The Analytical Approach

Edited by Claude A. Lucchesi

Assuring the Quality of Honey Is It Honey or Syrup?

Honey, a natural product of limited supply and relatively high price, traditionally has been a target for adulteration. As a result, bulk honey markets are being lost to mixtures and substitutes in the form of sugar cane and corn-derived syrups. When such mixtures are appropriately labeled, there is no legal violation. However, the evidence of widespread mislabeling of these materials as pure honey represents a fraud to the consumer. The adulteration of honey with various sweet syrups without fear of detection is a great threat to the integrity of the honey markets, to the economic resources of beekeepers, and to the production of the more than 11.5 billion dollars worth of agricultural crops that depend on the honeybee for pollination.

The corn processing industry now produces a new low-cost sweetener, high fructose corn syrup (HFCS), in great amounts by making use of advances in bound enzyme technology. It has been estimated (*1*) that the food industry will use four billion pounds of HFCS annually by 1980, with per capita consumption reaching 18.2 lb, 14% of the total nutritive sweetener consumption. HFCS production involves the enzymatic isomerization of a portion of the glucose in conventional corn syrup to the sweeter sugar, fructose. The resulting syrup is then refined with activated carbon and ion-exchange treatment, and yields the two monosaccharide sugars, glucose and fructose, with only slight amounts of other materials.

A research program designed to develop methods for the detection of honey adulteration by HFCS was initiated at the Eastern Regional Research Center early in 1975. We considered several approaches with the hope that some would result in methods for HFCS detection in honey, regardless of improved production methods of this sweetener by the corn processing industry.

The Analytical Approach

Two general analytical approaches can be taken in attacking a problem such as the detection of mixtures of honey and HFCS.

Approach One: Identification of a constituent or property of the adulterant and detection of its presence in suspect honeys. Pure honeys would be shown either not to possess the chosen characteristic or to possess it at a much lower level.

Approach Two: Identification of a constituent or property of honey that is always present at a certain level. The addition of an adulterant without the characteristic would lower the concentration of the constituent or the value of the property.

Tests are available to detect the traditional adulterants of honey, including conventional corn syrup and commercial invert syrup (*2*). Approach One was used in the development of the official methods for identifying each syrup in honey. Conventional corn syrup (primarily glucose) contains appreciable amounts of higher molecular weight glucose polymers not present in honey, and these are detected by paper and thin-layer chromatography. Inverted cane syrups (consisting primarily of glucose and fructose) are detected in honey through the presence of hydroxymethylfurfural (HMF), which is present at significantly higher levels in acid-inverted cane syrups than in pure honeys.

Approach Two is of no value for the detection of adulterated honey because of the wide variability among the known constituents of honey. The most authoritative study of United States honey composition (*3*) illustrates the variability encountered. In that study, all commercially significant domestic honey types and blends

were analyzed, and the results can be considered truly representative of domestic honey composition with regard to the components determined. The concentration ranges for the major constituents of honey (490 samples analyzed) and of HFCS samples from a major commercial source are given in Table I. The ranges indicate that a considerable amount of a sweetener like HFCS, with its sugar composition resembling honey, could be added without the mixture exceeding normal honey concentration limits. The increased moisture content can be compensated for easily by a clever manipulator. In the survey (*3*) the presence of other honey constituents over a wide range precluded the use of dilution of a honey property for detecting adulteration. Approach Two was used for just two possibilities examined in our research, unsuccessfully both times, as indicated in Table II.

The composition of honey depends upon two most important factors, the floral source and the composition of the nectar. Less important are certain external factors, including climate and differences in processing. All factors contribute to the variability of honey composition and to its enormous complexity [22 minor di- and trisaccharides have been identified (*4*)] and make most approaches to the detection of adulteration unworkable.

Early in our research program we had to learn as much as possible about the properties and composition of HFCS from the various commercial sources. Our goal was to find a constituent or property common to the various HFCS samples but not found in any honeys. Little information regarding the minor HFCS constituents that may come through the rigorous refining process was available in the literature, and examination of significant numbers of representative honeys with respect to any candidate HFCS components was required. It was our expectation that no single test would serve the objective of this search, and the need to screen large numbers of suspect samples would require relatively simple indicator tests; confirmatory analysis could be more complex. Furthermore, tests, to obtain legal standing, would have to be subjected to formal collaborative testing by independent laboratories, under the auspices of the Association of Official Analytical Chemists.

