer of carbon dioxide to ribulose biphosphate in these plants is quantitative, no additional isotope fractionation results. Because of this, δ¹³C values of C₄ plants are nearer that of source atmospheric carbon dioxide.

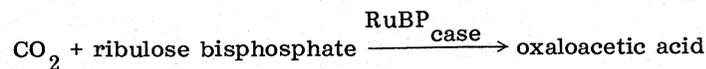
In light of the discovery of the C₄ cycle, it was possible to rationalize the values of δ¹³C in plants reported in an early survey (Craig, 1953). Two additional comprehensive surveys were conducted on car-

Table 2 Summary of Plant Photosynthesis

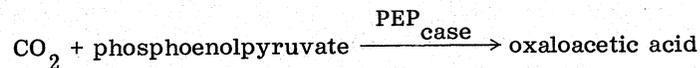
Light reaction: For ATP, NADPH generation in plant chloroplasts

Dark reaction: For carbon dioxide fixation and sugar formation

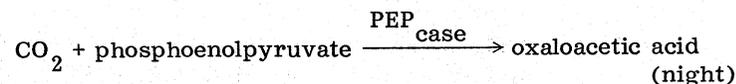
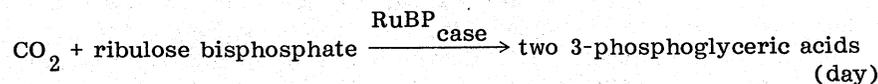
C₃ (Calvin) pathway, initiated by:



C₄ (Hatch-Slack) pathway, initiated by:



CAM (crassulacean acid metabolism), initiated by:



bon isotope ratios in plants (Bender, 1971; Smith and Epstein, 1971). These confirmed that all plants are "lighter" in carbon (more ^{12}C) than is the atmospheric carbon dioxide (-7 per mil) they use and that plants using C_4 photosynthesis invariably possess less negative values than those of C_3 plants. Now, rather than classifying plants into photosynthetic categories by examining leaf anatomy, $\delta^{13}\text{C}$ analysis could be used. It was suggested (Smith and Epstein, 1971) that one could distinguish between sucrose obtained from sugarcane (C_4 plant) and sucrose from sugar beet (C_3 plant). Chemically, of course, the sugars are identical and their distinction is otherwise impossible. This new concept provided the impetus for numerous applications of the method to food adulteration problems, some of which are described in this chapter.

B. CAM Plants

Like C_4 plants, crassulacean acid metabolism plants form a C-4 acid as the initial photosynthetic product. CAM plants are unique in that they accumulate this and other acids in the dark. Many succulent plants, such as pineapple, have CAM photosynthesis, and in the light, the C-4 acid malate is decarboxylated and the carbon dioxide produced is fixed by the C_3 cycle. The $\delta^{13}\text{C}$ values of CAM plants are determined primarily by the relative proportions of carbon dioxide fixed at night by phosphoenolpyruvate and by ribulose biphosphate carboxylase during the day (Sternberg et al., 1984). So CAM plants possess a wide range of values, from about -10 to -20 per mil, depending on the relative amounts of day and night carbon dioxide fixation.

C. Carbon Dioxide Diffusion Effects

Since a range of $\delta^{13}\text{C}$ values exists for representatives of both C_3 and C_4 plant categories, it is obvious that factors in addition to differential isotope effects of the initial photosynthetic enzymes are involved. Measurements of $\delta^{13}\text{C}$ in plants grown under controlled environmental conditions support the use of an expression (Farquhar et al., 1982) to predict $\delta^{13}\text{C}$ values when certain variables are known. A useful expression (Farquhar et al., 1982) for C_3 plants is

$$\delta^{13}\text{C}_{\text{plant}} = \delta^{13}\text{C}_{\text{atmosphere}} - a - (b - a) \frac{C_i}{C_a}$$

where the constant a (4.4 per mil) accounts for the diffusivity of $^{12}\text{CO}_2$ relative to $^{13}\text{CO}_2$ and b (29 per mil, assumed constant) is the isotopic discrimination by ribulose biphosphate carboxylase. Measurement of C_i and C_a (concentrations of carbon dioxide in leaf intercellular spaces and in the atmosphere, respectively) allows solution of the equation. So factors that effect diffusion of carbon dioxide from the atmosphere to the leaf mesophyll will modify C_i/C_a and the plant's value of $\delta^{13}\text{C}$.

