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Ion chromatographic methods for the detection of starch hydrolysis products in ruminal digesta

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ABSTRACT

Dionex high-performance ion chromatographic methods were evaluated for separation and quantitation of plant sugars and starch digestion products in the ruminal digesta of cattle. Mono- and disaccharides were eluted from a Dionex CarboPac PA1 column with sodium hydroxide used isocratically or as a pH gradient. Maltooligosaccharides which had a degree of polymerization (DP) less than 30 glucose residues were eluted in 60 min by a sodium hydroxide eluent containing a sodium acetate gradient. Carbohydrates were detected amperometrically. Responses were linear ($r^2 > 0.99$) for glucose, disaccharides and maltooligosaccharides (DP < 8). Precipitation and solid-phase extraction methods were evaluated for clean-up of samples of feedstuffs, ruminal contents, and bacterial culture fluids. Perchloric acid precipitation hydrolyzed sucrose but did not affect recoveries of cellobiose, isomaltose or maltose. Ethanol in concentrations of 79 and 86% precipitated maltooligosaccharides having chain lengths larger than 14 and 9 glucose residues, respectively. Maltooligosaccharide recoveries from solid-phase extraction columns varied with maltooligosaccharide size and column packing. Recoveries were >94% for short chains (DP < 6) eluted from phenyl-substituted columns and variable for all oligosaccharides eluted from C_{18} columns. Applications of these methods are presented and include: (1) detection of sugars in ruminant feed, (2) monitoring changes in ruminal sugars after feeding and (3) monitoring changes in extracellular sugars and oligosaccharides in the culture fluids of the ruminal bacterium, *Bacteroides ruminicola*.

INTRODUCTION

Starch is a major component of the diets of commercially raised beef and dairy cattle in the U.S.A. The initial digestion of starch occurs in the rumen where the indigenous microbial community hydrolyzes and ferments starch to volatile fatty acids (VFA). These acids are used by the animal for its metabolism and growth [1]. Our laboratory is examining starch digestion in cattle, monitoring the changes in low-molecular-weight carbohydrates and maltooligosaccharides resulting from starch hydrolysis in the rumen. The ruminal digesta samples in our studies are complex, containing bacteria, protozoa, fungi, plant materials, inorganic ions, short chain carboxylic acids and protein. High-performance ion chromatography (HPIC) with pulsed amperometric detection (PAD) affords us a convenient means of monitoring ruminal carbohydrates with minimal sample processing. It specifically detects both reducing and non-reducing sugars without chemical derivatization and its sensitivity is as low as 50 n*M* [2]. Moreover, carboxylic acids and most inorganic ions do not cause interference with saccharide detection [2]. The chemical and electrochemical

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details of this methodology and its advantages over other methods for the analysis of saccharides in soil and other complex samples have been discussed previously in detail [3–6].

This report describes some minor modifications of existing HPIC methods [2] for the separation and quantitation of various sugars and maltooligosaccharides resulting from starch hydrolysis. Sample clean-up procedures are evaluated for recovery of glucose and maltooligosaccharides. Finally, these methods are applied for analysis of the carbohydrate content of complex biological samples, including feedstuffs, ruminal fluid and ruminal bacterial culture fluids.

EXPERIMENTAL

Instrumentation

A Dionex 4000i ion chromatograph system (Dionex, Sunnyvale, CA, U.S.A.) was used and equipped with a pulsed amperometric detector, autosampler and either a 4270 integrator or Auto Ion 450 data system. The PAD consisted of a gold working electrode and a silver–silver chloride reference electrode. The applied potentials were $E_1 = 0.10$ V, $E_2 = 0.6$ V and $E_3 = -0.80$ V with pulse durations of 300, 120 and 300 ms, respectively. Carbohydrates were separated on a Dionex HPIC-AS6 anion-exchange column (10 μm; 250 × 2 mm I.D.) with a HPIC-AG6 guard column (50 × 2 mm I.D.) unless otherwise indicated. Eluents were sparged continuously with helium and passed through an ATC-1 ion trap at a flow-rate of 1 ml/min. Prior to injection, the 25- and 50-μl sample loops were flushed with 500 μl of sample prefiltered through the 20-μm frit of the autosampler vial cap.

Materials

Perchloric acid, sodium hydroxide, potassium hydroxide, acetic acid and sodium acetate were analytical grade of Mallinckrodt (Paris, KY, U.S.A.). Ethanol was obtained from Aaper (Shelbyville, KY, U.S.A.) and acetonitrile was the B and J Brand from Baxter, Burdick and Jackson (Muskegon, MI, U.S.A.). The C_{18} solid-phase extraction (SPE) columns (3 ml column volume) were obtained from Analytichem (Harbor City, CA, U.S.A.). The C_2 and phenyl SPE columns (3 ml) were from J. T. Baker (Phillipsburg, NJ, U.S.A.). Filtered, 18 MΩ deionized water (NanoPure II, Barnstead, Boston, MA, U.S.A.) was used for all eluent and reagent preparations.

Carbohydrate standards and their preparation

Carbohydrate standards were purchased from the following suppliers: Mallinckrodt, (glucose, sucrose); Sigma (St. Louis, MO, U.S.A.) (cellobiose, 2-deoxyribose, fucose, fructose, isomaltose, maltooligosaccharide G4-G10, sorbitol, xylose); Fisher Scientific (Fair Lawn, NJ, U.S.A.) (maltose); Calbiochem (San Diego, CA, U.S.A.) (maltotriose); Boehringer Mannheim, (Germany) (malto-tetraose, -pentaose, -hexaose, -heptaose); Difco Labs. (Detroit, MI, U.S.A.) (soluble starch).