Establishing tests for HFCS in honey proved more challenging than earlier methods for the traditional adulterants, since HFCS is simpler, more closely resembles honey composition with regard to major components, and is more highly refined. Complicating the problem is the fact that methods of HFCS production are continuing to evolve, and trace constituents found to be unique in present syrups may be eliminated by new refining processes. Accordingly,

Table I. Concentrations of Major Constituents of Honey and High Fructose Corn Syrup (HFCS)

Constituents	Honey Range (%)	Honey Mean (%)	HFCS Range (%)	HFCS Mean (%)
Moisture	13.4–22.9	17.2	23–29	26
Fructose	27.2–44.3	38.2	30–42	36
Glucose	22.0–40.7	31.3	31–35	33
Di-, tri-, and higher saccharides	4.6–23.3	10.1	3.1–5.7	4.4
Other		3.2		0.6

Table II. Approaches for Detection of Mixtures of High Fructose Corn Syrup and Honey

Method	Constituent determined	Found to be applicable
Approach One		
Differential scanning calorimetry	Organic compounds	No
Gas-liquid chromatography	Isomaltose/maltose ratio	Yes
Gel filtration, affinity chromatography	Polysaccharides	No
High-pressure liquid chromatography	Monosaccharide (psicose)	No
Immunodiffusion	Polysaccharides, proteins	No
Stable carbon isotope ratio analysis	$^{13}C/^{12}C$ ratio	Yes
Thin-layer chromatography	Dextrins, polysaccharides	Yes
Turbidimetry (with concanavalin A)	Polysaccharides	No
Approach Two		
Atomic absorption spectroscopy	Sodium/potassium ratio	No
Colorimetry	Proline	No

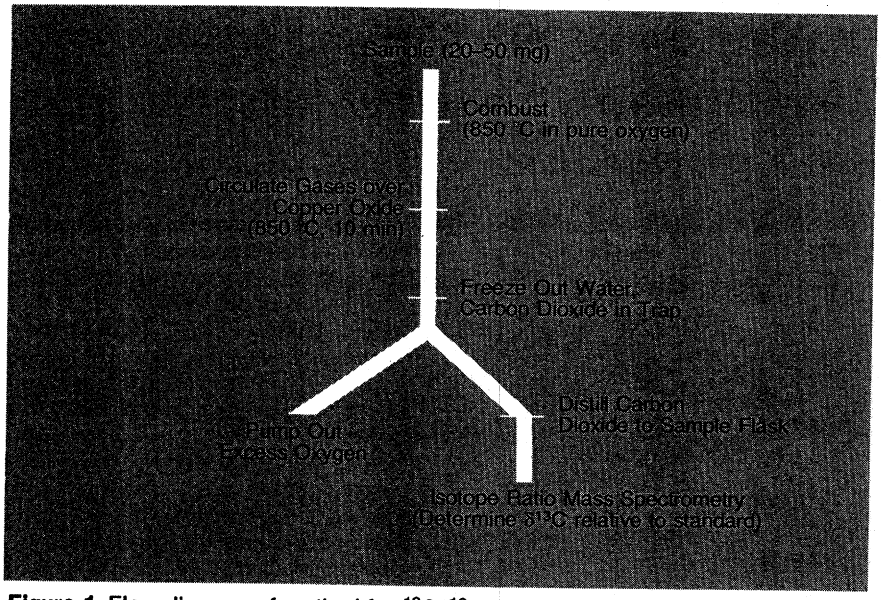

Figure 1. Flow diagram of method for $^{13}C/^{12}C$ determination

we applied Approach One to the problem of detecting HFCS in honey in eight separate investigations (Table II); three proved useful.

Evaluation of Various Approaches

Three approaches to the problem involved determining lower molecular weight ions and molecules and were not valid for judging honey purity. A suggestion had been made (*5*) that examination of the sodium/potassium ratio was useful because HFCS is refined by ion-exchange treatment, and the original cations present in HFCS are replaced by sodium. Honey has long been known to be relatively poor in sodium but rich in potassium. A paper evaluating literature data (*6*), however, demonstrated that the sodium/potassium ratio is of little use as the sole parameter because of the extreme variability of these elements in honey. The convenience of atomic absorption analysis would have made this a very attractive approach, but this variability precluded its use.

