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Determination of carbohydrates in fermentation processes by high-performance liquid chromatography

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Summary. HPLC is a universal, fast, accurate and selective method for the quantification of carbohydrates during fermentation processes. HPLC is not affected by complex constituents of fermentation media, such as meat extract, soybean meal or distillers solubles. The detection limit of the different investigated carbohydrates by refractive index monitoring ranges between 20 and 40 mg/l using a cation-exchange resin and between 50 and 100 mg/l using amino- or diol-bonded phases.

Introduction

Carbohydrates are the most abundant compounds found in nature and the analysis of sugars and sugar mixtures used as substrates in microbial growth is of considerable importance in fermentation technology.

The application of HPLC for analysis of carbohydrates in complex and chemically defined fermentation media has a lot of advantages compared to traditional methods. Analysis by HPLC is a rapid, selective and quite convenient method, while wet chemical methods incur interferences from other saccharides (Chaplin and Kennedy 1986). Enzymatic determination as a preferentially used method for quantification of sugars is sometimes falsified by interfering substances in the culture broth of microorganisms. In addition, enzymatic tests are restricted to just one compound and the specific determination of disaccharides demands their cleavage into monosaccharides (Bergmeyer 1974). Gas-liquid chromatography (GLC) normally requires derivatization before analysis and often has difficulties in the appearance of multiple peaks in the chromatogram (Gianetto et al. 1986). This may be a problem if several similar sugars have to be separated and analysed. Therefore, the introduction of amino-bonded phases, diol-bonded phases and ion-exchange resins has led to many different new applications using HPLC for carbohydrate determination (Linden and Lawhead 1975; Brons and Olieman 1983; Rajakylä and Paloposki 1983; Du Toit et al. 1984).

The present study has been undertaken in order to show that carbohydrate quantification using HPLC with refractive index detection offers a rapid analysis method for a large spectrum of saccharides requiring a minimum of sample preparation. We can show that also carbohydrates in complex fermentation media may be analysed with high reproducibility.

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Material and methods

Chromatographic conditions. Three HPLC columns were used: (1) Nucleosil-NH_2 (5 µm) 125 mm × 4.6 mm internal diameter (ID), guard column 20 mm × 4.6 mm ID (Grom, Ammerbuch, FRG). The eluent was acetonitrile/water (75 + 25) and the flow rate 1 ml/min at room temperature; (2) LiChrosorb-Diol (5 µm) 250 mm × 4.6 mm ID, guard column 20 mm × 4.6 mm ID (Grom). The eluent was acetonitrile/water (75 + 25) and the flow rate 1 ml/min at room temperature; (3) Aminex HPX-87C ion exchange column (9 µm) 300 mm × 4 mm ID, guard Carbo-C cartridge 30 mm × 4 mm ID (Bio-Rad, München, FRG). The eluent was water and the flow rate 0.3 ml/min at 85° C.

HPLC equipment. An HP 1050 isocratic pump, HP 1037A refractive index detector thermostated at 40° C, HP 3396A integrator (Hewlett-Packard, Waldbronn, FRG), an MSI 660 autosampler (Kontron, München, FRG), and an HPLC column heater (Bio-Rad) were used.

Chemicals. Acetonitrile (HPLC grade) was obtained from Merck (Darmstadt, FRG). Water was purified by means of a Milli-Q-system (Millipore, Eschborn, FRG).

Table 1. Carbohydrate selectivity of amino-bonded, diol-bonded and cation-exchange HPLC columns

Carbohydrates	HPLC column				
	Nucleosil-NH_2		LiChrosorb-Diol		Aminex HPX-87C
	Retention time (min)	k'^b	Retention time (min)	k'^b	Retention time (min)
Monosaccharides					
Arabinose	5.17	1.87	5.40[a]	0.58	7.67
Fructose	5.55	2.08	6.32[a]	0.85	7.82
Glucose	6.30	2.50	6.74	0.97	6.14
Sugar alcohols					
Mannitol	6.31	2.51	6.83	1.00	9.95
Inositol	11.80	5.56	9.06	1.65	7.65
Disaccharides					
Sucrose	10.26	4.70	8.27	1.42	5.24
Maltose	11.10	5.17	9.06	1.65	5.29
Lactose	12.68	6.04	9.39	1.75	5.41

[a] Double peak
[b] Column capacity factor

Sample pretreatment. The fermentation samples were centrifuged for 2 min at 10000 *g*. The supernatants (10 µl) were injected automatically onto the HPLC column.