All mono-, di- and oligosaccharides were prepared as aqueous stocks and stored at −15°C. Working stocks, diluted in deionized water, were stored at 4°C. Standard solutions used for calibration were prepared to represent the concentration ranges expected to be found in the samples to be analyzed. Soluble starch (0.5 mg/ml) was prepared in deionized water daily by boiling for less than 1 min in a capped tube.

Elution of mono- and disaccharides

Glucose, fructose, sucrose, isomaltose, cellobiose and maltose were separated isocratically with a 150 m*M* sodium hydroxide eluent on a CarboPac PA 1 (250 × 4 mm I.D.) column and CarboPac PA guard column (25 × 3 mm I.D.). A more complex mixture of sorbitol, fucose, 2-deoxyribose, sucrose, glucose, xylose, mannose, fructose, isomaltose and maltose was separated by a pH gradient on the AS6 column. Sodium hydroxide and acetic acid concentrations were changed linearly from 0 to 100 and 1.5 m*M*, respectively, over 40 min and then maintained for 20 min (flow-rate, 1 ml/min). Sodium hydroxide (500 m*M*) was delivered (1 ml/min) into the postcolumn eluent stream prior to the detector cell.

Elution of maltooligosaccharides

Glucose, maltose and the maltooligosaccharide standards [degree of polymerization (DP) < 8] were eluted in 18 min by a 125 to 300 m*M* sodium acetate linear gradient in 150 m*M* sodium hydroxide (flow-rate, 1 ml/min). Maltooligosaccharides (DP < 30) in soluble starch were eluted within 60 min by a 125 to 375 m*M* sodium acetate gradient. Column equilibration required 2 and 5 min for the 18- and 60-min gradients, respectively.

Ethanol precipitation

Ethanol (95%) was added to soluble starch solutions (final concentrations, 48, 79 and 86%) and held overnight at room temperature. Ethanol insoluble components were removed from solution by centrifugation (12 000 *g*, 15 min, 20°C) and were redissolved in deionized water. The ethanol soluble carbohydrates were recovered from the supernatant by rotary evaporation and redissolved in deionized water for analysis. Concentrations of oligosaccharides in these redissolved fractions were compared to those measured in the original soluble starch solution.

Perchloric acid precipitation

Ruminal fluid samples (1 ml) were added to prechilled tubes containing 48 μl of 70% (v/v) perchloric acid and held on ice for 30 min. The tubes were centrifuged (12 000 *g*, 15 min, 4°C) and stored at 4°C for a minimum of 3 days. Just prior to HPIC analysis, 48 μl of a 45% (w/v) potassium hydroxide solution was added to the samples for removal of excess perchloric acid. The resulting precipitate was removed by centrifugation (12 000 *g*, 15 min, 25°C) and the supernatant was assayed for sugar content by the isocratic HPIC method.

To test whether disaccharides commonly found in ruminal digesta were hydrolyzed during this procedure, we made a comparison of disaccharide recoveries before and after potassium hydroxide addition. Sucrose, isomaltose, cellobiose and maltose were diluted 1:10 in supernatants of ruminal fluid samples (previously shown to contain no detectable sugar) following the samples' precipitation with perchloric acid. Final concentrations of the added sugar solutions were 20 m*M* for each disaccharide except cellobiose (10 m*M*). Potassium hydroxide was added to the control tubes prior to the addition of the sugar. For tests of the effects of storage in perchloric acid, potassium hydroxide was added 24 and 120 h after the addition of the sugar. Recoveries of the sugars in rumen fluid were compared to those of sugars diluted similarly in water, without added perchloric acid or potassium hydroxide. All treatments were run in triplicate.

Solid-phase extraction

The disposable C_2-, C_{18}- and phenyl-SPE columns were conditioned with 2.5 ml acetonitrile followed by 5 ml deionized water. A mixture of glucose, isomaltose, maltose and seven other monosaccharides (2 ml of 672 μM total sugar concentration) was eluted with 3 ml of water. The soluble starch solutions (0.5 ml of a 5 mg/ml sample) were eluted with 5 ml of water.

RESULTS AND DISCUSSION

Separation of mono- and disaccharides

Glucose, fructose and four disaccharides (sucrose, isomaltose, cellobiose and maltose) were well separated by a 150 mM sodium hydroxide eluent (Fig. 1). The detector response was linear for 1.5–50 μM for glucose, fructose and cellobiose, 3–100 μM for sucrose and isomaltose, and 6–100 μM for maltose. When a detector setting of 3 mA was used, and 5 concentrations of each sugar were tested, the calculated slopes of the standard curves ranged from 1.01 to 1.03 area counts $(\cdot 10^{-5})/\mu M$ and the y-intercepts ranged from -0.48 to -0.04 area counts $(\cdot 10^{-5})$. The r^2 values were all greater than 0.99.

Although the isocratic separation method was appropriate for eluting the carbohydrates in feedstuffs and ruminal fluid (see Applications 1 and 2), it did not adequately resolve glucose, maltose, isomaltose and cellobiose from the more complex mixture of sugars found in cultures of ruminal bacteria (see Application 3).

