Analysis and Quantitation of the Carbohydrates in Honey Using High-Performance Liquid Chromatography

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Carbohydrase enzymes present in honey catalyze transglucosylation reactions which result in the formation of over 20 structurally similar oligosaccharides. These oligosaccharides make up a small percentage of the total carbohydrates in honey. The combination of these two facts makes the analysis and quantitation of these minor oligosaccharides difficult. Methodology was developed using high-performance liquid chromatography to qualitatively and quantitatively analyze 20 of the minor oligosaccharides present in four unifloral Canadian honeys.

INTRODUCTION

Honey is one of the most complex mixtures of carbohydrates produced in nature. The major carbohydrates in honey are glucose and fructose, which account for 65–75% of the total soluble solids in honey and 85–95% of honey carbohydrates (White, 1979). The remaining carbohydrates are a mixture of at least 11 disaccharides (Siddiqui and Furgala, 1967; Siddiqui, 1970; Low and Sporns, 1988), 11 trisaccharides (White and Hoban, 1959; Siddiqui and Furgala, 1968), and several higher oligosaccharides.

Several methods have been attempted to analyze the total carbohydrate profile of honey. These include a variety of chromatographic methods such as paper chromatography (Partridge, 1949), thin-layer chromatography (Tate and Bishop, 1962), gas–liquid chromatography (GLC) (Echigo, 1970), and high-performance liquid chromatography (HPLC) [for a review, see Honda (1984)]. All of these methods can be utilized to separate and quantitate the major carbohydrates in honey; however, the analysis of the more complex minor carbohydrates has proven much more difficult.

The method of choice for the analysis of structurally similar carbohydrates such as those present in honey is GLC. GLC analysis of carbohydrates is very popular due to the high sensitivity obtained by the flame ionization detector (FID). With FID, detection limits for oligosaccharides are in the order of 40 ppb (Low and Sporns, 1988). There are, however, two main drawbacks with the use of GLC to analyze carbohydrates: (1) The sugars have to be in a single tautomeric form or multiple peaks will occur from one sugar. (2) Since carbohydrates are not volatile, they must be derivatized before GLC analysis. The most common method of derivatizing carbohydrates for GLC analysis was developed by Sweeley et al. (1963) using trimethylchlorosilane and hexamethyldisilazane.

Due to these problems, the use of HPLC for carbohydrate analysis has received a great deal of attention. Relatively little sample preparation is required, and the carbohydrates can be isolated after separation for further analysis. Gel permeation (Whistler and Anisuzzaman, 1980), reverse-phase (McGinnis et al., 1986), and silica (Linden and Lawhead, 1975) columns have been used for HPLC analysis of carbohydrates, but the most popular are amino-bonded silica (Truong Van Den et al., 1986) and

fixed-ion resin (Thomas and Lobel, 1976) columns. The amino-bonded silica columns use acetonitrile/water as the mobile phase and are probably the most common resin used today for the separation of carbohydrates. However, these stationary phases suffer from poor resolution of structurally similar carbohydrates and loss of amino groups due to cleavage of the silyl ether bonds (Shaw, 1988). The fixed-ion resins use water as the mobile phase, but also suffer from poor resolution of structurally similar carbohydrates. In addition, these stationary phases are less efficient unless elevated column temperatures are used and are highly compressible due to low cross-linking.

Separation and detection of the minor oligosaccharides in honey by HPLC is difficult for two main reasons. The first problem is due to oligosaccharides with structural similarities. Siddiqui and Furgala (1967, 1968) found that the oligosaccharides present in honey comprised mainly disaccharides which are either glucose–glucose or glucose–fructose linked. The other major problem is that the concentration of the minor oligosaccharides in honey is low, making detection difficult. To some degree, detection problems have been alleviated by the development of new detectors for HPLC, such as mass detectors (MacRae and Dick, 1981) and electrochemical detectors (Hughes and Johnson, 1982), and design improvements in refractive index (RI) detectors. However, detection limit problems still exist which cannot be alleviated by simply concentrating the honey sample. Increasing the concentration of honey not only increases the concentration of these oligosaccharides but also results in a dramatic increase in glucose and fructose which swamp the active sites in the column, inhibiting oligosaccharide analysis.

