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Quantitation of carbohydrate sweeteners and organic acids in human oral fluid using HPLC analysis

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Bestimmung von Zuckern und organischen Säuren in menschlicher Mundflüssigkeit unter Verwendung der HPLC-Analyse

Summary: A sensitive high performance liquid chromatography (HPLC) assay was developed for the qualitative and quantitative determination of carbohydrate sweeteners and organic acids in oral fluid. To separate these compounds, an ion-moderated partition resin HPLC column (Aminex HPX-87H) was used. All components of the HPLC system were interconnected using stainless steel capillary tubing. Isocratic elution with 0.01 N sulfuric acid provided the profile of both compound classes. The compounds were detected using a refractive index detector. The method employed computerized data collection and integration (Omega-2 system) with a detection sensitivity of 0.1 µg compound per HPLC assay (80 µl). This method is useful in caries research, because it detects minute amounts of sugars and organic acids in oral fluid during clearance studies of various foods in the mouth.

Zusammenfassung: Eine empfindliche HPLC-Methode wurde für die qualitative und quantitative Analyse von Kohlehydraten und organischen Säuren in Mundflüssigkeiten (Speichel und Zahnplaque) entwickelt. Für die Trennung dieser Verbindungen wurde eine Aminex HPX-87H (Bio-Rad) Chromatographie-Säule verwendet. Alle Komponenten des HPLC-Systems waren mit Edelstahl-Kapillaren verbunden. Die isokratische Elution beider Verbindungs-Klassen erfolgte mit 0,01 n Schwefelsäure. Alle Verbindungen wurden mit einem RI-(Refraktion-Index-) Detektor gemessen. Die Ergebnisse wurden mit einem PC-gestütztem Auswertesystem automatisch gesammelt und integriert. Die zeitbedingte Abnahme der Konzentration von Kohlehydraten im Munde einerseits und die Produktion von organischen Säuren durch Bakterien der Mundhöhle andererseits können mit dieser empfindlichen HPLC-Methode bis zu einer Genauigkeit von 0,1 µg Substanz per Analyse (80 µl) bestimmt werden.

Key words: Sugars; carbohydrates; lactic acid; oral fluid; HPLC; glucose; sucrose

Schlüsselwörter: Zucker, Kohlenhydrate, Milchsäure, Speichel, HPLC-Analyse, Glukose, Saccharose

Introduction

During salivary oral clearance studies of various snack foods it was necessary to detect and quantify minute amounts (range 0.1 to 5 µg/ml) of

carbohydrates (maltotriose, maltose, sucrose, lactose, glucose, fructose), organic acids (lactate, formate, acetate, propionate), and ethanol within a single oral fluid sample. Because a large number of samples had to be analyzed, detection of these compounds using a HPLC technique was the method of choice. Necessary requirements for HPLC were; 1) separation of all compounds using a single column; 2) detection of all compounds in a single detector; and 3) a high sensitivity of detection. It was desirable to have a non-toxic mobile phase for this system.

Materials and methods

A liquid chromatographic system was constructed from commercially available components and interconnected with stainless steel capillary tubing (1/16" OD × 0.007" ID; Alltech Associates, Deerfield, Illinois, USA).

Pump

A Liquid Chromatograph Series 3B dual pump (Perkin-Elmer Corp., Norwalk, Connecticut, USA) was used. The mobile phase used was 0.01 N degassed and nitrogen purged sulfuric acid (Fisher Scientific Co., Fair Lawn, New Jersey, USA). The system was run at a flow rate of 0.6 ml/min, which generated a pressure of 700 to 800 psi (maximum pressure set to 1000 psi).

Column

A 300 × 7.8 mm ion exclusion column Aminex HPX-87H (Bio-Rad Laboratories, Richmond, California, USA) was employed. The main column was preceded by a 40 × 4.6 mm guard column of the same type. The columns were maintained at 30 °C in a Column Oven LC-100 (Perkin-Elmer Corp.). On-stream injections of 80 µl per sample were made through a loop injector (Rheodyne, 175 µl sample loop).

Detector

A Differential Refractometer R401 (Waters Associates, Morristown, New Jersey, USA), set to positive polarity and run at an attenuator setting of 32x was used. The refractive index of the eluate was determined at room temperature. The reference cell was filled with 0.01 N sulfuric acid.

