High-Performance Liquid Chromatography Analysis of Nectar and Pollen of Strawberry Flowers

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The major sugars and free amino acids in eight strawberry cultivars were analyzed by high-performance liquid chromatography. The total proteins were determined with a Technicon autoanalyzer. The levels of fructose, glucose, and sucrose ranged from 1.54 to 3.96, 1.36 to 3.18, and 0.89 to 2.72 mg/mL, respectively, but the ratio of sucrose to fructose and glucose remained relatively stable (0.28–0.45). Essential amino acids in pollen ranged from 22.3 to 37.1% of total free amino acids content. The coefficient of variation of the individual free amino acids and proteins ranged from 26 to 86% among the cultivars. None of the variables analyzed could individually be related to the differential attractiveness of strawberry cultivars to honey bees.

Nectar and pollen are major rewards to insect pollinators. However, despite their entomological and botanical importance, relatively few chemical analyses of nectar and pollen of economic plants are available.

Wykes (1952) analyzed the composition of nectar in 80 plant species and found that the proportion of sugars tends to remain constant for any one species. Percival (1961) analyzed the nectar of 889 species of some 50 varieties of angiosperms by paper chromatography technique. She concluded that all nectars could be classified into sucrose dominant, balanced (with equal amounts of sucrose, glucose and fructose), and fructose and glucose dominant. She found that fructose- and glucose-dominant nectars seem to be associated with "open" flowers having unprotected nectars. Her research also confirmed Wykes (1952) conclusion that most species appear to have nectar of constant composition.

Baker and Baker (1975, 1979, 1981) extensively studied sugars, amino acids, and other chemicals of nectar in an attempt to understand the foraging behavior of insect pollinators. They postulated that there were similarities in sugar ratios between plants with the same pollinators, honey bees generally prefering nectar that is hexose rich or dominant (ratio of sucrose to hexose between 0.1 to 0.499 and less than 0.1, respectively).

Zauralow (1983) analyzed the composition of sugars in nectars of several apple and cherry cultivars. The results indicated that there was no significant variation in ratios of sugars between samples taken from the same cultivars. In contrast, levels of sugars in nectars from different cultivars varied significantly. In the case of apple tree cultivars, sucrose varied from 37.2 to 82.6%, glucose from 3.3 to 32.9%, and fructose from 6.5 to 46.6% total sugars. The study also suggested a positive correlation between the number of bee visits and concentration of sucrose in nectar.

Pollen is virtually the only source of essential amino acids in the diet of honey bees. DeGroot (1953) established that pollen contains all 10 essential amino acids (i.e., methionine, lysine, phenylalanine, isoleucine, tryptophan, arginine, histidine, leucine, threonine, and valine) required by honey bees. These amino acids account for about 40% of the total protein content of pollen. Proline is usually the major amino acid found in pollen, reaching levels as high as 1.65% in free form (Bathurst, 1954).

Analyzing 107 different pollen for free amino acids, Biederdorf et al. (1961) have shown that the maximum number of free amino acids, 19, occurred in Western cottonwood pollen and more than half of the species tested had more than 10 free amino acids.

Recently Gilliam et al. (1980) analyzed total amino acids and protein levels in pollen from flowers of nine citrus cultivars. Their results indicated that the levels of protein in pollen from different cultivars ranged from 6.2 to 20.7%. The percentage of individual amino acids was relatively constant, with a difference of less than 3% among nine cultivars. Pollen analyzed contained all the essential amino acids. Aspartic acid and glutamic acid were found in highest concentrations.

To our knowledge, no results of extensive chemical analysis of nectar and pollen of strawberry flowers are available. The only data are those by Shaw et al. (1954) who reported that on 3 consecutive days strawberry nectar contained 28, 36, and 26% total sugar and by Petkov (1965) who found that in different years an average of 0.6–0.8 mg of nectar of 26–30% total sugar concentration was secreted per flower.

Connor (1972) and Bagnara and Vincent (1988) observed that strawberry cultivars differ in their attractiveness toward insect pollinators. Therefore, the purpose of this study was to analyze and compare nectar and pollen of eight strawberry cultivars for their sugar, amino acid, and protein content by high-performance liquid chromatography (HPLC). Such information could be useful to understand the difference in attractiveness between strawberry cultivars and also to genetically engineer more attractive cultivars to pollinators.