Early in our research, we were optimistic that a useful test might be the determination of the monosaccharide sugar psicose, which in early production lots of HFCS had been reported (*7*) to be present at levels to 1% because of base-catalyzed isomerization of fructose. This sugar is not present in honey, and HPLC methods were available for its detection in standard mixtures with the major honey sugars, fructose and glucose. The presence of psicose in HFCS, however, indicated to the corn sweetener manufacturers that the process was not ideal, and they were successful in producing syrups free of this sugar and other byproducts considered detrimental to the quality of their product. This was our first experience with a potential method being eliminated because producers were obtaining better (from their viewpoint), more highly refined syrups. This was not our last such experience because, in a sense, we were shooting at a moving target.

The amino acid proline is present in unusually high levels in honey and absent from HFCS. Consequently, proline was measured in 740 honey samples (*8*) to determine whether it is present over a sufficiently narrow concentration range to permit the use of Approach Two. A convenient colorimetric method is available for such a test, but the wide range of values found precluded the use of proline determination as an indicator of honey purity.

Several methods were designed to reveal differences between the minor macromolecular (polysaccharide and protein) fractions of honey and HFCS. In one approach, we constructed an autoanalyzer to determine the molecular weight distribution of polysaccharides from honey and HFCS. Initially, this approach appeared to be very promising. In a second method based on the differences in the polysaccharide fractions from honey and from HFCS, we took advantage of the unusually high degree of branching present in polysaccharides from some HFCS samples. The soybean lectin, concanavalin A, associates with terminal glucose units in branched polysaccharides through a multivalent interaction and leads to precipitation and quantification by turbidity. Interesting results were obtained (*9*) but, as with the other macromolecular approaches, were rendered useless for the adulteration problem when HFCS containing no high molecular weight carbohydrate polymers became commercially available.

An immunochemical approach wherein rabbits were injected with HFCS materials and tested for elicitation of antibodies was attempted. HFCS polysaccharides were conjugated with bovine serum albumin and keyhole limpet hemocyanin and, after being administered to rabbits, produced immune sera, which were isolated by scientists at the Western Regional Research Center of the U.S. Department of Agriculture. Unfortunately, interaction between the immune sera and the injected materials was not inhibited by HFCS polysaccharides. Efforts to prepare a protein concentrate from HFCS for preparation of an immune serum in rats and rabbits were unfruitful. Considering the standard immunodiffusion techniques available, we were disappointed that these sensitive and convenient approaches to the problem did not work.

We also attempted to apply differential scanning calorimetry to the adulteration problem. However, the virtually identical profiles obtained for honey and HFCS again reflected the similarity in composition of the two products.

Stable Carbon Isotope Ratio Method

Clearly, we needed to identify a property of HFCS that would not be affected by new refining processes but would be characteristic of products derived from the corn plant. The breakthrough came when we evaluated the ratios of the stable isotopes of carbon in representative samples of honey and HFCS. This approach had been used (*10*) in detecting the illegal addition of cane sugar to Israeli citrus juice, and it was suggested (*10*) that the $^{13}C/^{12}C$ ratio might be useful in detecting fraudulent substitutions of various types of plant products with corn-derived materials. Methods for detecting the adulteration of maple syrup with cane sugar (*11*) and the adulteration of natural bean vanillin with synthetic vanillin (*12, 13*) had utilized $^{13}C/^{12}C$ analysis.

The reasons for differences in $^{13}C/^{12}C$ ratios among members of the plant kingdom are now beginning to be understood and result primarily from the three different pathways by which carbon dioxide is fixed into organic compounds via photosynthesis. In plants using the classic Calvin (C_3) cycle, the primary photosynthetic product is the 3-carbon acid, 3-phosphoglycerate. In plants using the Hatch-Slack (C_4) cycle, the initial products of carbon dioxide fixation are the 4-carbon acids oxalacetate, malate, and aspartate. Plants in a third category, crassulacean acid metabolism, have the enzymatic capability for initially fixing carbon dioxide by

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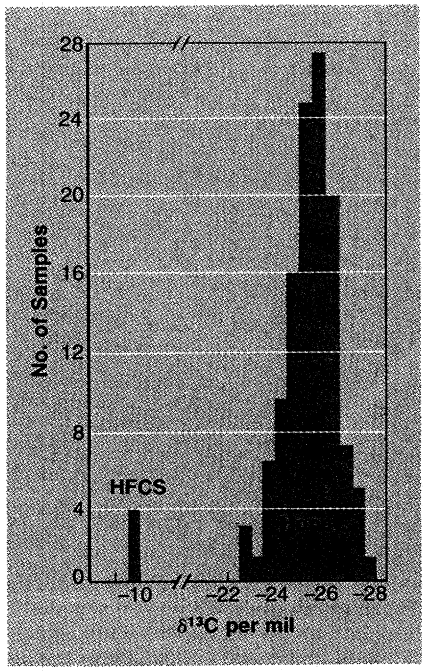