Standardization, data collection, and integration

The concentration of standard compounds was 1 µg/1 µl. For the standardization curve; 5, 10, and 15 µl of the following compounds were injected: maltotriose, maltose, sucrose, lactose, glucose, fructose, lactic acid, formic acid, acetic acid, propionic acid, and ethanol (Sigma Chemical Co., St. Louis, Missouri, USA). After injection of the standard compound sample the integrator interface was activated. The obtained retention times are detailed in Table 1. Each HPLC analysis of standard compounds or extracted oral fluid sample (injection volume 80 µl) was run for 24 min. The chromatographic data were collected and simultaneously integrated using an Omega-2 (Perkin-Elmer, Omega-2 software V 2.5) computerized (Epson Equity I+ computer with interface board and high resolution Amdek monitor) data station connected to an Epson LQ-500 printer. The sensitivity of the computerized integration method allowed for a detection sensitivity of 0.1 µg substance per HPLC assay.

Procedure to obtain oral fluid samples

Oral fluid samples (composed of dental plaque, saliva, and food remnants) were absorbed from five different caries-susceptible tooth sites using sterile paper points

(Absorbent Points, No. 2717 coarse, Johnson & Johnson, E. Windsor, New Jersey, USA). With the aid of self-locking dental pliers individual paper points were introduced between the interproximal surfaces of; the last two teeth present in the lower right quadrant, the interproximal area of the last two teeth of the upper left quadrant, the occlusal surface of the last tooth present in the lower left quadrant, the occlusal surface of the last tooth present in the upper right quadrant, and finally, on the lingual surface of the last tooth present in the lower right quadrant. Each paper point was allowed to fully absorb the oral fluids, and finally, all 5 points used were pooled in a single snap-cap vial (1.5 ml, No. 72.690, Sarstedt, Princeton, New Jersey). On average, the five paper points had absorbed approximately 60 mg of oral fluid. The oral specimens were immediately frozen in a Revco freezer (Revco Inc., West Columbia, South Carolina, USA) at −75 °C.

Extraction of carbohydrates and organic acids

The vials with the five frozen paper points were thawed at room temperature. To each vial, 1.0 ml of distilled water was added. The vial was then vigorously shaken at high speed in a Vortex mixer (Genie 2, Scientific Industries Inc., Bohemia, New York, USA) for 10 s. After 10 to 20 min the sample was shaken again for 10 s. The supernatant was transferred into a 3-ml syringe and then filtered through a 0.2-μm membrane filter (Acrodisc LC13, Gelman Sciences, Ann Arbor, Michigan, USA). The filtrate was kept cold and processed the same day by HPLC. After use, the sample was stored, frozen, in a Revco freezer. After an examination period of more than 3 months, the extracted and filtered samples remained stabile and chemically unchanged, withstanding repeated freezing and thawing. No change in the chemical composition of the samples was observed due to the possible presence of bacterial or mammalian enzymes in the oral fluid sample. Therefore, it was not

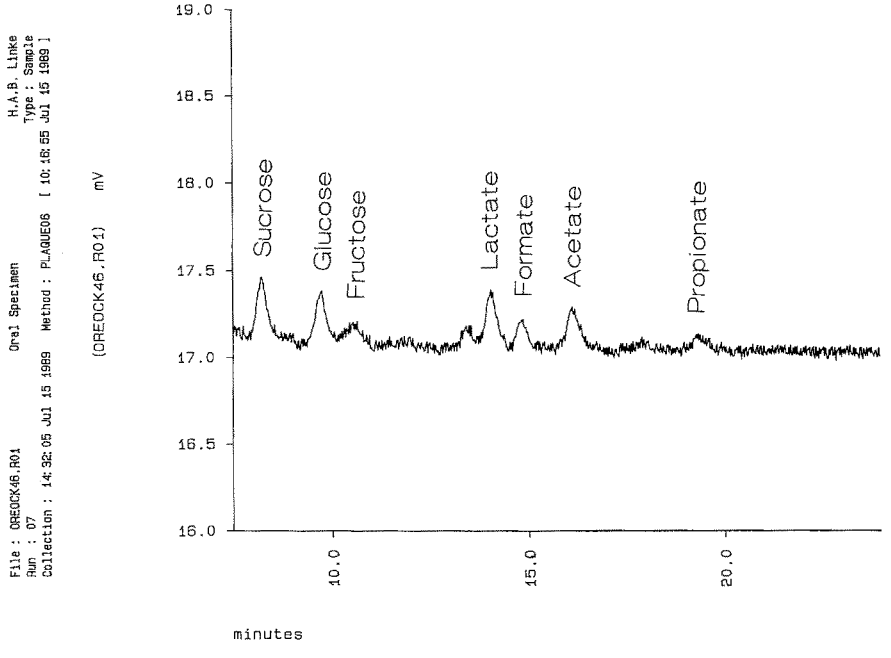

Fig. 1. HPLC analysis of an oral fluid sample 30 min after eating a cookie (for parameters see Materials and Methods).