MATERIALS AND METHODS

The nectars and pollen analyzed in this study were sampled from the following strawberry cultivars (*Fragaria* x *ananassa* Duch.): Catskill, Confitura, Elvira, Gorella, Korona, Redcoat, Scott, and Veestar. The plants were grown in the field at l'Acadie, Quebec (45° N, 18′ N:73° N, 20′ W). The first day of flowering, primary flowers were cut from the plant and transported immediately to the laboratory. Nectars were collected during the 1984, 1985 blooming periods, and pollen was collected during those of 1984–1986.

Preparation of Nectar Extracts. Due to storage problems with 1984 samples, only 1985 samples were analyzed. Nectar solutions were obtained by soaking 200 flowers of each cultivar in 400 mL of distilled water for 30 min. The solution was then filtered and stored at −20 °C. Prior to analysis, nectar samples were concentrated to 5 mL by the freeze-drying technique and preservative (25 mg of cycloheximide and 25 mg of chloramphenicol in 20 mL of distilled water) was added (0.1 mL/5 mL of

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extract). Sample cleanup was done on a 3-mL Sep-Pak C-18 disposible column (Waters) according to the procedure of Richmond et al. (1981). Prior to HPLC injection samples were filtered through an AcroLC13 0.45-μm filter pack (Gelman) to remove any particulate material present.

Preparation of Pollen Extracts. Since some of 1984 samples showed a high level of contamination with extraneous matter, additional samples were collected in the 1986 season for free amino acid and protein analysis. Pollen was collected by shaking 50 flowers of each cultivar into sterile petri dishes. The samples were then weighed and stored at −20 °C. Prior to analysis, pollen was finely ground in a mortar and a sample of approximately 2 mg was weighed. Extraction of free amino acids with simultaneous precipitation of proteins was done by adding 200 μL of 0.2 N perchloric acid to a 1-mL test tube containing pollen. The content of the test tube was vortexed and centrifuged (Beckman Accuspin FR) for 15 min at 2500 rpm. A 100-mL portion of clear supernatant was removed for HPLC analysis and filtered on Acrodisk 0.45-μm filter pack (Gelman), freeing the sample from precipitated proteins and other particulate material.

HPLC Analysis of Sugars. Sugar analysis of nectar was done on a Varian HPLC, Model Vista 5500, with a differential refractometer detector (Varian Series RI–3) and a Rheodyne injector with 10-μL sample loop. The detector signal was recorded on a Shimadzu C-R3A integrator. Varian MicroPak NH_2-10 column (30 cm × 4.5 mm) fitted with a NH_2-10 guard column (4 cm) was used with a mobile phase of acetonitrile and water (70:30) at 1.7 mL/min.

Standard solutions of fructose, glucose, and sucrose were prepared with Aldrich high-purity standards. The concentrations prepared ranged from 0.02 to 0.1%, which corresponded to the concentration range of sugars in sample solutions. Peak height measurements were used to quantify sugars in nectar, and linear regression equations were established for each compound. In addition, nectar samples were screened for less frequently occurring sugars such as maltose, galactose, raffinose, and melezitose. A mobile phase of acetonitrile and water (72:28) was used to enhance the separation of these sugars. The reproducibility of the analytical method was assured by analyzing each sample in triplicate. Concentration of sugars was expressed as milligrams per milliliter of nectar solution since the volume of collected nectar was unknown.