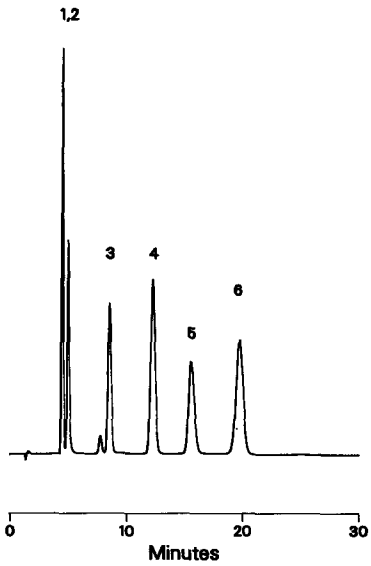

Fig. 1. Isocratic elution of 50 μl of a mixture of mono- and disaccharides by sodium hydroxide. The sugars (and concentrations) in the mix, in order of their elution, were: 1 = glucose (4.6 μM); 2 = fructose (4.2 μM); 3 = sucrose (4.2 μM); 4 = isomaltose (9.3 μM); 5 = cellobiose (4.6 μM); 6 = maltose (5.6 μM). Peaks 4, 5 and 6 are disaccharides which differ only in their respective α-1,6, β-1,4 and α-1,4 glucose linkages.

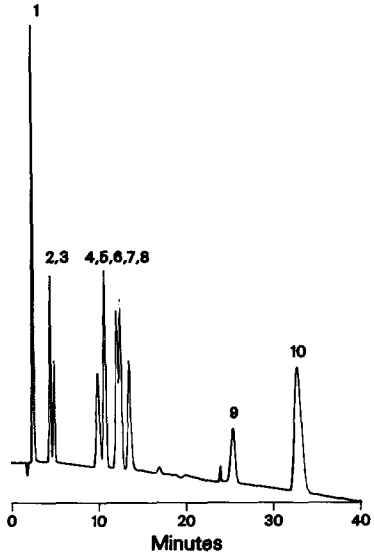

Fig. 2. pH gradient elution of 25 μl of a complex mixture of mono- and disaccharides. The sugars (and concentrations) in the mixture, in order of their elution, were: 1 = sorbitol (500 μM); 2 = 2-deoxyribose (500 μM); 3 = fucose (500 μM); 4 = sucrose (500 μM); 5 = glucose (500 μM); 6 = xylose (500 μM); 7 = fructose (500 μM); 8 = isomaltose (292 μM); 9 = cellobiose (500 μM); 10 = maltose (500 μM).

Accordingly, we adapted a gradient method [2] for the separation of a complex mixture of bacterial and plant sugars likely to be found in these samples [7].

In our hands, the gradient described by Dionex (3.5 mM sodium hydroxide–0.1 M acetic acid for 15 min, increasing to 50 mM sodium hydroxide–1.5 mM acetic acid over the next 10 min [2]) did not resolve our mixture of 10 mono- and disaccharides. Resolution was achieved (Fig. 2) when the elutions conditions were changed to a simple 40 min linear gradient of 0 to 100 mM sodium hydroxide–1.5 mM acetic acid. The elution order was sugar alcohols, deoxy sugars, mono- and disaccharides except for sucrose which preceded glucose. The pH gradient required post-column delivery of sodium hydroxide to eliminate the pH effect on the detector. The lowest detector setting providing maximum sensitivity was 10 mA under these conditions. Thus, this method resolved more sugars than the isocratic method at the expense of sensitivity. Glucose sensitivity was 10-fold lower than that of the isocratic method. The linear detection range of both glucose and isomaltose was 14–1100 μM. The ranges for cellobiose and maltose were, respectively, 15 to 580 μM and 7 to 550 μM. The slopes of the standard curves estimated for 5 different concentrations of standards of all four sugars ranged from 0.5 to 0.65 area counts ($\cdot 10^{-5}$)/μM. The y-intercepts ranged from 5.0 to 11.1 area counts ($\cdot 10^{-5}$) and the r^2 values were greater than 0.99.

Separation of maltooligosaccharides

A gradient of increasing sodium acetate concentration at constant pH eluted and separated glucose, maltose and the maltooligosaccharides in order of increasing glucose polymerization. The gradient conditions specified by Dionex [2] (250 mM acetate for 1 min, increasing to 500 mM acetate in 8 min and then maintaining for 7

more minutes) did not provide adequate resolution of the soluble starch oligomers in our samples. We adjusted both the initial and final eluent concentrations and the gradient slope to reproduce the resolution demonstrated by Dionex. By extending the gradient run time to 60 minutes, we were able to increase the resolution of larger oligomers from 22 glucose residues [2] to 30 residues (Fig. 3). This elution method required a 10 mA detector setting for minimal baseline drift due to the gradient. Standard curves were linear (r^2 values > 0.99) for glucose (2.5 to 25 μg/ml) for maltose and maltotriose (2.5 to 100 μg/ml) and for the maltooligosaccharide standards (DP = 3–7; 2.5 to 200 μg/ml). Calculated slopes of standard curves were 0.10, 0.10, 0.11, 0.13, 0.15, 0.17 and 0.19 area counts ($\cdot 10^{-5}$)/μM for glucose, maltose, maltotriose, maltotetraose, maltopantaose, maltohexaose and maltoheptaose, respectively, while intercepts were 0.54, 1.3, 0.77, 0.43, 0.49, 0.35 and 0.40 area counts ($\cdot 10^{-5}$), respectively.