Figure 2. Distribution of $\delta^{13}C$ values for honey and HFCS

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the C_4 pathway and then shuttling it into the C_3 system. All plants are slightly lighter in ^{13}C than the carbon dioxide of the atmosphere, and Calvin (C_3) plants discriminate to a greater extent than do Hatch-Slack (C_4) or crassulacean acid metabolism plants. The $^{13}C/^{12}C$ ratios of a sample are reported as per mil (‰) deviations from a limestone standard and are defined as:

$$\delta^{13}C \text{ (per mil, ‰)} = \left(\frac{^{13}C/^{12}C \text{ sample}}{^{13}C/^{12}C \text{ standard}} - 1\right) \times 10^3$$

These values are determined by isotope ratio mass spectrometry after complete sample combustion to carbon dioxide. A flow diagram of the procedure is shown in Figure 1. Calvin (C_3) plants have $\delta^{13}C$ values of −22 to −33‰, and Hatch-Slack (C_4) plants have values from −10 to −20‰ (*14, 15*). Crassulacean acid metabolism plants have intermediate $\delta^{13}C$ values.

Corn, sugar cane, sorghum, and other grasses native to the tropics fall into the Hatch-Slack category with $\delta^{13}C$ values in the upper range. Pure HFCS then would have a similar value. It was our hope that all honey samples would be in the range expected for Calvin plants and that this might be a characteristic of all flowering, nectar-bearing honey sources. To test this, we selected 84 samples from our collection of pure honeys to represent all commercially important United States honey sources from wide geographical areas. We analyzed 35 imported honey samples from 15 countries, with geographical latitude the primary consideration in their selection. Details of the method and results of $\delta^{13}C$ analysis of these samples are described elsewhere (*16–18*); the distribution of values are diagrammed in Figure 2. The coefficient of variation for all honey samples was 3.86%, the smallest yet encountered for any constituent or physical property of honey. Mixtures of HFCS and honey would be expected to have $\delta^{13}C$ values equal to the sum of the fractional contribution of each. This was found to be the case.

A collaborative study of the method was conducted (*17*) by seven laboratories, each testing four prepared honey–HFCS mixtures and a pure honey. Because of the excellent agreement among the collaborators, the Association of Official Analytical Chemists has adopted the method as official first action for handling cases of honey adulteration by HFCS. The beauty of this method is that it is noncircumventable and will apply regardless of new refinements in HFCS production. The only requirement is that these syrups continue to be produced from corn or other Hatch-Slack (C_4) plants. This procedure is now being used by regulatory agencies and by the honey industry for self-policing. The $\delta^{13}C$ values indicate to them whether samples are pure or adulterated honeys. The upper (least negative) limit for authentic honey may be set with any desired degree of certainty; Table III indicates the confidence with which a honey of a certain $\delta^{13}C$ value may be considered pure. A sample with a value less negative than −21.5‰ can be classified as adulterated.

Screening Methods Developed

The carbon isotope ratio test is moderately expensive. Only a few laboratories possess the required instrumentation, and none is in the regulatory field. A need existed for more routine tests that could be conducted

Table III. Probability of $\delta^{13}C$ Value of Authentic Honey Sample Being Lower Than a Stated Limit

Probability of a sample lower than limit	(%)	Limit $\delta^{13}C$ (per mil, ‰)
5 of 6	84.1	−24.4
43 of 44	97.7	−23.4
769 of 770	99.87	−22.5
24 999 of 25 000	99.996	−21.5