A relatively new method of carbohydrate separation was introduced by Rocklin and Pohl (1983) using anion-exchange chromatography. By use of this method of separation, carbohydrates are ionized in a high pH eluant and separation occurs due to variations in pK_a values of the carbohydrates in the alkaline mobile phase. These stationary phases achieve remarkably good resolution of structurally similar carbohydrates using simple aqueous buffers.

In this paper, we introduce methodology employing HPLC to separate 20 structurally similar carbohydrates. Using anion-exchange chromatography in conjunction with a pulsed amperometric detector, we qualitatively and quantitatively analyzed the minor oligosaccharides present in four honeys of known botanical origin.

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Table I. Retention Time (T_r), Response Factor (RF), and Weight Percent of the Carbohydrates in Alfalfa, Alsike, Canola, and Trefoil Honey Samples (Average of Three Replicates)

peak	carbohydrate	T_r, min	RF	wt % Alfalfa	wt % Alsike	wt % Canola	wt % Trefoil
1	neotrehalose	7.8 ± 0.3	1.19	trace	trace	trace	trace
2	glucose	13.7 ± 0.1	2.55	33.9 ± 0.4	35.6 ± 0.4	40.0 ± 0.4	33.2 ± 0.4
3	fructose	15.5 ± 0.1	2.56	37.0 ± 0.4	38.5 ± 0.4	36.2 ± 0.4	37.1 ± 0.4
4	melibiose	19.9 ± 0.1	1.07	ND[a]	ND	ND	ND
5[b]	isomaltose maltulose	24.9 ± 0.3	0.52	0.30 ± 0.01	0.31 ± 0.01	0.12 ± 0.01	0.35 ± 0.01
6	sucrose	28.2 ± 0.4	1.09	1.18 ± 0.09	0.90 ± 0.04	0.046 ± 0.008	0.55 ± 0.03
7	kojibiose	30.0 ± 0.4	0.92	0.36 ± 0.01	0.36 ± 0.05	0.37 ± 0.05	0.49 ± 0.02
8[c]	turanose gentiobiose	37.9 ± 0.4	1.04 4.29				
9	palatinose	39.3 ± 0.4	3.49	0.067 ± 0.001	trace	trace	trace
10	melezitose	43.2 ± 0.5	0.93	0.038 ± 0.002	0.056 ± 0.003	0.041 ± 0.001	0.047 ± 0.001
11	isomaltotriose	47.6 ± 0.5	1.06	0.038 ± 0.002	0.028 ± 0.005	trace	trace
12	nigerose	51.2 ± 0.4	2.14	0.30 ± 0.01	0.34 ± 0.02	0.23 ± 0.01	0.40 ± 0.01
13[d]	maltose 1-kestose	53.2 ± 0.5	1.32	1.18 ± 0.05	1.00 ± 0.02	0.76 ± 0.05	1.11 ± 0.04
14	theanderose	55.4 ± 0.6	0.92	0.38 ± 0.04	0.34 ± 0.01	0.067 ± 0.011	0.103 ± 0.001
15	laminaribiose	57.2 ± 0.7	3.10	0.10 ± 0.01	trace	0.053 ± 0.002	0.132 ± 0.005
16	isopanose	60.3 ± 0.5	1.23	ND	0.10 ± 0.01	ND	trace
17	erlose	61.4 ± 0.4	0.63	3.43 ± 0.20	3.97 ± 0.22	0.26 ± 0.01	2.86 ± 0.20
18	panose	63.9 ± 0.5	0.86	0.080 ± 0.003	trace	0.025 ± 0.002	0.090 ± 0.003
19	maltotriose	66.4 ± 0.6	1.35	0.10 ± 0.01	0.087 ± 0.011	0.023 ± 0.001	0.059 ± 0.004
20	laminaritriose	71.1 ± 0.5	3.10	trace	ND	ND	ND

[a] ND, not detected. [b] Calculated as isomaltose. [c] Concentration of turanose and gentiobiose varies in honey and therefore cannot be calculated. [d] Calculated as maltose.