III. APPLICATION OF $^{13}\text{C}/^{12}\text{C}$ RATIO VARIATIONS TO FOOD ANALYSIS

Numerous applications of stable carbon isotope ratio measurements that relate to food and nutritional problems have been reported. Food applications have been the subject of previous reviews (Winkler and Schmidt, 1980; Bricout, 1982; Krueger, 1984), and the use of isotopes as tracers in nutritional investigations has been comprehensively reviewed (Matthews and Bier, 1983). Analysis of human tissue samples for $\delta^{13}\text{C}$ values has been useful for estimating proportions of C_3 and C_4 plant sources in human diets (Gaffney et al., 1978). Also, the use of $\delta^{13}\text{C}$ measurements in reconstructing the diets of prehistoric humans has been the subject of productive investigations, and this topic has been reviewed (van der Merwe, 1982).

A. General Comments

Economic incentives to adulterate foods and beverages with less expensive substances have been significant for many years. Honey, for example, has been a target since early times, with adulterants such as starch, glucose, sucrose, and more recently, cane invert syrup, high fructose corn syrup (HFCS), and beet invert syrup. Presently, the early adulterants of honey are easily detectable, because they contain either a substance not found at all in pure honeys or a higher proportion of a given substance than does pure honey. For example, acid-converted invert syrups contain 2-hydroxymethylfurfural in higher levels than the range normally found in pure honeys.

With the advent of enzyme-converted invert syrups and HFCS, adulteration strategies became much more sophisticated, and equally sophisticated procedures were required to detect the addition of these to honey, juices, and other foods that were labeled "pure." The foods are very complex compared to those highly refined adulterants, and no compounds could be identified that were unique to the relatively simple adulterants. These adulterants are inexpensive compared to the target foods, and they are available in a variety of sucrose/glucose/fructose or glucose/fructose ratios. So syrups with sugar ratios in the range found in the pure food can be obtained. As a result, researchers are challenged to identify differences between the adulterants and the target foods.

B. Orange and Lemon Juices

It was fortuitous that the major adulterants of honey, orange juice, and apple juice were, until quite recently, HFCS and cane invert syrups. Both these products are derived from C_4 plants, while the juices and the great majority of honeys are derived from C_3 plants. Since the act of processing plant sources in foods results in minimal further fractionation of isotopes, it could be anticipated that $\delta^{13}\text{C}$ measurements

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would be extremely helpful in detecting these forms of adulteration. The most important food applications of $^{13}\text{C}/^{12}\text{C}$ ratio values have been in detecting mixtures of materials derived from C_3 and C_4 plants. Because of the wide variation in composition of each of the target foods due to factors such as climate, location, and genetic variability of source plants, approaches that involve trying to define the food in terms of composition and then classifying it as adulterated when it deviates from this definition have met limited success. The adulterant being used at a particular time should also be considered, and its unique features (physical, chemical, biological) examined.

In 1974 the results of a survey of $\delta^{13}\text{C}$ values in 42 samples of genuine French and Israeli oranges were reported (Nissenbaum et al., 1974). The possibility of detecting the addition of cane sugar was demonstrated, since values of -25.0 and -24.3 per mil were obtained for French and Israeli juices, respectively. Beet and cane sucrose gave values of -24.3 and -12.2 per mil, respectively; since beet sugar is used almost exclusively in Israel, no application of the method was realized. Further studies were later initiated elsewhere.

A report that appeared in 1977 demonstrated the use of $\delta^{13}\text{C}$ values in the detection of maple syrup adulterated with cane sugar (Hillaire-Marcel et al., 1977). Mixtures of maple (C_3 source) and cane (C_4 source) sugars possessed values between those of the pure materials. Our research was initiated when it became apparent that honey was being adulterated with HFCS. Honey originates in the nectar from a large number of plant families. For the carbon isotope approach to be useful, commercially significant honeys have to be generated from C_3 floral sources. Samples from throughout the world were analyzed, and all had $\delta^{13}\text{C}$ values characteristic of C_3 plants (Doner and White, 1977; White and Doner, 1978). As a result, honey adulteration with HFCS could be detected. Since then, a South African honey from a CAM source was found with an elevated value (Ziegler et al., 1979).