HPLC Analysis of Amino Acids. Free amino acid analysis was performed on Varian HPLC, Model Vista 5500, with a UV-200 detector and a Rheodyne injector with 10-μL loop. All amino acids were derivatized with ninhydrin reagent (Blackburn, 1968) and detected at 570 nm for primary amino acids and 440 nm for secondary amino acids. The ninhydrin derivatization system was composed of a PCR pump and a stainless steel reaction coil heated in a block to 125 °C. A Varian MicroPak amino acid column (15 cm × 4 mm), 9-μm poly(styrene-divinylbenzene) in sodium form, was used for separation of amino acids. The separation was optimized by using two buffers in a continous gradient. Buffer 1 was 0.2 M sodium citrate (pH 3.25), and buffer 2 was 1.0 M sodium citrate (pH 7.4). To obtain a satisfactory separation of all 18 amino acids, a modified Varian (1981) procedure was used. The programs controlling the pump and detector conditions are presented in Table I. Pierce standard containing 18 amino acids from protein hydrolysate at 2.5 μM/mL (except for 2-cystine at 1.25 μM/mL) was diluted with 0.1 N HCl to a working amino acid standard of 0.063 or 0.125 μM/mL, depending on concentration of amino acids in analyzed sample. In order to eliminate variations due to instrument response and extracting efficiency among the samples, an internal standard was used for quantification of amino acid levels. Concentration of internal standard, norleucine, was 0.2 μM/mL both in standard and in sample extracts. Amino acids were identified by comparing their retention times in the sample to the one in the standard. Quantification was done by comparing the peak height of each amino acid in standard to the one in the sample with adjustment for the internal standard factor (IS factor = norleucine peak height in standard/norleucine peak height in sample). Duplicate analyses of each sample were carried out.

Table I. HPLC Procedures Used To Analyze Amino Acids

		pump program			
time, min	wavelength, nm	buffer 1, %	buffer 2, %	0.2 N NaOH, %	flow, mL/min
	Program 1: Total Time 10 min at 40 °C				
0	570	100	0	0	0.3
8	570	100	0	0	0.3
12	570	94	0	6	0.3
	Program 2: Total Time 50 min at 70 °C				
0	570	94	0	6	0.3
6	440				
8	570	82	0	18	0.3
12	570	70	0	30	0.3
12.1	570	0	100	0	0.3
45	570	0	100	0	0.3
46	570	0	50	50	0.3
47	570	100	0	0	0.3

Technicon Analysis of Protein. Proteins in pollen samples were determined as total nitrogen by using a Technicon block digestor and automatic Technicon autoanalyzer II C plus continuous flow system. Digestion of pollen samples was based on the slightly modified method of Isaak and Johnson (1976). A 0.3-mL portion of digestion mixture (1.5% Se in H_2SO_4) was added to each sample in a 2-mL heating vial and left overnight for predigestion. Then, 1.5 mL of 30% H_2O_2 solution was added dropwise and the sample was boiled for 1 h at 370 °C in a block heater. The cooled digest was made up to 2 mL with distilled water and the nitrogen content determined with a Technicon autoanalyzer. Determination of nitrogen was based on a colorimetric method (Technicon Instruments Co., 1977) in which ammonia salicylate complex (formed in the reaction of ammonia, sodium nitroprusside, and sodium hypochlorite) was buffered at pH 12.8–13 and then read at 660 nm. All samples were analyzed in duplicate. The accuracy of the analytical method was assured by analyzing NBS standard (citrus leaves) whose nitrogen content is 2.86%. The crude protein content in pollen samples was calculated with the Kjeldahl factor of 5.6 as recommended by Rabie et al. (1983).

RESULTS AND DISCUSSION

Fructose, glucose, and sucrose were identified in all the nectar samples. The linear regression equations determined for the standards in the 0.02–0.1% range were highly significant, having correlation coefficients (peak area vs amount) of 0.999, 0.999, and 0.998 for fructose, glucose, and sucrose, respectively. Analysis of nectar for the less common sugars, such as galactose, maltose, raffinose, and melezitose (Percival, 1961), gave negative results. The results of quantitative determinations of sugars are shown in Table II. Typical chromatograms of sugars in the standard and a nectar sample are presented in Figures 1 and 2. The separation between the sugars was complete, facilitating accurate quantification. Under these chro-

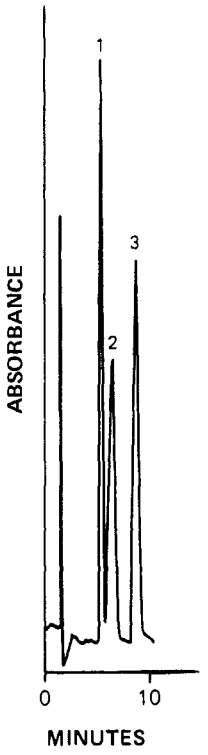

Figure 1. Chromatogram of sugars in standard solution: (1) fructose, (2) glucose, (3) sucrose. HPLC conditions: MicroPak NH_2-10 column (30 cm × 4.5 mm); mobile phase acetonitrile–water (70:30); flow rate 1.7 mL/min, attenuation 3×.