necessary to add chemical enzyme inhibitors, such as azide, to the extract. Blind water extraction of the paper points yielded no carbohydrates or compounds which would interfere with the HPLC assay. Standard compounds added to an oral fluid extract were fully recovered. Figure 1 shows a typical HPLC assay plot of an oral fluid sample, obtained 30 min after eating a cookie.

Results and discussion

Peak retention times of standard compounds and their corresponding HPLC peak area are summarized in Table 1. The separation of most carbohydrates and organic acids was excellent, except for the disaccharides maltose, sucrose, and lactose. Their peak retention times were

Table 1. Peak retention times of standard compunds and their corresponding HPLC peak area*.

Compound (10 µg/10 µl injected)	Retention time (minutes)	HPLC peak area** (Omega-2 system)
Maltotriose	7.509	441 871
Maltose	8.171	435 586
Sucrose	8.245	679 400
Lactose	8.352	450 628
Glucose	9.667	462 248
Fructose	10.504	400 874
Lactate	13.947	46 956
Formate	14.768	196 252
Acetate	16.141	233 981
Propionate	19.291	341 208
Ethanol	21.275	205 786

*) 300 × 7.8 mm Aminex HPX-87H ion exclusion column (Bio-Rad Laboratories); mobile phase 0.01 N sulfuric acid; flow rate 0.6 ml/min at 30 °C; Differential Refractometer R 401 (Waters)
**) Sensitivity of detection 0.1 µg compound (in 80 µl)

Table 2. Sucrose content of oral fluid (30 min after ingestion of 25 g sucrose).

Volunteer	Ingestion time (s)	Amount (mg)[a] of oral fluid	HPLC peak area[b]	Sucrose (µg) in oral fluid	% Sucrose in oral fluid
1*	160	87.5	6 174	1.135	0.0013
2	470	81.0	59 408	10.925	0.0135
3	720	87.5	81 538	14.995	0.0171
4	590	89.0	26 144	4.808	0.0054
5	515	94.5	41 863	7.699	0.0081
6	460	92.0	42 003	7.724	0.0084
7	1155	81.0	53 499	9.838	0.0121
8	430	85.5	39 822	7.323	0.0086
Average	562.5	87.25	43 806	8.056	0.0093

*) Volunteer with high salivary flow;
[a]) Total amount of oral fluid obtained from volunteer;
[b]) From 80 µl injected oral fluid extract.

Table 3. Carbohydrate and organic acid content of oral fluid (30 min after eating 25 g of various foods)*.

Food	Maltose	Sucrose	Glucose	Fructose	Lactate	Formate	Acetate	Propionate
Chocolate bar	–	45 619	94 146	29 184	40 858	40 240	28 963	15 468
Potato chip	92 201	–	137 860	9 381	14 902	55 525	43 667	7 546
Cookie	–	44 284	89 480	21 871	34 772	53 482	38 642	20 013
Sugar cube	–	43 806	73 249	47 796	51 602	31 262	39 681	11 633
Raisin	–	51 306	173 718	127 026	71 599	22 195	39 938	12 914
Jelly bean	–	453 006	219 122	61 963	41 924	16 213	44 553	8 360

*) Data expressed in HPLC peak area. Injection volume 80 µl or oral fluid extract per food sample (for preparation of sample, see Materials and Methods section). Data averaged from eight volunteers for each food.

observed in the narrow range from 8.171 to 8.352 min, with an error margin of 0.050 min. Table 2 displays the sucrose content of oral fluid 30 min after ingestion of 25 g sucrose by eight volunteers. The sucrose data were found to be similar in all volunteers. However, the effect of certain uncontrollable physiological parameters, such as salivary flow, was clearly visible. The average amount of oral fluid collected in this experiment using the five paper point technique was 87.25 mg per sampling. Data on the carbohydrate and organic acid content of oral fluid taken 30 min after eating 25 g of various foods are presented in Table 3. Each individual HPLC peak area shown had been averaged from eight volunteers. The food "jelly bean" produced the highest amount of carbohydrates in oral fluid. Maltose was detected only in the food "chocolate bar". In this experiment (30 min sampling time) no lactose or ethanol was found in oral fluid.