MATERIALS AND METHODS

The honey carbohydrate standards were obtained from the following sources: D-glucose and sucrose (α-D-glucopyranosyl β-D-fructofuranoside) were obtained from BDH Chemicals. D-Fructose was obtained from Aldrich Chemical Co., Inc. Kojibiose [O-α-D-glucopyranosyl-(1→2)-D-glucopyranose] was obtained from Koch-Light Laboratories Ltd. Neotrehalose (α-D-glucopyranosyl β-D-glucopyranoside) was a gift from Dr. I. R. Siddiqui, Agriculture Canada. Laminaribiose [O-β-D-glucopyranosyl-(1→3)-D-glucopyranose] and laminaritriose [O-β-D-glucopyranosyl-(1→3)-O-β-D-glucopyranosyl-(1→3)-D-glucopyranose] were gifts from Dr. E. Reese of the U.S. Army Natick Research and Development Laboratories. Maltulose [O-α-D-glucopyranosyl-(1→4)-D-fructose] and nigerose [O-α-D-glucopyranosyl-(1→3)-D-glucopyranose] were synthesized (Hicks et al., 1983) in our laboratory. Palatinose [O-α-D-glucopyranosyl-(1→6)-D-fructose], gentiobiose [O-β-D-glucopyranosyl-(1→6)-glucopyranose], isomaltose [O-α-D-glucopyranosyl-(1→6)-D-glucopyranose], melibiose [O-α-D-galactopyranosyl-(1→6)-D-glucopyranose], maltose [O-α-D-glucopyranosyl-(1→4)-D-glucopyranose], turanose [O-α-D-glucopyranosyl-(1→3)-D-fructose], maltotriose [O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucopyranose], isomaltotriose [O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→6)-D-glucopyranose], and melezitose [O-α-D-glucopyranosyl-(1→3)-O-β-D-fructofuranosyl-(2→1) α-D-glucopyranoside] were obtained from Sigma Chemical Co. 1-Kestose [O-α-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(1→2) β-D-fructofuranoside], erlose [O-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl β-D-fructofuranoside], and theanderose [O-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl β-D-fructofuranoside] were gifts from Dr. S. Chiba, Department of Agriculture, Hokkaido University. Panose [O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→4)-D-glucopyranose] and isopanose [O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→6)-D-glucopyranose] were synthesized (Wolfram and Koizumi, 1967) in our laboratory.

Alfalfa, Alsike, Canola, and Trefoil honey samples were obtained in the following manner. Four-frame nuclei with 1 kg of bees, a queen, 1 frame of brood and 3 empty combs were placed in the middle of a field with a minimum of 8 ha (20 acres). All the floral sources were in full bloom during the honey collection. The frames were collected 7–10 days later, and the honey was extracted by using a 2-frame hand extractor. Samples were stored at −20 °C until required for analysis. Botanical or-

Table II. Relative Percentages (w/w) of Glucose, Fructose, Total Oligosaccharides and H_2O in Alfalfa, Alsike, Canola, and Trefoil Honey Samples

honey	% glucose[a]	% fructose[a]	% oligosaccharides	% H_2O[a]
Alfalfa	33.9 ± 0.4	37.0 ± 0.4	7.5	19.6 ± 0.6
Alsike	35.6 ± 0.4	38.5 ± 0.4	7.5	17.1 ± 0.6
Canola	40.0 ± 0.4	36.2 ± 0.4	2.0	18.9 ± 0.6
Trefoil	33.2 ± 0.4	37.1 ± 0.4	6.2	21.9 ± 0.6

[a] Average of three replicates.

igin of each honey was confirmed by pollen analysis (Low et al., 1989).