Table II. Sugar Content in the Nectar of Strawberry Flowers

	sugar content,[a] mg/mL				
cultivar	fructose	glucose	sucrose	total	S/F + G
Catskill	2.65	1.97	1.49	6.11	0.32
Confitura	3.96	3.18	2.72	9.86	0.38
Elvira	2.41	2.41	1.92	6.73	0.40
Gorella	2.64	0.92	1.04	4.60	0.29
Korona	1.54	1.36	0.89	3.79	0.31
Redcoat	2.15	2.48	1.29	5.92	0.28
Scott	2.57	2.78	2.18	7.53	0.41
Veestar	2.01	2.19	1.88	6.06	0.45
mean	2.49	2.16	1.68	6.33	0.36
CV,[b] %	28.3	34.1	36.7	29.2	17.8

[a] Mean value, triplicate analysis. [b] Coefficient of variation.

matographic conditions the detection limit for the sugars was 20 μg/mL. The concentration of fructose ranged from 1.54 to 3.96 mg/mL, glucose from 0.92 to 3.18 mg/mL, and sucrose from 0.89 to 2.72 mg/mL, which agree with Zauralow's (1983) findings. The total sugar concentration also varied significantly: 3.79–9.86 mg/mL. Cultivar Confitura showed the highest level of individual sugars, while Korona contained the lowest level of fructose and sucrose and Gorella the lowest levels of glucose. Although the level of individual sugars varied, the ratio of sucrose to fructose and glucose for each nectar remained relatively constant. It ranged from 0.29 to 0.45, thus qualifying all the analyzed nectars as hexose rich. The above findings confirmed the results of Wykes (1952), indicating that nectar of any one species would tend to have a constant proportion of sugars and the results of Baker and Baker (1979) who postulated that honey bees, the major pollinator of strawberry plants, prefer hexose-rich nectar. Differences in the cultivar attractiveness of floral nectar are known to occur (Stith, 1970; Butler et al., 1972; Zauralow, 1983). Zauralow (1983) reported a positive correlation between the number of bee

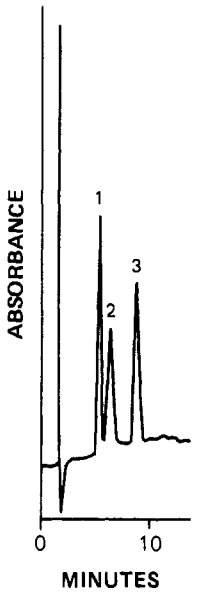

Figure 2. Chromatogram of sugars in strawberry nectar solution: (1) fructose, (2) glucose, (3) sucrose. HPLC conditions: MicroPak NH_2-10 column (30 cm × 4.5 mm); mobile phase acetonitrile–water (70:30); flow rate 1.7 mL/min, attenuation 3×.

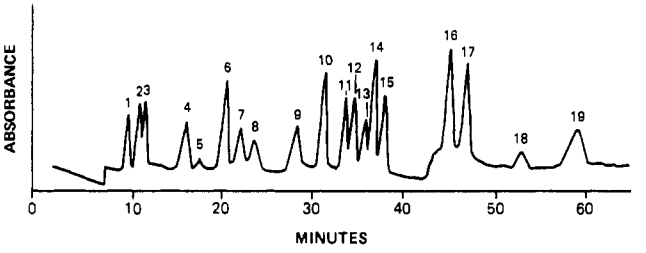

Figure 3. Chromatogram of amino acid standard solutions: (1) asparagine, (2) threonine, (3) serine, (4) glutamine, (5) proline, (6) glycine, (7) alanine, (8) cystine, (9) valine, (10) methionine, (11) isoleucine, (12) leucine, (13) norleucine, (14) tyrosine, (15) phenylanaline, (16) lysine, (17) histidine, (18) arginine. HPLC conditions: MicroPak column (15 cm × 4 mm); mobile phase buffer A (0.2 M sodium citrate, pH 3.25), buffer B (1.0 M sodium citrate, pH 7.4); flow rate 0.3 mL/min; detection wavelength 570 nm (primary amino acids), 440 nm (secondary amino acids).