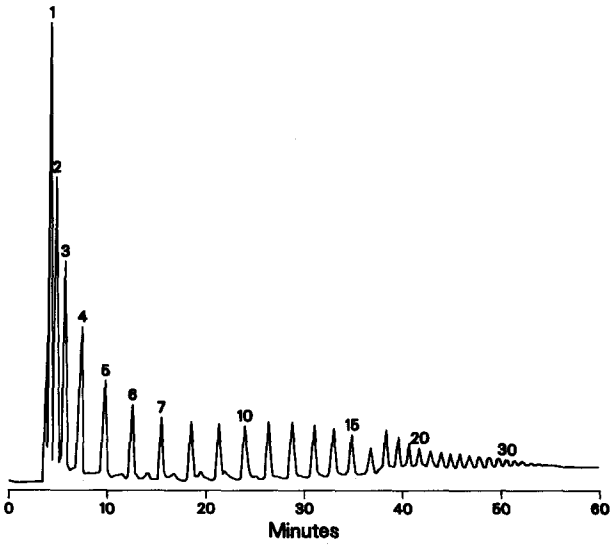

Fig. 3. Sodium acetate gradient elution of 50 μl of soluble starch oligosaccharides (0.5 mg/ml). Peak number represents the DP of α-1,4 linked glucose. Minor peaks between the major peaks are maltooligosaccharides having α-1,6 linkages [8]. The sample load was 250 μg soluble starch.

Sample clean-up by ethanol precipitation

Ethanol is used commonly to fractionate soluble sugars and polysaccharides from complex mixtures [9–11], but the solubility of the intermediate maltooligosaccharides have not been reported. Since individual standards (DP > 7) were not commercially available and the soluble starch prepared by Difco Manufacturers contained a wide range of maltooligosaccharides (Fig. 3), we used this soluble starch preparation to evaluate the solubility of these oligosaccharides in 48, 79 and 86% ethanol.

When the supernatant fractions were evaporated, the residues, like the air-dried precipitates, were readily dissolved in water. However, the sum of the maltooligo-

saccharides measured in these fractions were 0–30% less than their amounts measured in the untreated soluble starch sample (Table I). This did not appear to be due to a systematic recovery error since recovery was: (1) highly variable within a treated soluble starch sample and (2) independent of oligomer size and alcohol concentration.

In general, the solubility of the starch oligosaccharides decreased with increasing chain length and increasing alcohol concentration. Oligosaccharides with less than 28, 15 and 10 glucose residues were detected solely in the supernatant fraction obtained from the 48, 79 and 86% ethanol treatments, respectively (Table I). Larger oligosaccharides ($14 < DP < 23$ for 79% ethanol treatment and $9 < DP < 19$ for 86%

TABLE I

PEAK AREA COUNTS OF MALTOOLIGOSACCHARIDES DETECTED IN SUPERNATANT AND PRECIPITATE FRACTIONS OBTAINED AFTER TREATMENT OF SOLUBLE STARCH WITH DIFFERENT CONCENTRATIONS OF ETHANOL

Oligosaccharide (DP)	Area counts ($\cdot 10^{-5}$) of oligomers after treatment[a]						
	Untreated	48% Ethanol		79% Ethanol		86% Ethanol	
		Super	Precip	Super	Precip	Super	Precip
1	218±3[b]	184	5.4	189	0	223	0
2	187±8	172	1.6	123	0	197	0
3	146±8	128	0	99	0	152	0
4	96±4	97	0	63	0	93	0
5	67±6	62	0	42	0	64	0
6	54±3	49	0	37	0	52	0
7	45±2	41	0	30	0	39	0
8	47±9	38	0	27	0	35	0
9	46±1	45	0	30	0	38	0
10	49±2	40	0	30	0	37	6.1
11	49±2	44	0	30	0	35	8.3
12	49±3	44	0	31	0	32	13
13	47±2	42	0	28	0	26	16
14	45±2	41	0	28	0	20	15
15	40±2	36	0	22	3.0	14	15
16	28±2	26	0	17	4.9	8.8	16
17	28±5	20	0	14	4.1	6.9	13
18	21±4	16	0	10	4.0	3.6	16
19	13±0.2	13	0	6.1	5.4	0	10
20	11±0.1	10	0	3.7	6.0	0	8.9
21	11±0.4	10	0	2.9	5.9	0	9.4
22	11±0.1	10	0	3.3	6.5	0	9.8
23	11±0.1	10	0	0	7.3	0	9.1
24	11±0.5	9.7	0	0	7.8	0	8.8
25	9.7±0.6	7.8	0	0	7.5	0	0
26	8.8±0.4	7.6	0	0	7.0	0	0
27	8.8±0	7.5	0	0	6.5	0	0

[a] Area counts of oligosaccharides detected in supernatant (super) and precipitate (precip) fractions recovered from 48, 79 or 86% ethanol treatment. See text for details.

[b] Mean of two determinations ± standard deviation.

ethanol treatment) were detected in both precipitate and supernatant fractions. The largest oligosaccharides 23 $\leqslant$ DP $\leqslant$ 27 and 19 $\leqslant$ DP $\leqslant$ 24) were detected only in the precipitate fractions of the 79 and 86% ethanol treatments, respectively. Thus, ethanol precipitation provided a selective means of retaining or removing oligomers of various lengths.