Sample Preparation. In preparation for oligosaccharide analysis, 1 g of each honey was dissolved in 19.0 g of HPLC grade H_2O and passed through a C-18 Sep-Pak cartridge (Waters Associates) and 3 cm³ of AG 501-X8 mixed bed resin, 20–50 mesh (Bio-Rad Laboratories). The samples were then passed through 3 cm³ of AG 1-X4 anion-exchange resin, 100–200 mesh, (Bio-Rad) to remove organic acids. The monosaccharides were removed from the honey by a procedure modified from Whistler and Durso (1950). The samples were stirred with 4.0 g of activated charcoal, 50–200 mesh (Fisher Scientific Co.) for 17 h at 4 °C. After mixing, the samples were placed on a 3.0 cm diameter column containing 4.0 g of 50/50 (w/w) mix of activated charcoal and Celite (Fisher). Approximately 99% of the monosaccharides were removed from the column by washing with 1 L of 0.1% (v/v) ethanol at room temperature at a flow rate of 10.0 mL/min. The remaining oligosaccharides were eluted from the column with 500 mL of a 60 °C solution of 50% (v/v) ethanol at the same flow rate. The filtrate was frozen at −70 °C and dried at 30 °C in a Lab Con Co freeze dryer (Freeze Dry 5). Ten milliliters of deionized water was added to the lyophilized honey samples. The samples were stored at −20 °C until required for analysis. Sample preparation for glucose and fructose quantitation was achieved by simple dilution of each honey with deionized water. All samples were passed through a 0.2-μm nylon 66 filter (Rainin Instrument Co.) to remove particulate matter. Ninhydrin and Bradford tests were carried out on the samples to ensure that no amino acids or proteins were present.

To ensure that no loss of oligosaccharides had occurred during the removal of glucose and fructose, a solution containing sucrose, maltose, and maltotriose was placed on the charcoal/Celite column. The column was washed with 0.1% ethanol at ambient temperature and 50% ethanol at 60 °C as described
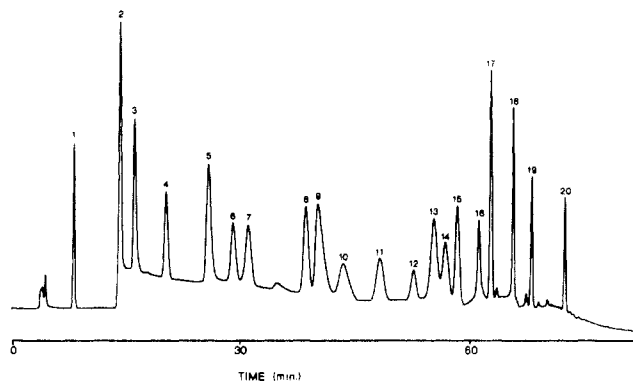
Figure 1. HPLC chromatogram of the 23 honey carbohydrate standards (see Table I for a list of the carbohydrates).

earlier, and both eluates were saved. HPLC analysis of the eluates revealed that greater than 99% of the sucrose, maltose, and maltotriose was recovered during the charcoal/Celite treatment.

HPLC Analysis of the Carbohydrates in Honey. The resulting samples were analyzed on a Dionex Bio LC 4000 gradient HPLC containing a 50-μL sample loop. Separation of the carbohydrates was carried out on two Dionex 10-μm Carbo Pac PA1 pellicular anion-exchange columns (4 × 250 mm) connected in series. The flow rate was 0.70 mL/min, and the carbohydrates were detected by a PAD (pulsed amperometric detector) with a gold electrode and triple pulsed amperometry at a sensitivity of 10K. The electrode was maintained at the following potentials and durations: $E_1 = 0.05$ V ($t_1 = 120$ ms); $E_2 = 0.80$ V ($t_2 = 120$ ms); $E_3 = -0.60$ V ($t_3 = 420$ ms). A postcolumn delivery system of 0.3 M sodium hydroxide (NaOH) at a flow rate of 0.80 mL/min was used to prevent base-line drift. The following gradient elution was used to achieve separation of the oligosaccharides: 0.1 M NaOH for 4 min; after 20 min, the mobile phase was 0.1 M NaOH and 0.03 M sodium acetate (NaOAc); after 50 min, the mobile phase was 0.1 M NaOH and 0.1 M NaOAc. This eluant was held for 10 min before a 0.3 M NaOH wash was used to remove the acetate ions from the columns. Following the wash step, the columns were reequilibrated with 0.1 M NaOH in preparation for the next injection. The carbohydrates eluting from the columns were plotted by a Spectra Physics Model 4290 integrator. Triplicate injections of all samples were carried out to ensure correct integration of the carbohydrates.

RESULTS AND DISCUSSION

The vast majority of nectars contain exclusively the sugars glucose, fructose, and sucrose (Baker and Baker, 1983). Research has shown that the minor oligosaccharides in honey arise from the transglucosylation activity of α- and β-glucosidases contributed by honeybees (White and Maher, 1953; Low et al., 1986). These carbohydrases transfer glucose moieties to other carbohydrates during the hydrolysis of sucrose, resulting in the formation of many complex oligosaccharides.