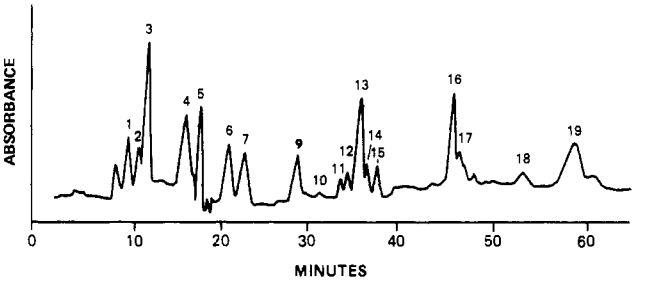

Figure 4. Chromatogram of amino acids in strawberry pollen solution: (1) asparagine, (2) threonine, (3) serine, (4) glutamine, (5) proline, (6) glycine, (7) alanine, (9) valine, (10) methionine, (11) isoleucine, (12) leucine, (13) norleucine, (14) tyrosine, (15) phenylalanine, (16) lysine, (17) histidine, (18) arginine. HPLC conditions: MicroPak column (15 cm × 4 mm); mobile phase buffer A (0.2 M sodium citrate, pH 3.25), buffer B (1.0 M sodium citrate, pH 7.4); flow rate 0.3 mL/min; detection wavelength 570 nm (primary amino acids), 440 nm (secondary amino acids).

visits and sucrose concentration in nectar of several varietes of apple and cherry trees. Although some cultivars in our study had been found to be more attractive to honey bees than others (Bagnara and Vincent, 1988), we found no direct correlation between quantitative sugar ratios in

nectar and cultivar attractiveness to the pollinators.

The typical calibration and sample chromatograms of amino acids presented in Figures 3 and 4 show that all amino acids were adequately separated. Since the mechanism of separation on the ion-exchange column is based on ionic interaction with a strongly acidic support, the acidic amino acids eluted first, followed by neutral and finally basic amino acids. This complex separation was effected by an increase in the elution buffer pH (from 3.25 to 7.40) and ionic strength (from 0.2 to 1.0 M). The wavelength change for proline was done automatically. Since time and resolution varied slightly for proline and glutamic acid from week to week, the timing for the wavelength changes was adjusted weekly. Also the concentration of internal standard, norleucine, was adjusted to 0.2 μM/mL so as not to affect the resolution of the adjacent amino acids. Under these chromatographic conditions the estimated limit of detection for the amino acids was 0.005 μM/mL. The time of the chromatographic run was approximately 55 min, which is typical for the ninhydrin derivatization system.

Table III presents the average content of free amino acids and protein in pollen over 2 consecutive years. The levels of individual amino acids differed by less than 10% CV between the duplicate results for the same year, but the differences were higher (less than 20% CV) between the results for 2 consecutive years. These variations could probably be attributed to several factors such as weather conditions, degree of contamination of pollen with extraneous matter, and the source of pollen.

The total free amino acids content varied from cultivar to cultivar (Table III), ranging from 37.9 μM/mg for Gorella to 105 μM/mg for Confitura. The percent essential amino acids present (de Groot, 1953), based on total amino acid content, was also reported since these amino acids play an important role in bee nutrition. They range from 22.3% for Korona to 37.1% for Catskill. All the pollen analyzed contained all the amino acids present in protein hydrolysate used as standard, except for cysteine. Proline was the major amino acid detected, as usually reported in studies dealing with other plant species (Bathurst, 1954). However, honey bees can synthesize proline from glucose (Lue and Dixon, 1967), and hence it is not essential in their diet. Tryptophan, one of the essential amino acids, was not detected. This amino acid is seldom limiting in the diet of animals, so it is not routinely determined (Gilliam et al., 1980). The concentrations of individual amino acids varied considerably among cultivars analyzed (Table III), ranging from 26% CV for histidine to 86% CV for glycine. Our results differed from those of Gilliam et al. (1980) who found the concentration of individual amino acids in nine citrus cultivars to be relatively constant. The results also show the consistently high concentrations of all the amino acids in pollen of Redcoat and Confitura. According to Stanley and Linskens (1974), concentration of all amino acids is considerably higher in the bound than free fraction. However, their distribution in both fractions tends to follow the same pattern. Therefore, on the basis of results obtained, an estimate can be obtained of the amino acid content in protein of pollen.