Sample clean-up by perchloric acid

Since our laboratory uses perchloric acid to deproteinate ruminal fluid samples for assays of bacterial fermentation acids, we tested the suitability of using this acid treatment for the analysis of small molecular weight plant carbohydrates. In preliminary analysis of perchlorate treated samples, we observed decreased retention times of the sugars and loss of column resolution. This was prevented by the removal of excess perchlorate by potassium hydroxide precipitation (Dionex, personal communication).

We tested the stability of disaccharide standards added to perchloric acid-treated ruminal fluid previously shown to contain no detectable sugars. When maltose, isomaltose and cellobiose standards were added to this acid-precipitated sample and stored at 4°C for 120 h, their measured concentrations upon potassium hydroxide addition (22.7 $\pm$ 2.2 m*M*, 21.9 $\pm$ 2.3 m*M* and 10.6 $\pm$ 1.5 m*M*, respectively) were comparable to measured concentrations of standards (27.5 $\pm$ 0.33 m*M*, 21.3 $\pm$ 0.7 m*M* and 10.9 $\pm$ 0.8 m*M*, respectively) stored in water at 4°C for 120 h. Continued storage of these potassium hydroxide-treated samples yielded significant glucose concentrations (8.6 $\pm$ 0.4) and lower concentrations of maltose (11.4 $\pm$ 0.5). No sugars appeared in ruminal fluid controls (no added sugars) following the same perchlorate–potassium hydroxide treatment. Thus, maltose was stable in acid but not in base while isomaltose and cellobiose were stable regardless of the storage conditions. No glucose or maltose was detected in acid-precipitated ruminal fluid to which a mixture of maltooligosaccharides (DP 4–10; 1 mg/ml total) was added after 7 days storage at 4°C. Although these data were supportive that hydrolysis of maltooligosaccharides to glucose did not occur, we have not confirmed that partial hydrolysis did not occur.

When sucrose was stored in acid-precipitated ruminal fluid at 4°C, both glucose and fructose were measurable (3.6 $\pm$ 0.3 m*M* and 2.0 $\pm$ 0.2 m*M*, respectively) after 24 h storage and increased to 15.2 $\pm$ 1.1 m*M* and 11.0 $\pm$ 0.7 m*M*, respectively, after 120 h storage. Sucrose concentrations concomitantly declined with storage in acid-precipitated ruminal fluid, dropping from 26.6 $\pm$ 0.8 m*M* immediately after its addition to rumen fluid to 17.3 $\pm$ 0.7 m*M* and 10.1 $\pm$ 0.7 m*M* when held for 24 h and 120 h at 4°C, respectively. Sucrose was stable in water at 4°C, measuring 21.2 $\pm$ 0.7 m*M* throughout the storage period. Thus, sucrose was not stable under these acid storage conditions.

Sample clean-up by solid-phase extraction

Solid-phase extraction is recommended for protein and organic compound removal from complex biological samples [2]. Recoveries of individual sugars (glucose, isomaltose, cellobiose and maltose) from C_{18} SPE cartridges were comparable, averaging 86 $\pm$ 3% and 96 $\pm$ 2% for two different column batches. This variability between batches may have been due to column aging [12] and should be monitored. The recoveries of the oligosaccharide standards also varied considerably between

TABLE II

PERCENTAGE OF SOLUBLE STARCH OLIGOSACCHARIDES ELUTED FROM SOLID-PHASE EXTRACTION COLUMNS

Oligosaccharide (DP)	Recovery from column packings[a]		
	C_{18}	C_2	Phenyl
1	91.98[b]	100	99.96[b]
2	75.96	96	95.96
3	74.98	99	95.98
4	73.98	99	98.99
5	70.99	98	94.98
6	62.94	99	64.92
7	60.90	116	61.98
8	42.72	91	90
9	39.51	120	101
10	30.60	103	102
11	24.42	97	98
12	17.0	96	98
13	10.0	104	99
14	0.0	86	99

[a] Expressed as a percent of the amount of the same oligosaccharide measured in the soluble starch sample before application on the SPE column.
[b] Duplicate determinations.

column batches and, to a lesser extent, with the hydrophobicity of the packing material (Table II). Recoveries of the soluble starch oligosaccharides having more than 8 glucose residues decreased with increasing chain length. The limited recoveries of the longer oligosaccharides could have been due to the interaction of these longer carbohydrate chains with the column bed or with each other [13]. Although organic solvents might increase recoveries of the oligomers from the SPE packing, they were not used since they interfered with detection of the carbohydrates. We were also concerned about the solubility of the longer oligomers in even low concentrations of organic solvent. Thus, the utility of solid-phase extraction for the quantitation of oligosaccharides was limited by saccharide size and column batch variability.

APPLICATIONS

The following shows our applications of the HPIC methods described above in our *in vitro* and *in vivo* studies of the effects of small molecular weight carbohydrates on bacterial digestion and fermentation of ruminal carbohydrates. Summaries of our methods and results are presented below.