The main goal of this investigation was to find a HPLC system to obtain separation of sugars and carboxylic acids in human oral fluid using a single column. The primary reson for choosing this analytical approach was the relatively low quantity of these compounds in oral fluid samples. HPLC analysis was found to be superior over chemical or enzymatic assay for the qualitative and quantitative determination of organic acids and carbohydrate sweeteners in oral fluid.

Previously, the carbohydrate content of oral fluid had been studied using the orcinol or gas-liquid chromatography (1) methods. Recently, glucose alone was determined in oral fluid spectro-photometrically (8) using Nelson's (4) arsenomolybdate, as modified by Somogyi (7). There are some excellent reviews available on the HPLC analysis of carboxylic acids (5) and carbohydrate sweeteners (6). As outlined in the introduction, none of the available HPLC methods were suitable for this described application.

Screening of the available HPLC carbohydrate separation columns led to the selection of Bio-Rad's Aminex columns. These types of columns are established ion moderation partition (IMP) resin-based HPLC columns. Aminex ion exchange resins are finely sized spherical beads of styrene-divinylbenzene copolymer with attached functional groups to optimize compound separation. Aminex columns are available for carbohydrate separation (HPX-87C) and for organic acid analysis (HPX-87H). The Aminex HPX-87C column used at 60 °C, with water as the mobile phase, produced excellent and reproducible HPLC separation of mono-, di-, and trisaccharides.

The analytical assay was accomplished at the same "run", using a refractive index detector on both compound classes (carbohydrates and organic acids), however, differences in peak areas occurred because of the distinct nature of refractive indices of the various compounds. The Aminex HPX-87C column was not useful for the separation of carboxylic acids. The specific chemical makeup of the packing material prevented separation of these compounds. Experimentation with the Aminex HPX-87H column, used at 30 °C in combination with 0.01 N sulfuric acid as mobile phase, led to excellent and reproducible separation of lactic, acetic, formic, and propionic acid, as well as ethanol. This column was also useful for the separation of mono-, di-, and trisaccharides. Use of the Aminex

HPX-87C column at temperatures of 60 °C or higher resulted in a better separation of disaccharides, but led to a breakdown of sucrose into glucose and fructose.

The use of common stainless steel tubing (1/16″ OD × 0.030″ ID) produced a large dead volume of the HPLC system, resulting in a lower sensitivity of the assay. However, by using stainless steel capillary tubing (1/16″ OD × 0.007″ ID) for the interconnection of the system components the sensitivity of the HPLC analysis was increased approximately tenfold.

Because the carbohydrate content of oral fluid (plaque, saliva, food remnants) varies to some extent within the human oral cavity, a serious problem arose during the development of the procedure to obtain and prepare a representative oral fluid sample for HPLC analysis. Organic acids are produced by bacterial fermentation of carbohydrates, mainly within dental plaque after food intake. The sampling technique using paper points as described under Materials and Methods was found to be superior. This sampling and preparation technique provided reliable and reproducible carbohydrate and organic acid data, a prerequisite for complex oral food clearance studies.

Because no interference was observed due to bacterial or mammalian enzymes in the oral fluid sample, use of chemical enzyme inhibitors, such as azide, was not necessary. More than 500 oral samples were analyzed using this HPLC method. Different techniques of data integration electronic (Omega-2, Chrom-Perfect) as well as manual, produced the same relative analytical results. As can be seen from Table 2 the quantitative data were affected by the physiology of the participating volunteers. Increased salivary flow and time needed to ingest the standardized food sample visibly influenced the analytical results of volunteer 1.

The new HPLC method was made almost fully automated by adding a computerized data collection and integration system. Commercially available hardware components were used in combination with Perkin-Elmer's data collection interface board and Omega-2 software (V 2.5). After manual injection of the sample the printout of the analyzed data was available after a 24-min running time. Some analytical data from the oral fluid samples were published earlier (2, 3). Figure 1 shows an original HPLC analysis of oral fluid 30 min after ingestion of a cookie. This plot is representative for the reported HPLC method, which provides a simple, reliable, and rapid analytical assay for sugars and organic acids in oral fluid.

In conclusion, this HPLC method allowed for separation and detection of both carbohydrate sweeteners and organic acids (and ethanol) in oral fluid. This task was accomplished using a single ion exclusion column in connection with a refractive index detector, with a detection sensitivity of 0.1 μg compound per HPLC assay.

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