Twenty-three carbohydrates previously identified in honey (Siddiqui and Furgala, 1967, 1968; Siddiqui, 1970; Low and Sporns, 1988) were pooled and analyzed by HPLC. The HPLC chromatogram of these standard carbohydrates is shown in Figure 1. By use of our methodology, 20 of the 23 honey carbohydrate standards were resolved. Each standard was injected separately, and the standard mixture was spiked with each individual carbohydrate to determine the response factor and confirm the identity of each oligosaccharide. Coelution of the carbohydrates isomaltose/maltulose (peak 5), turanose/gentiobiose (peak 8), and maltose/1-kestose (peak 13) occurred during the optimal conditions developed for oligosaccharide separation.

Table I lists the retention time (T_r), response factor (RF), and weight percent of the carbohydrates identified in each
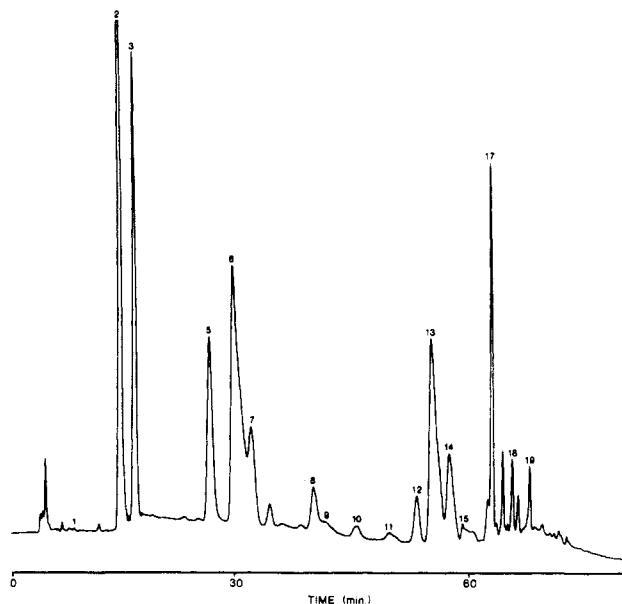
Figure 2. HPLC chromatogram of the carbohydrates in Alfalfa honey (see Table I for a list of the carbohydrates).
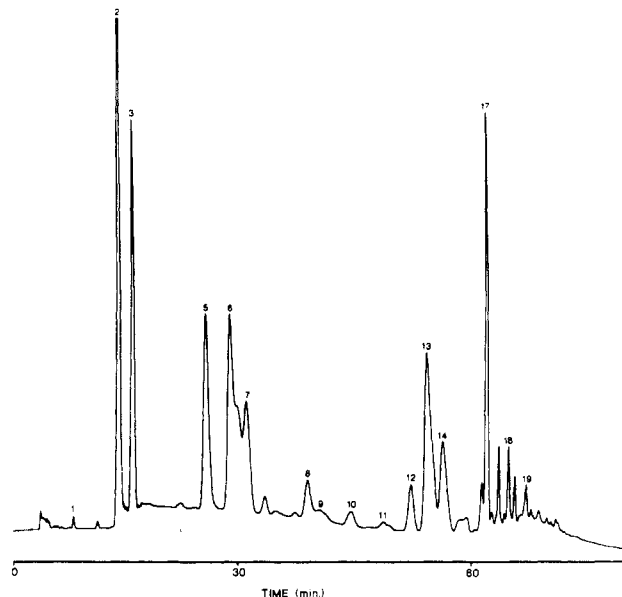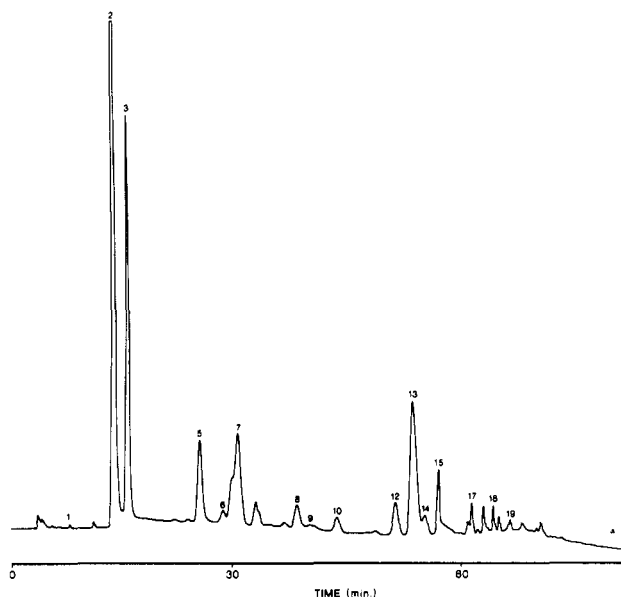