(1) Carbohydrate profiles of feedstuffs

We have compared ground corn against a ground corn–molasses mixture (90:10) to determine the impact of molasses on ruminal sugar concentrations and other ruminal fermentation variables in cattle. The feedstuffs were first analyzed for sugar content. Triplicate 3-g samples of the two feedstuffs were extracted in alcohol

by the AOAC method [14]. The alcohol solution was removed by evaporation under a stream of nitrogen. The residue was resuspended in water and particulate was removed by centrifugation (12 000 *g*, 5 min, 25°C). The supernatant solution was diluted 200-fold and analyzed (25 μl) by the isocratic elution HPIC method (Fig. 4).

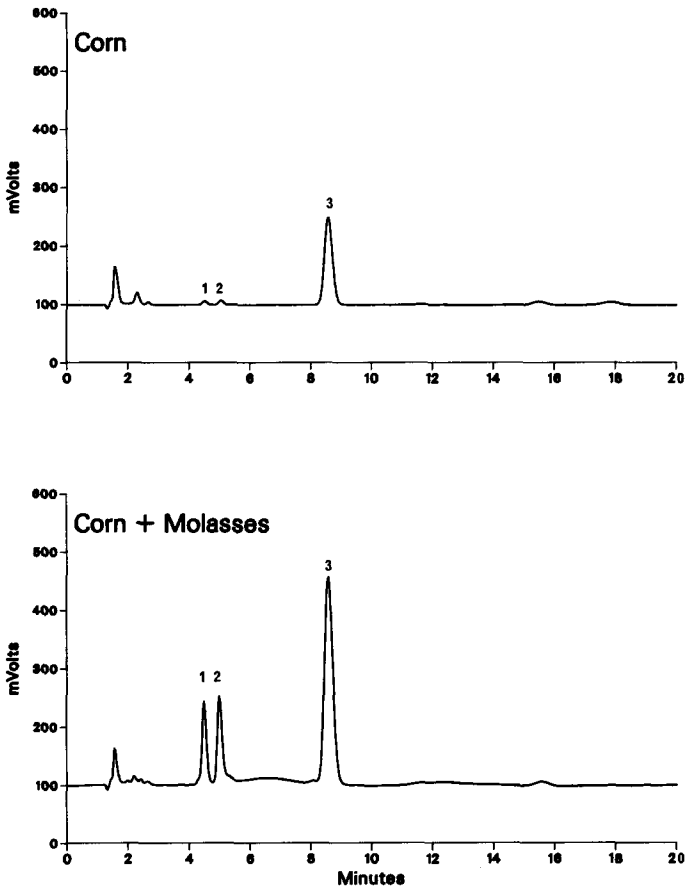

Fig. 4. Chromatograms of alcohol-soluble mono- and disaccharides in feedstuffs. Glucose (peak 1), fructose (peak 2) and sucrose (peak 3) were extracted in ethanol from either ground corn or a mixture of ground corn–molasses (90:10) and were eluted isocratically with sodium hydroxide.

Fig. 4 shows chromatograms of the alcohol-soluble carbohydrates detected in the feeds. Ground corn contained (on a dry weight basis) 1.3 ± 0.01% sucrose and equal amounts (0.03 ± 0.01%) of glucose and fructose. The corn–molasses mixture contained 3.2 ± 0.4% sucrose, 0.8 ± 0.03% glucose and 0.4 ± 0.03% fructose. Molasses contains roughly 30% sucrose [14]. The measured sugar concentration of the 10% molasses-ground corn mixture was 4.4% as expected since the measured concentrations of sugars in the ground corn were 1.3% and the molasses would have contributed an additional 3% sugar.

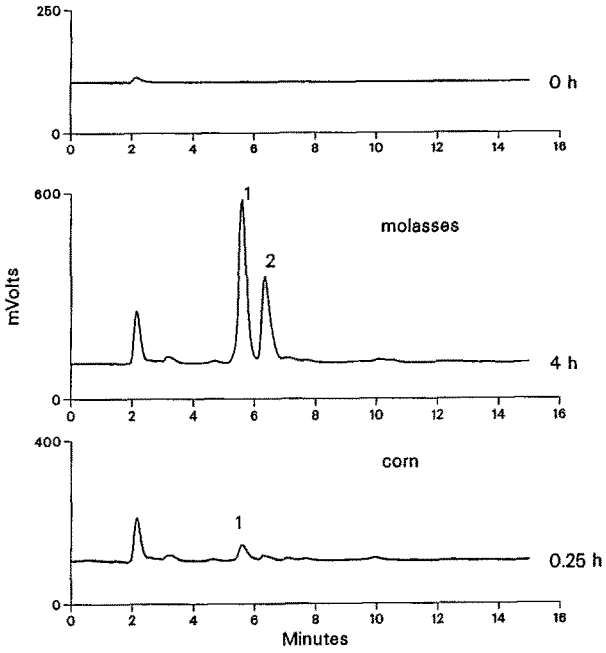

Fig. 5. Typical chromatograms of sugars in ruminal fluid. Glucose (peak 1) and fructose (peak 2) were eluted isocratically. Plots shown are from 50 μl of a 1:20 dilution of perchloric acid, potassium hydroxide-treated ruminal fluid sample collected prior to dosing (0 h) with a feedstuff; 4 h after dosing animals with a ground corn–molasses mixture (4 h, molasses) and 0.25 h after dosing with ground corn (0.25 h, corn).