Orange and lemon juices come from single plant families, long known to be C_3 plants. So it was quite predictable that adulteration with corn and cane sugars could be detected. It was necessary to determine the range of $\delta^{13}\text{C}$ values in juices from the important producing areas to establish the sensitivity of this approach. In the United States it was likely that HFCS and cane invert syrups would be used as adulterants of orange juice. HFCS, in particular, became widely available in the early 1970s and was relatively inexpensive. Values of $\delta^{13}\text{C}$ in 42 samples of pure juices from California, Florida, Texas, Arizona, Mexico, Spain, and South Africa were determined (Doner and Bills, 1981). The mean value for all samples was -24.5 per mil, and the coefficient of variation was just 2.41 per mil, the smallest variation yet encountered for any juice component or physical property. These results and those reported earlier (Nissenbaum et al., 1974), as well as results for apple juice (Doner et al., 1980), are summarized in Table 3 and in Figure 1. The uniformity in $\delta^{13}\text{C}$ values enhances the detectability of C_4 adulter-

Table 3 Summary of $\delta^{13}\text{C}$ Values in Orange and Apple Juices

Sample	Location	Number of Samples	Mean (per mil)	Range (per mil)	SD
Orange juice	United States	38	-24.5	-23.4 to -25.6	0.591
Orange juice	France	-	-25.0	-	-
Orange juice	Israel	-	-24.3	-	-
Orange juice	Mexico	2	-24.3	-23.4 to -25.2	-
Orange juice	Spain	1	-23.9	-	-
Orange juice	South Africa	1	-24.8	-	-
Apple juice	United States	40	-25.3	-22.5 to -27.9	1.275

Sources: Orange juices, Doner and Bills (1981) and Nissenbaum et al. (1974); apple juices, Doner et al. (1980).

ants such as HFCS and cane invert syrups ($\delta^{13}\text{C}$ values near -10 per mil) in orange juice. The measurements were made on total orange juice solids; it was subsequently shown (Parker, 1982) that detectability of C_4 adulterants is enhanced by measuring values in the whole juice, pulp, and soluble fraction. Since HFCS resides mostly in the soluble fraction, differences between $\delta^{13}\text{C}$ in this fraction and the pulp (juice source) are sensitive indicators of low levels of adulteration.

To demonstrate that independent laboratories obtain similar values of $\delta^{13}\text{C}$ values for replicate samples, a blind interlaboratory comparison study was conducted (Doner and Bills, 1982), employing procedures established by the Association of Official Analytical Chemists (AOAC). Five samples containing various proportions of orange juice and HFSC were analyzed by seven laboratories and the agreement among collaborators was excellent (Table 4), resulting in the method being officially sanctioned. The procedures to detect honey, apple juice, and orange juice adulteration by stable carbon isotope ratio analysis were the first AOAC official final action methods to employ mass spectrometry, starting with honey (White and Doner, 1978).

Because of the natural variability in $\delta^{13}\text{C}$ values among pure juices, detection of adulteration by this method is qualitative rather than quantitative. This is because the value for the pure juice before adulteration cannot be determined. For example, if a pure juice with a $\delta^{13}\text{C}$ value of -25.6 per mil was adulterated to a level of 30% with HFCS ($\delta^{13}\text{C} = -9.7$ per mil), the value for the mixture would be reduced to -21.8 per mil. If, on the other hand, a pure juice with

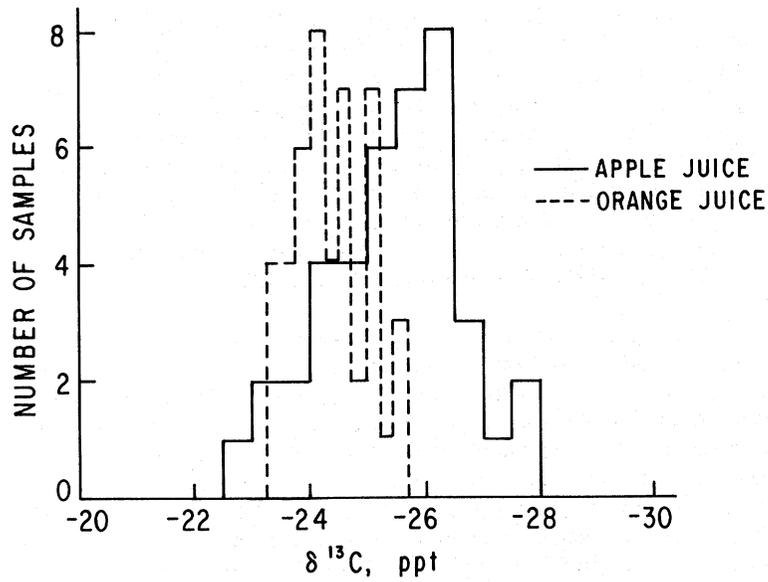


Figure 1 Distribution of $\delta^{13}\text{C}$ values among 42 pure orange juices and 40 pure apple juices, plotted from data of Doner and Bills (1981) and Doner et al. (1980), respectively.