Results

Separation of carbohydrate standards

The selectivity of different stationary phases for the separation of monosaccharides, disaccharides and sugar alcohols is shown in Table 1. The amino-bonded phase has the best separation selectivity for all the carbohydrates investigated, demonstrated by the differences in retention times and k' values, respectively. With the exception of glucose and mannitol eluting in one peak, all compounds are separated in one chromatographic run. The same limitation is given using the diol-bonded phase; additionally, fructose and arabinose are eluted as double peaks. In contrast to these columns, the cation-exchange resin lacks separation selectivity for different carbohydrates, because arabinose, fructose and inositol elute in the same peak, as well as sucrose, maltose and lactose.

The HPLC analyses of carbohydrate standard solutions using an amino-bonded phase and an optimized solvent composition are shown in Fig. 1. The quantification of compounds is faciliated by a linear response in the range of analytical interest. With refractive index monitoring, linearity is achieved in a concentration range between 0.1 g/l and 20 g/l, such carbohydrate concentrations being commonly used in complex or chemically defined fermentation media.

Figure 2 shows the correlation between glucose and sucrose concentrations and peak areas and its dependence on the stationary phase. The detectability with a differential refractometer is very much a function of the mobile phase and is influenced by other variables such as solute and column plate count. Because the detection limit is defined as a signal-to-noise ratio of 2, the mini-

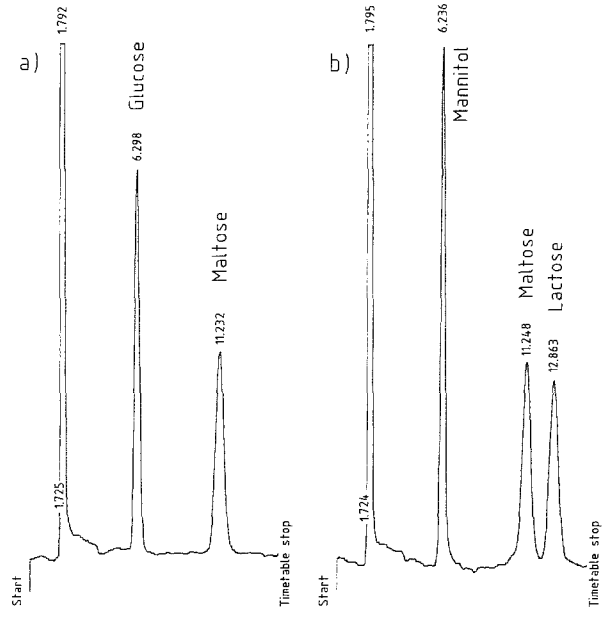

Fig. 1. HPLC separation of carbohydrate standards with amino-bonded phase and refractive index monitoring: **a)** glucose, maltose (10 mg/ml); **b)** mannitol, maltose, lactose (10 mg/ml)

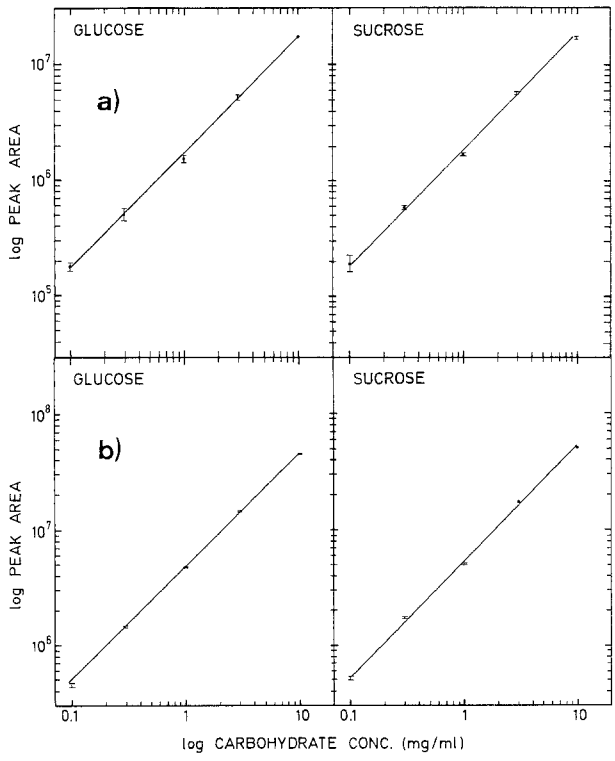

Fig. 2. Linear dynamic range of carbohydrate determination by HPLC: **a)** separation with amino-bonded phase; **b)** separation with cation-exchange resin