(2) Ruminal carbohydrate profiles

The feedstuffs described above were delivered into the rumen in four equal doses (1.6 kg/dose/cow) at hourly intervals. Ruminal fluid samples were collected hourly, frozen immediately in a dry ice–aceton bath and stored at −20°C. The samples were prepared for analysis by centrifugation (2000 *g*, 4°C, 30 min), deproteinated with perchloric acid and stored at 4°C. Immediately prior to analysis potassium hydroxide was added as described above. Samples were diluted 20-fold and analyzed by the HPIC isocratic method.

Typical chromatograms and changes in total ruminal sugar concentrations (glucose and fructose) are shown in Figs. 5 and 6. Carbohydrates were not detected in the samples collected before feeding (Fig. 5, 0 h). Carbohydrates were barely detectable in the ruminal fluid of the animals fed the ground corn diet (low sugar content; Fig. 5, 0.25 h and Fig. 6, closed bars) compared to those observed in the ruminal fluids of the animals fed molasses-ground corn mixture (high sugar content; Fig. 5, 4 h and Fig. 6, open bars). One hour after the last portion of feed was introduced into the rumen, the average concentration of ruminal sugar (glucose and fructose) was 44 m*M*. This value is comparable to a 46 m*M* theoretical value based on the amount of sugars detected in the molasses–ground corn mixture (see Application 1), the average amount of total feed given to induce acidosis (5.7 kg/animal, $n = 4$ cattle) and an estimated ruminal volume of 30 liters [16]. The sugars that were measured were glucose and fructose which may have been present in the ruminal fluid as sucrose

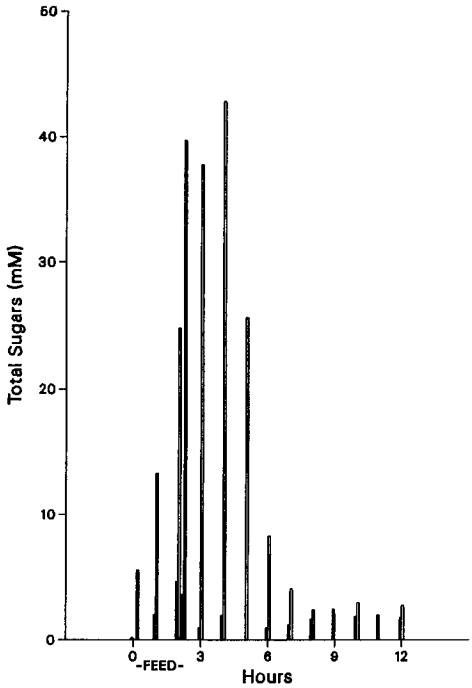

Fig. 6. Postprandial changes in dietary carbohydrates in the rumen. Equal doses of a feedstuff were delivered through a fistula into the rumen at 0, 1, 2 and 3 h. Sugars in perchloric acid, potassium hydroxide-treated ruminal fluid samples were separated by the HPIC isocratic method. A bar graph representing the sums of ruminal glucose and fructose concentrations measured at each sampling time in animals dosed with either ground corn (closed bars) or a 90:10 mixture of ground corn–molasses (open bars) is shown.

before perchloric acid precipitation. It is likely that complete hydrolysis of sucrose occurred during the lengthy storage time (up to 3 months for these samples) in acid.

Extracellular carbohydrates in culture supernatant

The purpose of this experiment was to measure the extracellular appearance of the hydrolysis products of amylopectin during growth of one of the more prominent species of amylolytic bacteria in the rumen, *Bacteroides ruminicola. B. ruminicola* strain 118B was grown at 37°C under strictly anaerobic conditions (100% CO_2) in batch cultures containing 0.2% amylopectin. Extracellular fluids were collected by centrifugation (12 000 *g*; 10 min; 4°C) at selected times during the growth cycle. Following alcohol precipitation and C_{18} SPE clean-up, the supernatant fluid was assayed by the acetate gradient method to characterize the appearance of maltooligosaccharides. Supernatants were also assayed by the pH gradient method for characterization of small molecular weight carbohydrates formed during growth.

Recoveries of maltooligosaccharide standards (G3–G7) in the extracellular fluid were 90 to 100% (data not shown). However, the identification of these carbohydrates was difficult due to variation of their retention times between chromatographic elutions. Retention times for the standards suspended in water were repro-

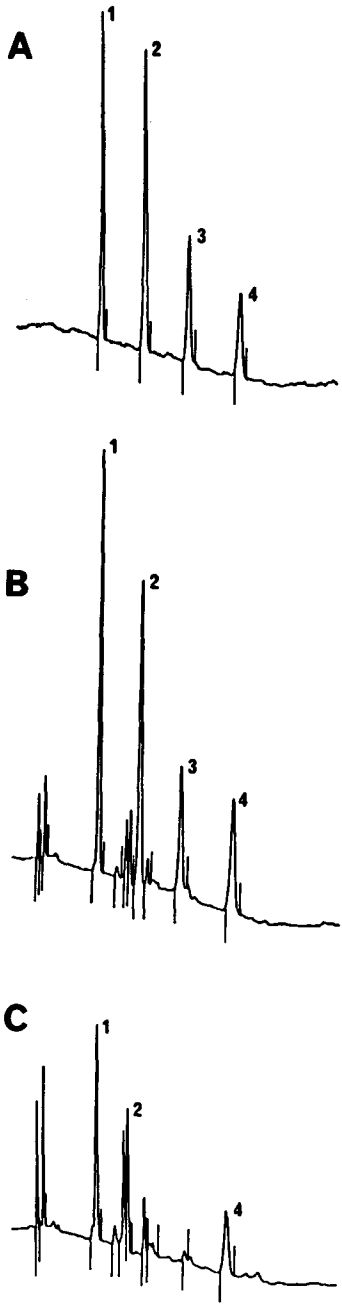

Fig. 7. Chromatograms of standards and sugars in the extracellular fluids of cultures of *Bacteroides ruminicola*. Glucose (peak 1), isomaltose (peak 2), cellobiose (peak 3) and maltose (peak 4) were separated by the pH gradient elution method and had elution times of 15.5, 24.5, 31.9 and 39.3 min, respectively. (A) Sugar standards prepared in water; (B) sugar standards added to extracellular fluids; (C) sugars in extracellular fluids without added standards.

