ANALYSIS OF SUGARS BY MICELLAR LIQUID CHROMATOGRAPHY WITH UV DETECTION

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SUMMARY

A micellar liquid chromatographic procedure is described for simultaneous determination of sugars previously derivatized with 4-aminobenzoic acid ethyl ester (4-ABEE). The optimized conditions, determined by use of the super-modified simplex method, were temperature 30°C, mobile phase containing 17.3 mM sodium dodecyl sulfate, 0.01 M phosphate buffer, pH 6.69, and 7.73% (v/v) ethanol, and UV detection at 307 nm. The 4-ABEE derivatives were separated in 10 min but a peak assigned to excess reagent appears at 13 min. Analytical data including linearity ($r > 0.9987$), limit of detection (35.47–81.74 $\mu g\,mL^{-1}$), precision ($RSD < 5.73\%$), and accuracy, measured by determination of recovery (>91%), support the usefulness of the proposed method for measuring sugars. When the proposed method was applied to the analysis of sugars in an infant formula and a sample of sorghum syrup the results were in good agreement with the content reported by manufacturer and with results obtained by refractive index detection.

INTRODUCTION

Carbohydrates are among the most abundant compounds in the plant world, and the analysis of sugars and sugar mixtures is of considerable importance to the food and beverage industries [1]. A variety of chromatographic systems including paper and thin-layer chromatography, gas–liquid chromatography with flame ionization or mass spectrometric detection, and high-performance liquid chromatography (HPLC) can be used to separate and analyze monosaccharides [1].

In HPLC, detection of unmodified carbohydrates can only be performed spectroscopically at 195 nm or by amperometric or refractive index detection, all of which are associated with low sensitivity [2]. Derivatization of carbohydrates serves multiple purposes [3]. Derivatization with

UV-absorbing or fluorescent molecules significantly enhances detection sensitivity of HPLC, capillary electrophoresis (CE), and polyacrylamide gel electrophoresis (PAGE). Most carbohydrates are hydrophilic and neutral. Derivatization with appropriate reagents changes these properties and can assist their resolution, e.g. it can endow them with charge to facilitate their electrophoretic separation or with hydrophobicity to enable their efficient resolution by reversed-phase HPLC or micellar electrokinetic chromatography (MEKC).

As recently reviewed by Lamari et al. [2] one of the most popular reactions used for derivatization of carbohydrates is reductive amination; a large variety of reagents, including UV active and fluorophore derivatization reagents such as 4-aminobenzoic ethyl ester (4-ABEE) [4–8], have been suggested for this purpose. Derivatization of reducing sugars with 4-ABEE is easy and requires no special equipment. The method is also highly sensitive and eliminates the doublet that can arise as a result of mutarotation of the free reducing end of the sugars. 4-ABEE-derivatized monosaccharides have been separated on an amino-bonded vinyl alcohol copolymer gel column [7], on C_{18} columns [4,6], and on narrow-bore C_8 columns [8].

Micellar liquid chromatography (MLC) has several advantages over other chromatographic methods, including low cost, low toxicity, low volatility, the possibility of simultaneous separation of ionic and non-ionic compounds, direct injection of biological fluids, and high separation selectivity, because of the involvement of many adjustable conditions [9].

The primary objective in the development of MLC separations is to optimize chromatographic performance by adjustment of such experimental conditions as temperature, type and concentration of organic modifier, pH, and concentration of surfactant [9]. HPLC separations are mainly modified by varying the composition of the mobile phase. Optimization is often effected by varying conditions one at a time, while keeping the others fixed, until an optimum is reached for each variable. But this is far from adequate and requires many experiments to be performed. This can be very time-consuming and, probably more important for complex chemical systems, interactions between the variables might mean that the optimum obtained will depend on the initial conditions chosen. Several different experimental designs have been developed to reduce the number of experiments and the amounts of chemicals used. The iterative regression optimization strategy is the most popular method for simultaneous optimization of different conditions in MLC separations [10–12]. The simplex method,

one of the most efficient optimization designs, was first introduced in HPLC separations by Morgan and Deming [13,14]. The modified simplex procedure was developed to overcome the disadvantages of the original simplex method [15] and has been used in MLC by Srijaranai and co-workers [16]. The super-modified simplex (SMS), which was introduced by Routh and co-workers [17], is a modification of the modified simplex method.