mum concentration of the different carbohydrates that can be adequately detected ranges between 50–100 mg/l using amino-bonded and diol-bonded phases, respectively, and 20–40 mg/l using the cation-exchange resin.

The accuracy of the method was tested by assaying the carbohydrate standards 20 times, as shown in Table 2. The reproducibility of both the peak areas and the retention times proved the system to be suitable for routine analysis.

Separation of fermentation samples

The aim of our investigations was to demonstrate that HPLC evaluation of carbohydrates may be performed both with discrete standard solutions and with fermentation media containing more than one carbohydrate compound, e.g. malt extract, which contains a mixture of maltose, glucose and sucrose. The efficiency of amino-bonded phase HPLC in the determination of carbohydrates in such complex media is compared with that of cation-exchange HPLC (Figs. 3, 4). The sugar components of malt extract cannot be determined by cation-exchange HPLC, because sucrose and maltose elute in the same peak, in contrast to amino-bonded phase HPLC.

Other complex media constituents, such as meat extract, soy bean meal or distillers solubles do not affect the HPLC evaluation of carbohydrates, particularly in the case of the amino-bonded phase.

Discussion

The quantification of carbohydrates in complex fermentation media by HPLC has been demonstrated to be a universal, rapid, accurate and selective method requiring a minimum of sample pretreatment. Although HPLC is not free from some problems. The disadvantages of amino- and diol-bonded phase HPLC are first the poor stability of the columns during routine analyses, being characterized by shortened retention times and complicated calibration procedures. The column lifetimes are limited to approximately 800 injections of biological samples. Second, the reproducibility and sensitivity is decreased compared to cation-exchange HPLC.

Cation-exchange HPLC shows advantages in reproducibility and detection sensitivity, due to the refractive index of water as the mobile phase, resulting in both the better response of the solute and signal-to-noise ratio. The requirement of column heating at 85° C demands a control of the column oven by the HPLC system, because the heating without eluent flow, e.g. in case of overpressure, leads to irreversible damage to the co-

Table 2. Relative standard deviation of peak areas and retention times[a]

	Glucose		Sucrose	
	Peak area	Retention time	Peak area	Retention time
Amino-bonded phase	1.62%	0.22%	1.23%	0.27%
Diol-bonded phase	1.55%	0.20%	0.22%	0.28%
Cation-exchange resin	0.21%	0.05%	0.10%	0.05%