ducible. Two oligosaccharides with retention times comparable to maltotetraose and maltohexaose were detected at low levels (2–42 μM) (data not shown) compared to glucose levels (50–300 μM, see below). The variability in retention times may have been due to the effects of the culture fluid components in the samples on the elution characteristics of the starch hydrolysis products. We have not identified what these factors are.

Fig. 7C illustrates the numerous peaks which were observed in chromatography of the amylopectin medium and shows why we needed the better resolving power of the gradient method. In contrast to the maltooligomers, the elution times of mono- and disaccharide standards in extracellular fluids were reproducible and recovery was 100% (Fig. 7A and B). Peaks having retention times identical to glucose, isomaltose and maltose showed the most dramatic concentration changes during the growth cycle of *Bacteroides ruminicola* and are shown in Fig. 8.

CONCLUSIONS

The isocratic HPIC method worked well for the analysis of mono- and disaccharides in feedstuffs and ruminal fluid samples. The saccharides of interest were in sufficient concentrations (mM) in our samples (Applications 1 and 2) so that interfering substances were diluted out. This was not the case for bacterial culture supernatants (Application 3) where saccharides in the bacterial medium were in concentrations (μM) equivalent to the saccharides of interest. Therefore, we adapted a gradient method to assure that glucose, isomaltose and maltose were well resolved from these other mono- and disaccharides. This pH gradient provided greater resolution than the isocratic method at the expense of sensitivity and total run time. An

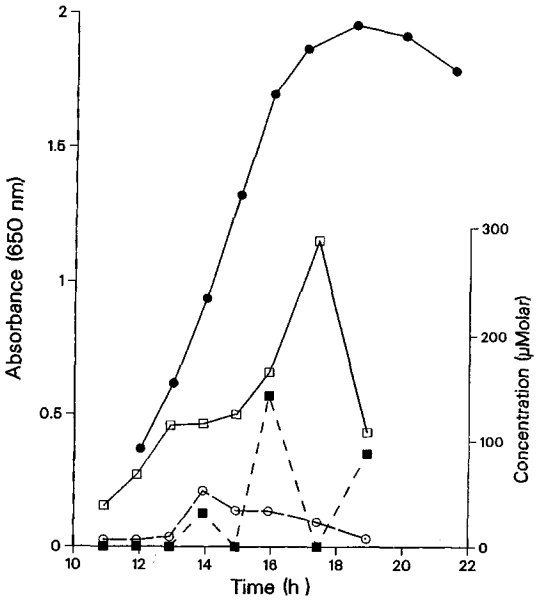

Fig. 8. Changes in concentrations of extracellular sugars during the growth of *Bacteroides ruminicola* in batch cultures containing amylopectin, a starch polysaccharide. Sugars were detected by the pH gradient method. A typical chromatogram is shown in Fig. 7C. Bacterial growth was monitored as the change in culture absorbance (650 nm). ● = Absorbance; □ = glucose; ■ = maltose; ○ = isomaltose.

acetate gradient method was used to monitor the appearance of maltooligosaccharide products of amylopectin hydrolysis. The retention times of the maltooligomer products of amylopectin in culture fluids were not as consistent as those of standards ($2 < DP < 7$) or soluble starch oligomers in spite of the clean-up procedures used (see below). However, we were able to follow the appearance of maltotetraose and -hexaose. Since both gradient methods required adjustments in eluent strength and gradient slope to obtain the same resolution as the published method, we suspect that differences in laboratory conditions and individual columns may have been the cause.

Three sample clean-up methods were examined. Ethanol provided a selective means for solubilizing mono-, di- and small maltooligosaccharides ($DP < 10$) and precipitating the large ($DP > 19$) maltooligomers. However, the alcohol needed to be removed from the sample before analysis because it was detected by the PAD and interfered with saccharide detection. Perchloric acid successfully deproteinated ruminal fluid samples, but it was necessary to remove excess acid by potassium hydroxide precipitation to maintain column integrity. Although perchloric acid did not affect the recoveries of cellobiose, maltose or isomaltose, it did hydrolyze sucrose. Maltose appeared to be hydrolyzed after potassium hydroxide addition. The solid-phase extraction columns gave the highest recoveries of the smaller maltooligosaccharides. Retention times of the maltooligosaccharides in bacterial culture fluids varied despite clean-up by a C_{18} solid phase extraction.

HPIC with pulsed amperometric detection offers both isocratic and gradient capabilities. In combination with its selectivity and sensitivity it is a powerful tool for the separation of a variety of mono-, di- and large maltooligosaccharides ($DP < 30$) in complex samples.

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