In this paper we report, for the first time, the separation and determination of sugars including glucose, xylose, arabinose, maltose, and lactose by MLC. The conditions, including sodium dodecyl sulfate (SDS) concentration, pH, and amount of ethanol in the mobile phase, were optimized by use of the SMS method. The optimized method was applied to the analysis of sugars in an infant formula and in a sample of sorghum syrup.

EXPERIMENTAL

Reagents

Analytical-grade glucose, maltose, lactose, xylose, arabinose, 4-aminobenzoic acid ethyl ester, sodium cyanoborohydride, HPLC-grade methanol, acetonitrile, SDS, sodium dihydrogen phosphate, sodium monohydrogen phosphate, and reagent grade ethanol, 1-propanol, 2-propanol, and 1-butanol all were purchased from Merck (Darmstadt, Germany). Doubly distilled, deionized water was used in all experiments.

A 500 mM stock solution of SDS was prepared in filtered water and diluted with water. Organic solvents and phosphate buffer were added to the mobile phase where necessary. All mobile phases and solutions were filtered through 0.45 μm pore size Nylon membranes (Millipore, Bedford, MA, USA).

Apparatus

Chromatography was performed with a Crystal 200 series HPLC pump (ATI Unicam, Cambridge, UK) equipped with a Rheodyne (Cotati, CA, USA) model 7125 manual injector with 20-μL loop, a PU4225 UV detector (Philips, Cambridge, UK), a RefractoMonitor IV refractive-index detector (LDC Analytical, FL, USA), a Kromasil 100-10NH$_2$ column (10 μm, 250 mm × 4.6 mm i.d.) from Hichrom (Berkshire, UK), a Spherisorb ODS analytical column (5 μm, 100 mm × 3.9 mm) from Unicam (Cambridge, UK), and RP-18 and amino guard columns (7 μm, 15 mm × 3.2 mm) from Applied Biosystems (San Jose, CL, USA). The analytical column was

water-jacketed and thermostatted with an NB-33722 Ultra-Thermostat (Colora, Lorch, Germany). The mobile phase flow rate was 1 mL min^{-1}. The dead volume was determined by injecting water. Chromatographic calculations were performed by means of a Unicam 4880 data-handling system.

Derivatization Procedure

The sugars were derivatized with 4-ABEE, at their reducing end, by the method of Wang et al. [18]. Each sugar, infant formula (Karicare, Nutricia, New Zealand), and sorghum syrup were separately weighed (500.0 mg) and dissolved in 5 mL water. These solutions were always freshly prepared. The solutions were diluted to the desired concentrations immediately before analysis. Each solution (50 μL) was mixed with 200 μL reagent mixture (prepared by mixing 1 mmol 4-ABEE, 35 mg sodium cyanoborohydride, 41 μL glacial acetic acid, and 350 μL methanol) and the mixture was heated at 80°C for 30 min. After cooling to ambient temperature 5 mL distilled water was added. The aqueous phase was extracted with 5 mL chloroform to remove excess 4-ABEE and the aqueous layer was subjected to HPLC analysis.

RESULTS AND DISCUSSION

Optimization of Temperature and Type of Organic Modifier

Although it is not usually possible to set valid rules for the effect of temperature on HPLC separations, the column temperature affects pressure, analysis time, and separation [19]. The lower efficiency of MLC compared with conventional reversed phase liquid chromatography (RP-LC) is because of the higher viscosity of the micellar mobile phase. Column efficiency could be improved at higher temperatures, because of faster mass transfer of solute between mobile and stationary phases. For several reasons, however, for example decomposition of sample or mobile phase, bubble formation, effect on temperature-dependent chromatographic equilibria, and increasing solubility of silica, efficiency may decrease with increasing temperature [19]. The basic criteria for determination of optimum temperature were the number of theoretical plates, N, and the asymmetry factor, B/A [20]; the effect of temperature on these was investigated using glucose (Glu) and maltose (Mal) derivatives as samples and 50 mM SDS as mobile

phase. From data listed in Table I it was apparent that the most appropriate temperature for separation of these compounds was 30°C.