Table 4 Interlaboratory Study of $\delta^{13}\text{C}$ Values (per mil) in Orange Juice/HFCS Mixtures

Laboratory	HFCS/orange juice ratio				
	0:100	30:70	45:55	60:40	75:25
1	-24.9	-20.2	-18.0	-15.5	-13.2
2	-25.9	-21.3	-19.2	-17.2	-14.8
3	-26.0	-21.3	-19.2	-16.7	-14.5
4	-24.7	-20.7	-19.0	-16.0	-14.2
5	-25.3	-21.5	-18.9	-16.6	-15.2
6	-26.1	-21.5	-18.8	-16.7	-14.5
7	-26.2	-21.2	-18.8	-16.4	-14.5

Source: Doner and Bills (1982).

a value at the higher end of the range (-23.4 per mil) was adulterated with the same level of HFCS, the value of the mixture would be -19.3 per mil. Because of this uncertainty, the upper limit of $\delta^{13}\text{C}$ for authentic juice may be set with any desired degree of certainty as the mean value for pure juices plus a multiple of their standard deviation. Using this approach, there is only 1 probability in 25,000 that a juice having a value less negative than -22.1 per mil (4 SD from mean) is pure and unadulterated. This type of interpretation for both orange and apple juices is shown in Table 5. It has been considered in relation to values of $\delta^{13}\text{C}$ in honey and nectars (White, 1980; White and Robinson, 1983).

It is interesting to note that citrus honeys possess slightly higher $\delta^{13}\text{C}$ values than all other honeys; their mean value of -23.8 per mil is close to that of the mean value for citrus juices. Since very similar values were reported for orange juices from Israel, France, the United States, Mexico, Spain, and South Africa, it can be anticipated that the $\delta^{13}\text{C}$ method can be used worldwide for the detection of added syrups derived from C_4 plants (Nissenbaum et al., 1974; Doner and Bills, 1981).

The application of stable carbon isotope ratio analysis to detecting adulteration of lemon juice is much more limited because citric acid accounts for more than 60% of the solids in lemon juice. Addition of this adulterant has been a long-standing problem. If citric acid were industrially produced by fermentation of C_4 plant sources, detection would be quite straightforward, since lemon is a C_3 plant. Citric acid, however, is produced by large-scale fermentations of beet and cane molasses, corn sugars, and more recently, paraffin hydrocarbons. Samples isolated from lemons and obtained from commercial sources were analyzed for $\delta^{13}\text{C}$ values (Doner, 1985). The citric acid from the lemons averaged -24.0 per mil in $\delta^{13}\text{C}$, and that from fermentation of beet sugar, corn sugar, and paraffin gave values of -25.2, -9.9,

Table 5 Probabilities of $\delta^{13}\text{C}$ Values of Authentic Orange and Apple Juices Being More Negative than a Stated Limit

Probability (%)	Limits (per mil)	
	Orange juice	Apple juice
5 of 6 = 88.33	-23.9	-24.1
43 of 44 = 97.73	-23.3	-22.8
769 of 770 = 99.87	-22.7	-21.5
24,999 of 25,000 = 99.997	-22.1	-20.2

Sources: Orange juices, Doner and Bills (1982); apple juices, Doner and Phillips (1981).

and -27.2 per mil, respectively. It is obvious that while citric acid from corn and cane sugar sources would be readily detected, that from the beet sugar would go undetected, and the addition of paraffin-derived citric acid would be indicated only if substantial amounts had been added.

Since petroleum products such as paraffin are devoid of ^{14}C , it is likely that addition of paraffin-derived citric acid would be detectable by liquid scintillation counting of this radioactive isotope. The "new" carbon in lemon citric acid would be expected to have a rather uniform level of ^{14}C , and a reduction in this level would suggest adulteration. This approach has been successfully undertaken in studies on the detection of synthetic ethanol in wines and spirits (McWeeny and Bates, 1980) and to distinguish natural and synthetic cinnamic aldehyde (Hoffman and Salb, 1980).