Figure 3. HPLC chromatogram of the carbohydrates in Alsike honey (see Table I for a list of the carbohydrates).

of the honey samples analyzed. The concentration of the carbohydrates was determined by using response factors calculated from the carbohydrate standards. Estimation of the concentration of isomaltose (peak 5) and maltose (peak 13) in honey can be achieved since the amount of maltulose and 1-kestose in honey is small relative to isomaltose and maltose (Low and Sporns, 1988; Siddiqui and Furgala, 1967, 1968). Quantitation of either turanose or gentiobiose (peak 8) could not be accomplished with this elution program.

The glucose and fructose concentration for each honey was determined by HPLC employing untreated (without charcoal/Celite chromatography) honey samples (Table II). In addition, the moisture content of each honey was determined by using the Karl Fischer titration method. The results from these analyses fall within the range of the 490 floral honeys analyzed by White et al. (1962).

Figures 2-5 are HPLC chromatograms of the carbohydrates in Alfalfa, Alsike, Canola, and Trefoil honey samples, respectively, following removal of >99% of the

Figure 4. HPLC chromatogram of the carbohydrates in Canola honey (see Table I for a list of the carbohydrates).
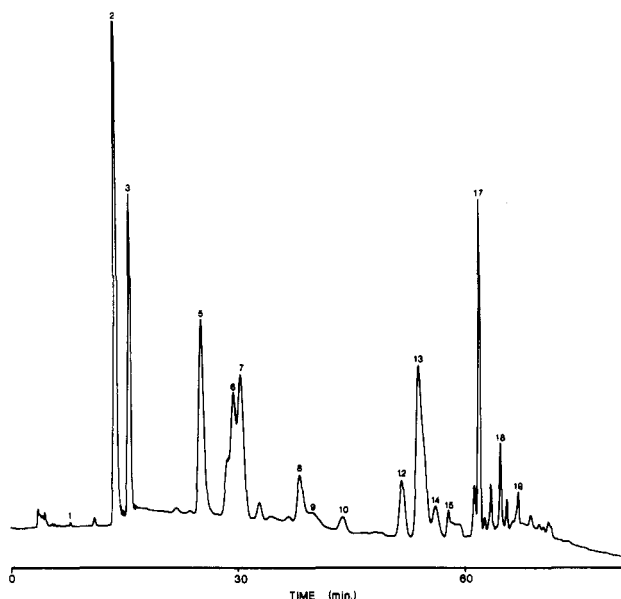
Figure 5. HPLC chromatogram of the carbohydrates in Trefoil honey (see Table I for a list of the carbohydrates).

glucose and fructose by charcoal/Celite chromatography. Although relative oligosaccharide concentration varies from one honey sample to the next, the overall oligosaccharide pattern does not differ significantly. Therefore, these oligosaccharide patterns could be used as "fingerprints" for honey authenticity. The largest variation in honey oligosaccharides is that found in Canola honey (Figure 4). Canola flowers contain very little sucrose (Low et al., 1988), which results in a significantly different carbohydrate profile. One example is an almost total absence of erlose (peak 17) which predominates in other unifloral honeys.

Concentration of the oligosaccharides using charcoal/Celite chromatography followed by HPLC analysis with anion-exchange chromatography in conjunction with a pulsed amperometric detection system affords the qualitative and quantitative analyses of structurally similar oligosaccharides. This methodology can be used to determine the minor oligosaccharides present in a variety of foods. These patterns can then be used as fingerprints for the authenticity of foods. We are currently inves-

tigating the oligosaccharide fingerprint profiles for a number of food products to test this hypothesis.

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