Table I

Effect of temperature on the chromatography of the 4-ABEE derivatives of glucose and maltose

Temperature (°C)	k		B/A		N	
	Glu	Mal	Glu	Mal	Glu	Mal
25	6.15	5.10	2.31	1.80	185	197
30	5.70	4.80	2.38	1.80	194	274
35	5.40	4.45	2.50	2.14	176	182
40	5.30	4.45	3.05	2.52	160	166

Addition of short-chained alcohols to the micellar mobile phase reduces the thickness of the film of surfactant molecules covering the stationary phase and, thus, enhances the efficiency [21]. The presence of the alcohol in the micellar mobile phase also alters the mechanism of retention by shifting the equilibria of the solutes from the stationary phase and the micelle toward the bulk aqueous phase, which leads to reduction of capacity factors [22,23]. N and B/A were again used to select the best organic modifier (Table II). The mobile phase was 50 mM SDS containing 5% (v/v) organic modifier and the column temperature was 30°C. The results showed that substantial improvement of chromatographic efficiency could be achie-

Table II

Effect of organic modifiers on chromatographic data for the 4-ABEE derivatives of glucose and maltose

Organic modifier	k		B/A		N	
	Glu	Mal	Glu	Mal	Glu	Mal
None	5.70	4.80	2.38	1.80	194	274
Methanol	4.60	3.97	2.13	1.67	265	476
Ethanol	4.22	3.40	2.02	1.76	473	522
Acetonitrile	3.54	2.97	2.05	1.88	424	369
1-Propanol	3.15	2.59	2.54	2.03	397	535
2-Propanol	3.45	2.88	2.97	2.62	204	207
Butanol	2.23	1.83	3.60	3.09	240	197

ved by addition of organic solvent. Ethanol and 1-propanol were the most suitable for efficient separation of these compounds; because of the high hydrophilicity of sugars better selectivity was observed when ethanol was used.

Simultaneous Optimization of Ethanol Content, pH, and SDS Concentration

The SMS program, which was written in GW-Basic, was used for optimization of the conditions regarded as most important for separation of the mixture of sugar derivatives – amount of modifier (ethanol), SDS concentration, and mobile phase pH. The chromatographic response function (*CRF*) given by eq. (1), was chosen as the criterion for the optimization process [24].

$$CRF = \sum_{i=1}^{n-1} R_i + n^a - b|T_A - T_L| + c(T_1 - T_0) \tag{1}$$

where R_i is the resolution of adjacent peak pairs, n is the number of peaks detected, T_A is a specified analysis time (30 min in this work), T_L and T_1

Table III

Progress of the SMS toward optimum conditions for analysis of the sugars

Experiment number	SDS concentration (mM)	Ethanol content (%, *v/v*)	pH	*CRF*
1	20	7	6	12.59
2	40	3	3	7.01
3	30	5	4	8.52
4	50	2	5	4.57
5	100	8	3.67	4.39
6	36	4.1	4.53	8.14
7	17.3	7.73	6.69	13.65
8	17.7	7.66	6.63	13.44
9	26.6	5.82	5.24	12.84
10	20	7	6	12.59
11	18.5	7.29	5.85	13.56
12	21.4	6.77	5.63	13.39
13	22.8	6.5	5.46	13.07
14	24.1	5.86	4.77	12.99

are the retention times of the last and first peaks, respectively, T_0 is a specified minimum retention time (8 min in this work), and a, b and c are operator-selectable weightings (3/2, 0.2 and 1, respectively).

The super-modified simplex program was started by introducing lower and upper boundaries for the three conditions ethanol concentration (1–10%), micelle concentration (10–100 mM), and pH (3–7). The initial simplex consisted of the first four vertices (one more than the number of variables). Experiments 1–4 in Table III show the values of the three initial simplex conditions. The experimental conditions and calculated *CRF* values are also presented in the table. Experiment 7 indicates the optimum conditions with the highest *CRF* value. All calculations were performed using worksheets. The simplex was halted at experiment 14, because there was no significant further improvement toward maximization of the *CRF* value, as shown in Fig. 1.
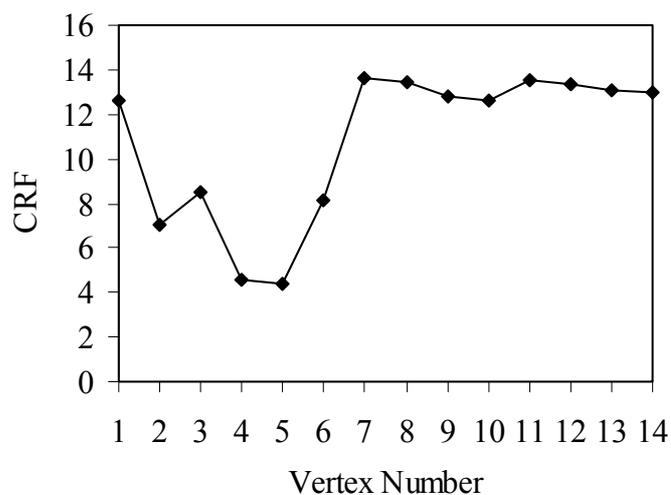