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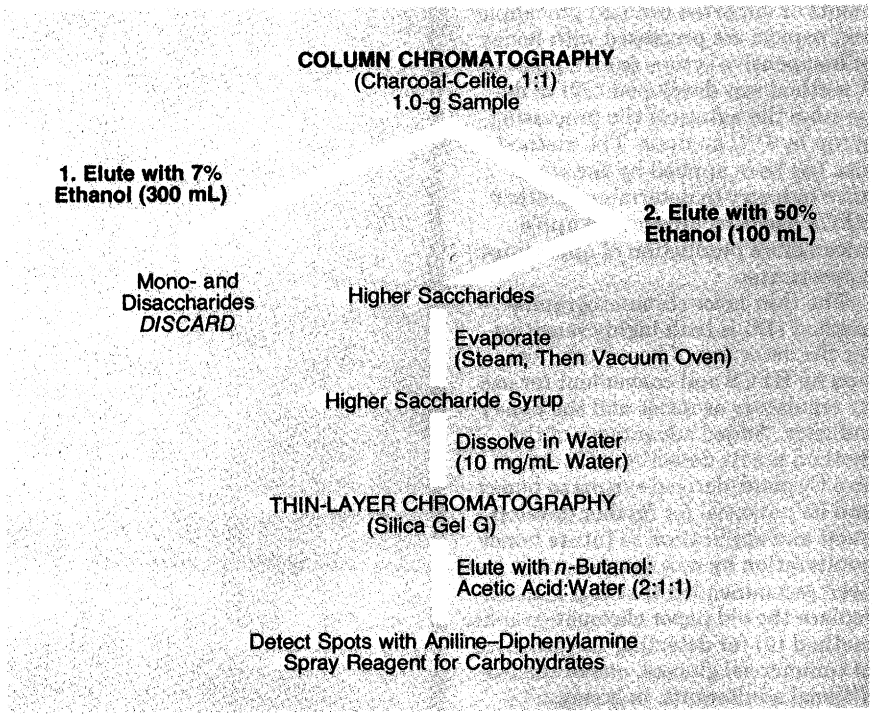

Figure 3. Thin-layer chromatographic test for honey adulteration

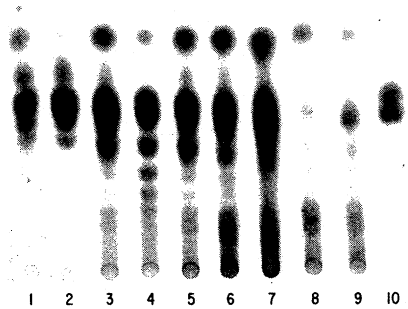

Figure 4. TLC plate of oligo- and polysaccharide fractions from honey, honey–HFCS mixtures, HFCS and standard trisaccharides

1, 2: Pure orange and clover honeys. 3–6: Mixtures of honey with 5, 10, 25, and 50% HFCS, respectively. 7: Mixture of honey with 5% conventional syrup. 8, 9: HFCS samples from two manufacturers. 10: Mixture of trisaccharides raffinose and melizitose

in ordinary laboratories for selecting samples sufficiently suspicious to justify the confirmatory isotope ratio test. Recently, we developed two such methods, one using thin-layer chromatography and the other gas-liquid chromatography.

The thin-layer chromatographic method (*19*) has been subjected to successful collaborative testing (*20*) and recommended for adoption as an official method of analysis. A flow diagram of the procedure is given in Figure 3. This very sensitive procedure involves isolation of a fraction containing oligo- and polysaccharides from both honey and HFCS by column chromatography on charcoal-Celite. After concentration, these fractions were examined by silica gel thin-layer chromatography; consistent differences between honey and HFCS fractions were revealed. Whereas pure honeys yielded only one or two blue-grey or blue-brown spots of R_f greater than 0.35, a series of spots or blue streaks extending from the origin characterized adulterated samples. The method detects HFCS and the traditional honey adulterants, even when present as 10% or less of the total mixture. An added advantage is that this procedure should detect in honey the presence of all starch-derived sugar syrups tested, regardless of the plant source. Figure 4 shows the differences in the chromatographic profiles of honey, HFCS, and adulterated mixtures. This procedure is being used routinely to screen samples, not only for HFCS but also for other adulterants of honey, including conventional corn syrup and inverted sucrose syrups.

A gas-liquid chromatographic method (*21*) based on the determination of maltose and isomaltose has been useful in our laboratory. However, in view of the small number of successful collaborative tests (*20*), it could not be recommended for adoption as an official method. The results of the maltose and isomaltose determinations in honey and in HFCS samples are given in Table IV. A discriminatory equation was developed from these data, and 81% of authentic honey samples and 78% of adulterated honey samples (as determined by $\delta^{13}C$ analysis) were correctly classified.

Table IV. Gas Chromatographic Determinations of Maltose and Isomaltose in Honey (80 U.S. Samples, 35 Imported Samples) and in HFCS (21 Samples)

	Maltose		Isomaltose	
	Mean (%)	SD	Mean (%)	SD
[illegible]	1.98	0.51	0.64	0.37
[illegible]	3.74	0.53	0.87	0.50
[illegible]	0.12	0.26	1.50	0.82

[illegible] Copyright 1979 Association of Official Analytical Chemists

Society Benefits

We are optimistic that awareness of these convenient new methods for detecting honey adulteration will minimize the threat to the integrity of honey markets. This will help protect the many thousands of beekeepers whose economic resources depend on confidence in the purity of their product. As a result, the population of honeybee colonies will be maintained at the high level so essential for the pollination of billions of dollars in food, feed, and fiber crops.