[a] Based on 20 replicate analyses; carbohydrate concentration = 3 mg/ml

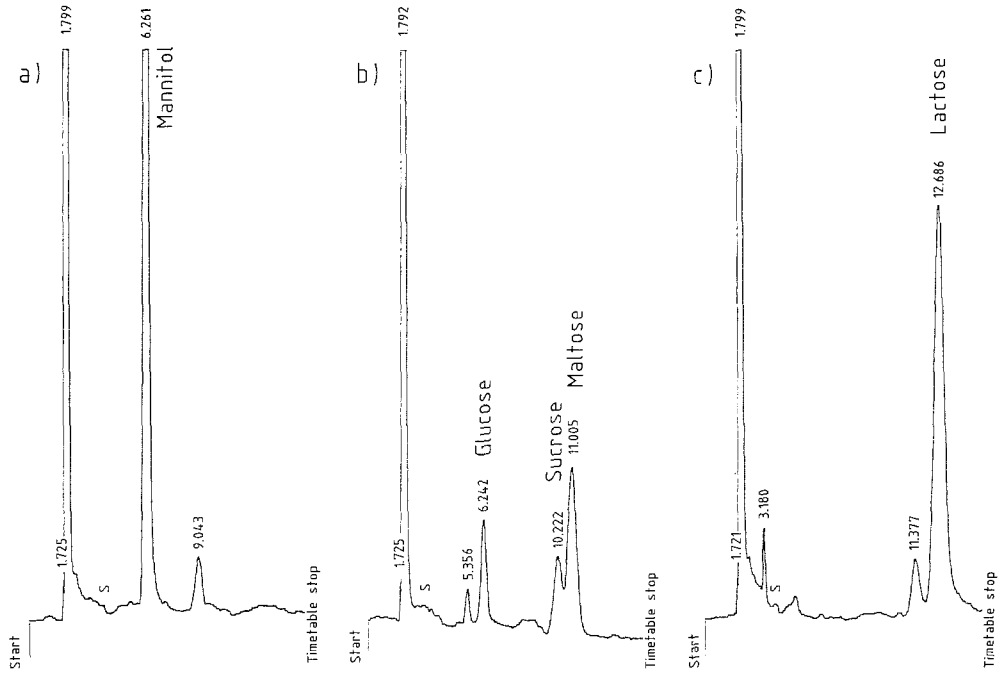

Fig. 3a–c. HPLC separation of carbohydrates in complex fermentation media with amino-bonded phase; **a** 20 g mannitol, 20 g soybean meal, 1 l tap water, pH 7.5; **b** 20 g malt extract, 10 g calcium carbonate, 1 l tap water, pH 7.2; **c** 20 g lactose, 20 g distillers solubles, 5 g sodium chloride, 1 g sodium nitrate, 1 l tap water, pH 7.2

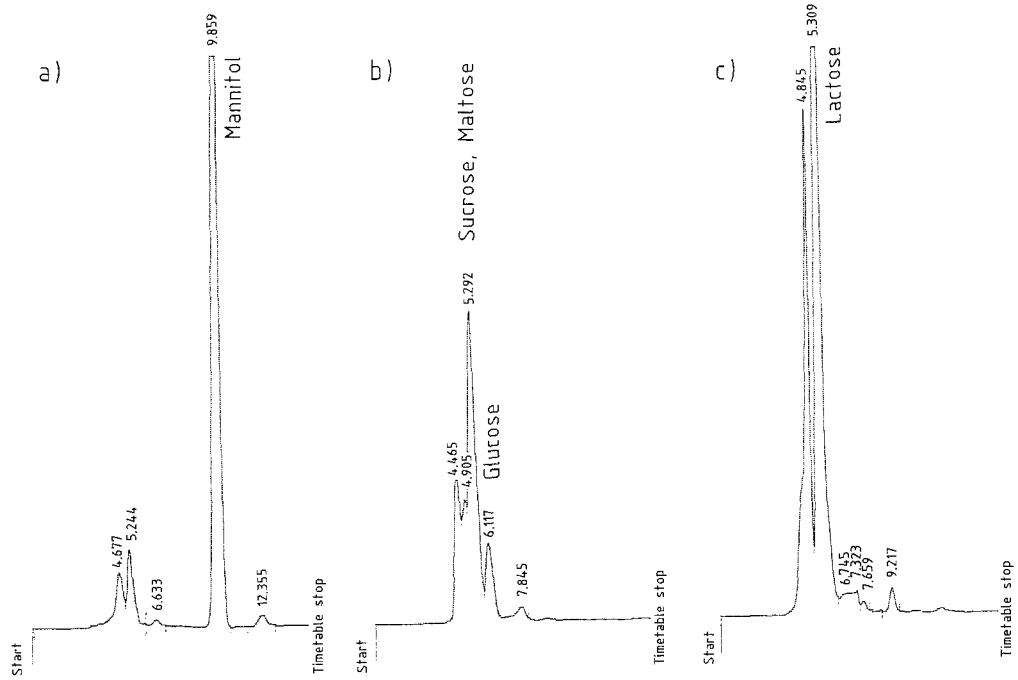

Fig. 4a–c. HPLC separation of carbohydrates in complex fermentation media with cation-exchange resin; same media composition as in Fig. 3

lumn. Further disadvantages of cation-exchange HPLC are given in the reduced selectivity to various carbohydrates, especially disaccharides, and in enhanced prime costs compared to amino- and diol-bonded silica columns.

Nevertheless, the advantages of these HPLC

techniques in the quantification of carbohydrates are obvious. We have successfully used this method for routine analyses in fermentation process control.

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