Fig. 1

CRF values corresponding to the experiment numbers during SMS optimisation

Figure 2 shows the chromatogram obtained under the optimum conditions (vertex 7). It can be seen that the disaccharides and monosaccharides have been separated in a reasonable time and with good resolution.
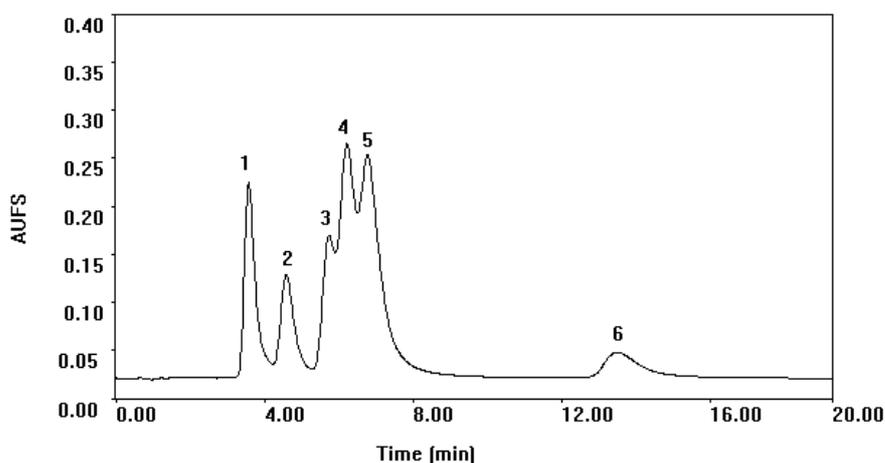
Fig. 2

Chromatogram obtained from 4-ABEE derivatives of the sugars. Column, Spherisorb ODS (5 μm, 100 mm × 3.9 mm); mobile phase, 17.3 mм SDS, 7.73% (*v/v*) ethanol, 0.01 м phosphate buffer pH 6.69 at 30°C; flow rate, 1 mL min^{-1}, injection volume, 20 μL; detection at 307 nm, AUFS = 0.5. Peaks: 1, lactose (0.50 mg); 2, maltose (0.50 mg); 3, glucose (0.25 mg); 4, arabinose (0.25 mg); 5, xylose (0.25 mg); 6, excess 4-ABEE

Validation

The linearity of the dependence of response on concentration was verified by triplicate analysis of nine standard solutions containing 0.125–20 mg mL^{-1} of each sugar, with xylose as internal standard (Table IV). The calibration plots for all the sugars revealed good correlation between the peak area and sugar concentration; the regression coefficients were al-

Table IV

Results from linear least-squares analysis of calibration data obtained from analysis of the sugars

Sample	Property				
	Linear range (mg mL^{-1})	Slope	Intercept	*R*	*LOD* (μg mL^{-1})
Lactose	0.125–20	0.0305	0.0079	0.9989	67.23
Maltose	0.125–20	0.0476	−0.0046	0.9990	81.74
Glucose	0.300–20	0.0863	−0.0264	0.9997	46.23
Arabinose	1.000–20	0.0731	−0.0014	0.9987	35.47

ways >0.9987. The precision of the method was investigated using a synthetic sample. Complete analysis of the sugars was performed in triplicate to calculate the average deviations as a measure of chromatographic reproducibility. The relative standard deviations obtained from analysis of the sugars are presented in Table V. The results show the precision of the proposed method, under the optimized conditions, is relatively high – relative standard deviations (*RSD*) were ≤5.73%.