Since its advent about 30 years ago, isotope ratio mass spectrometry has been a powerful tool, particularly in the realm of basic research. Now it has been applied to a major problem for the food and agricultural industries. Undoubtedly, numerous applications will be forthcoming as more is learned regarding natural variations in $^{13}C/^{12}C$ ratios and ratios of other stable isotopes among plants and their derived products.

The adulteration of natural vanilla extract with synthetic vanillin has been revealed by $\delta^{13}C$ measurements (*12, 13*); detection will hopefully result in this practice being discouraged. More recently, U.S. Customs authorities have been confronted with the problem of determining whether ship-

ments of imported candied pineapple and papaya are processed with honey or inexpensive syrups from C_4 plants. A method was developed (*22*) to determine the nature of the processing syrup by $\delta^{13}C$ analysis. The method also has been applied by the apple juice industry to determine whether HFCS has been mixed with apple juice before production of apple juice concentrates.

The thin-layer chromatographic method (*19*) is both highly sensitive for the detection of honey adulteration by HFCS and convenient for use by regulatory agencies and the honey industry. Added advantages of this method are its detection of inexpensive C_3 plant-derived syrups in honey and its potential for further development and application to future honey adulteration by new sweeteners. It has been recommended that this method replace the old paper chromatographic method (*2*) for detecting the presence of commercial glucose, one of the traditional adulterants, in honey.

References

(1) *Chem. Eng. News,* **54** (17), 13 (1976).
(2) "Official Methods of Analysis," 12th ed., sections 31.134–31.136, 31.138–31.139, Association of Official Analytical Chemists, Washington, D.C., 1975.
(3) J. W. White, Jr., M. L. Riethof, M. H. Subers, and I. Kushnir, "Composition of American Honeys," Tech. Bull., U.S. Dept. Agric. No. 1261, 1962.
(4) L. W. Doner, *J. Sci. Food Agric.,* **28** (5), 443–56 (1977).
(5) R. S. Shallenberger, W. E. Guild, Jr., and R. A. Morse, *N.Y. Food Life Sci.,* **8** (3), 8–10 (1975).
(6) J. W. White, Jr., *Bee World,* **58** (1), 31–5 (1977).
(7) J. M. Newton and F. K. Wardrip, "Symposium: Sweeteners," G. E. Inglett, Ed., Chap. 8, pp 87–96, Avi Publ., Westport, Conn., 1974.
(8) J. W. White, Jr., and O. N. Rudyj, *J. Apic. Res.,* **17** (2), 89–93 (1978).
(9) L. W. Doner, *J. Agric. Food Chem.,* **26** (3), 707–10 (1978).
(10) A. Nissenbaum, A. Lifshitz, and Y. Stepek, *Lebensm. Wiss. Technol.,* **7,** 152–4 (1974).
(11) C. Hillaire-Marcel, O. Carro-Jost, and C. Jacob, *J. Inst. Can. Sci. Technol. Aliment.,* **10** (4), 333–5 (1977).
(12) J. Bricout and J. C. Fontes, *Ann. Falsif. Expert. Chim.,* **716,** 211–5 (1974).
(13) P. G. Hoffman and M. Salb, *J. Agric. Food Chem.,* in press, 1979.
(14) M. M. Bender, *Phytochemistry,* **10,** 1239–44 (1971).
(15) B. N. Smith and S. Epstein, *Plant Physiol.,* **47,** 380–4 (1971).
(16) L. W. Doner and J. W. White, Jr., *Science,* **197,** 891–2 (1977).
(17) J. W. White, Jr., and L. W. Doner, *J. Assoc. Off. Anal. Chem.,* **61** (3), 746–50 (1978).
(18) J. W. White, Jr., and L. W. Doner, *J. Apic. Res.,* **17** (2), 94–9 (1978).
(19) I. Kushnir, *J. Assoc. Off. Anal. Chem.,* in press, 1979.
(20) J. W. White, Jr., I. Kushnir, and L. W. Doner, *ibid.*
(21) L. W. Doner, J. W. White, Jr., and J. G. Phillips, *ibid.*
(22) L. W. Doner, D. Chia, and J. W. White, Jr., *ibid.*