The results of pollen analysis for protein are also presented in Table III. The mean recovery of protein nitrogen from NBS standard (105.8%) confirmed the accuracy of reported results. The mean protein content of pollen over 2 consecutive years was 32% total weight, which is within the range reported by Barbier (1970) for pollen. The percent of protein varied among the cultivars (28% CV), with Redcoat containing the highest level (42.4%) and

Table III. Free Amino Acid and Protein Content in Pollen of Strawberry Flowers

	amino acid content,[a] mM/mg																			
	essential amino acids								other amino acids											
cultivar	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Ala	Asp	Cys	Glu	Gly	Pro	Ser	Tyr	Val	total	% EAA[b]	% protein[a,c]
Catskill	2.04	3.75	1.22	1.06	4.75	0.42	0.36	2.29	2.37	1.22	ND[d]	1.32	0.97	17.6	6.96	0.68	2.19	49.2	37.1	23.4
Confitura	2.66	4.68	1.22	1.41	6.85	1.46	1.79	3.71	8.18	4.04	ND	5.60	3.19	45.5	10.1	1.33	3.61	105	30.4	22.9
Elvira	2.49	2.92	1.04	0.82	4.18	0.42	1.66	1.63	7.12	2.50	ND	3.26	2.70	38.8	5.78	0.84	2.33	78.5	28.4	36.2
Gorella	1.24	1.82	0.78	0.82	2.33	N.D.	0.51	1.11	2.88	1.80	ND	1.79	1.18	10.4	9.37	0.50	1.38	37.9	30.3	40.7
Korona	1.12	4.17	1.15	1.39	4.05	0.24	1.74	4.10	4.61	4.82	ND	6.03	2.86	47.0	13.3	1.32	3.44	101	22.3	36.9
Redcoat	1.77	4.88	1.88	1.80	6.35	0.33	2.70	4.52	6.45	4.62	ND	5.58	10.1	28.5	15.9	2.21	4.52	102	30.1	42.4
Scott	2.19	4.14	0.95	1.36	5.11	0.61	1.14	3.17	5.15	2.82	ND	3.36	1.93	38.3	12.3	1.30	2.14	85.9	27.7	39.7
Veestar	2.13	3.82	1.13	1.24	4.60	0.31	1.48	1.93	5.47	3.77	ND	5.50	4.12	24.4	10.8	1.35	2.66	74.7	29.5	19.6
mean	1.96	3.77	1.17	1.24	4.78	0.54	1.42	2.81	5.28	3.20		4.06	3.38	31.3	10.6	1.19	2.78	79.4	29.5	32.7
CV,[c] %	28	26	28	27	29	78	53	44	38	41		46	86	42	31	45	36	31	14	28

[a] Mean value, duplicate analyses and two seasons. [b] Essential amino acids, percent of the total free amino acids. [c] Percent of total pollen weight. [d] Not detected. [e] Coefficient of variation.

Veestar the lowest (19.6%). However, the values of protein content may have been overestimated because of the presence of significant levels of free amino acids and nucleic acid in pollen (Stanley and Linskens, 1974). In fact, the use of a Kjeldahl nitrogen factor of 5.6 to estimate the protein content assumes that there is a minimal level of nonprotein nitrogen in pollen.

It was found for the first time that nectar and pollen of eight strawberry cultivars differ in their sugar and free amino acid contents. None of these variables was individually related to the differential attractiveness of the strawberry cultivars involved (Bagnara and Vincent, 1988). More analysis is to be done, notably on amino acids of the nectar and volatile compounds of strawberry flowers, to attempt to understand the behavior of pollinators visiting strawberry blossoms.

ACKNOWLEDGMENT

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Registry No. Arg, 74-79-3; His, 71-00-1; Ile, 73-32-5; Leu, 61-90-5; Lys, 56-87-1; Met, 63-68-3; Phe, 63-91-2; Thr, 72-19-5; Ala, 56-41-7; Asp, 56-84-8; Glu, 56-86-0; Gly, 56-40-6; Pro, 147-85-3; Ser, 56-45-1; Tyr, 60-18-4; Val, 72-18-4; fructose, 57-48-7; glucose, 50-99-7; sucrose, 57-50-1.

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