Table V

Results from studies of the recovery of sugars added to an infant formula

Sample	Concentration added (mg mL^{-1})	Concentration found (mg mL^{-1})	*RSD* (%)	Recovery (%)
Lactose	0.00	7.74	0.76	–
	2.00	9.60	1.71	98.06
	10.00	16.99	2.10	97.28
Maltose	2.00	1.82	4.36	91.00
	10.00	10.89	5.73	108.93
Glucose	2.00	2.00	3.50	100.00
	10.00	10.74	5.00	107.39
Arabinose	2.00	2.08	2.01	104.00
	10.00	9.97	5.31	99.74

Detection limits, defined as the quantities producing a peak height signal three times the background noise, were determined by use of external standards. The values obtained are listed in Table IV.

Validation of the recovery of the method was achieved by analysis of the infant formula sample spiked at two different concentrations. The average recoveries obtained, which ranged between 97% and 122%, testify to the accuracy of the method (Table V). There is good agreement between the amount of lactose measured by use of this method (61.92%) and the amount reported by manufacturer (54.00%).

Sorghum Syrup Analysis

Sorghum syrup is a natural sweetener made by processing juice squeezed from the seed or stalks of certain types of sorghum (*Sorghum bicolor*) called sweet sorghum or sorgo. Sweet sorghum is grown for syrup or forage, whereas most other sorghums, commonly referred to as milos or kafirs, are grown for grain. Sweet sorghums resemble grain sorghum at

maturity except they are approximately three times taller, reaching a height of 4 m or more [25]. To evaluate the applicability of the proposed method, sorghum syrup was analyzed and results were compared with those obtained by HPLC on an NH_2 column with refractive index detection and 75% acetonitrile in water as mobile phase. As is apparent from Table VI, there is good agreement between the results obtained by use of the proposed method and those obtained by conventional HPLC. Figure 3 shows a typical chromatogram obtained from natural sorghum syrup.

Table VI

Results from determination of sugars in sorghum syrup

Compound	Amount measured by conventional HPLC (%)	Amount obtained by MLC (%)
Glucose	32.1 ± 0.2^a	33.7 ± 1.7
Maltose	11.9 ± 0.9	9.6 ± 0.6

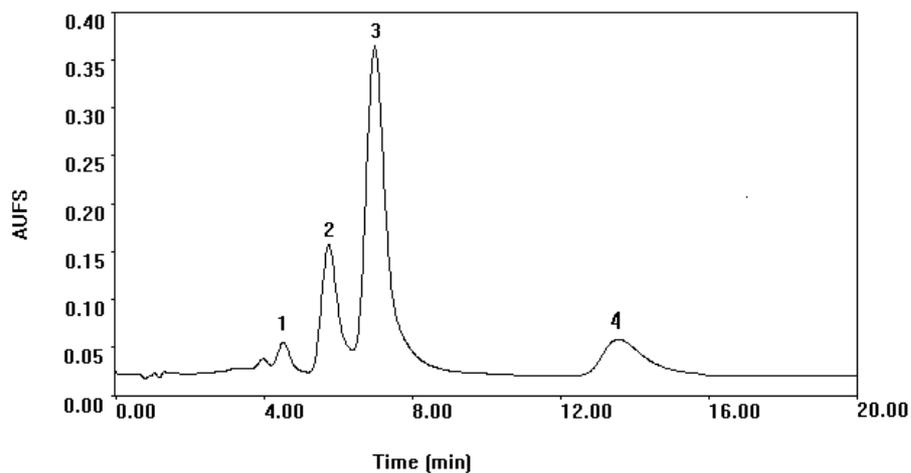[a]Results are means $\pm$ SD ($n = 3$)

Fig. 3

Chromatogram obtained from 4-ABEE derivatives of sugars from sorghum syrup. Peaks: 1, maltose; 2, glucose; 3, xylose; 4, excess 4-ABEE. Conditions as for Fig. 2.

CONCLUSION

Application of the SMS optimization procedure and the advantages of MLC resulted in successful separation and determination of sugars with good sensitivity and less use of toxic reagents in a reasonable time. The results of the work confirmed the usefulness of the proposed method for analysis of sugars in food samples. The method is environmentally sound and uses little toxic organic solvent.

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