Sugars would probably be added along with citric acid when preparing adulterated lemon juice, to maintain Brix/acid ratios appropriate for lemon juice. A fraction consisting mainly of sugars results after removing the citric acid as its calcium salt from lemon juice. Determination of $\delta^{13}\text{C}$ values in this fraction can be used as an indicator of cane invert syrup or HFCS addition.

C. Apple Juice

In 1978 market surveys revealed that a significant proportion of apple juice in the New England area contained added sugar, although it was labeled as pure juice. This adulteration was suggested by $\delta^{13}\text{C}$ analysis of market samples, and the topic received attention in the popular press (Beaton and Gold, 1979). As with honey and the other juices, incentives to adulterate apple juice with inexpensive sugar syrups are significant, since adulteration at the 50% level can reduce the manufacturer's costs by 40%. Apple had long been known to be the product of a C_3 plant, so elevated $^{13}\text{C}/^{12}\text{C}$ ratios for pure juices would not be anticipated. Baseline values were determined for $\delta^{13}\text{C}$ in pure juices, representing the important growing areas and varieties, and the C_3 character of apples was confirmed (Doner et al., 1980). The mean $\delta^{13}\text{C}$ value for 40 samples was -25.4 per mil, and the coefficient of variation was 4.88%. No correlation was noted between $\delta^{13}\text{C}$ value and growth location or apple variety. The results are summarized as part of Table 3, and in Figure 1 the relative distributions of values for apple and orange juices are represented.

A collaborative study was initiated, in which five samples of apple juice that had been adulterated to various levels with HFCS were submitted for analysis to six independent laboratories. The agreement among collaborators was satisfactory, and the method was approved as an official AOAC final action (Doner and Phillips, 1981). The probability limits for $\delta^{13}\text{C}$ values in pure apple juices appear in Table 5.

The apple juice industry rapidly responded to the availability of the new test procedure, and it was applied in random nationwide market surveys. As a result, adulteration of apple juice with inexpensive cane- and corn-derived sugars was markedly reduced.

IV. CONCLUSION

The presence of inexpensive sugars derived from the C₄ plants sugar-cane and corn can be detected in various C₃ plant foods such as apple and orange juices, and honey. Ratios of ¹³C/¹²C, which are higher than normal in such adulterated products, are precisely determined by stable isotope ratio mass spectrometry. The elevated δ¹³C ratios in C₄ plants and their derived products are due mainly to differential fractionation of source carbon dioxide during the initial carbon fixation step in photosynthesis. In C₃ plants the discrimination favoring ¹²CO₂ by the initial enzyme, ribulose biphosphate carboxylase, is to the extent of about 29 per mil, while the initial photosynthetic enzyme in C₄ plants, phosphoenolpyruvate carboxylase, shows little or no discrimination for the isotopic forms of carbon dioxide. Variable diffusivity of carbon dioxide from the atmosphere to leaf mesophyll accounts for some further differences among C₃ plants.

The applicability of the stable carbon isotope ratio procedure is assured when it can be determined that the food is derived from a C₃ plant and the adulterant from a C₄ plant, and vice versa. This can sometimes be determined for single-source foods by consulting the surveys (Craig, 1953; Bender, 1971; Smith and Epstein, 1971) of plant values for δ¹³C. These have been determined for edible parts of 35 fruits (Krueger et al., 1986), and of these, only pineapple, prickly pear (CAM sources), and possibly coconut were not derived from C₃ plants. To establish the sensitivity for detecting C₄ products in the C₃ fruits, the range and variability in δ¹³C for multiple samples of most fruits remains to be established.

The success of stable carbon isotope ratio analysis in detecting cane syrups and HFCS in various foods has led to modifications of adulteration strategies. There is evidence that beet invert syrup, a product derived from a C₃ plant, is being used to circumvent detection by δ¹³C measurement. Addition of this material to orange juice can be detected, since significant differences exist in the carbon-bound deuterium-hydrogen (D/H) and ¹⁸O/¹⁶O ratios in the sucrose from beet and that from orange juice (Doner et al., 1987). The magnitude of the difference, especially in deuterium ratios, suggests that this method will find further application.

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