Journal of Chromatography, 282 (1983) 169–177
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 131

IMPROVEMENTS IN SENSITIVITY AND RESOLUTION WITH THE CYANOACETAMIDE REACTION FOR THE DETECTION OF CHROMATOGRAPHICALLY SEPARATED REDUCING SUGARS

TIMOTHY D. SCHLABACH* and JOHN ROBINSON
Varian Instrument Group, Walnut Creek Instrument Division, 2700 Mitchell Drive, Walnut Creek, CA 94598 (U.S.A.)

SUMMARY

Fluorescence detection of reducing sugars with the cyanoacetamide reaction can be greatly enhanced by elevating the temperature of the postcolumn reaction. The reaction exhibits a several-fold enhancement when the temperature is increased from 105 to 135°C. This increase in signal is not accompanied by a change in baseline noise, so that 5- to 10-fold improvements in sensitivity are realized for ribose and glucose, respectively. For a desired sensitivity level, higher temperature permits shorter reaction times and reaction coils that have smaller diameters. Substantial improvements in band broadening are possible by use of 0.01-in. I.D. reaction coils instead of the 0.02-in. I.D. coils used in previous work.

The cyanoacetamide reaction is also compatible with sugar separations on amino columns. Reducing the borate concentration permits substantial amounts of organic modifiers, such as acetonitrile, to be used in the mobile phase without incurring precipitation of borate salts. Gradient separation of reducing sugars is also feasible on the amino column with cyanoacetamide-reaction detection.

INTRODUCTION

Several reactions have been employed in the detection of sugars after chromatographic separation. Both colorimetric[1,2] and fluorimetric[3,4] reactions have been developed, primarily as alternatives to the classical detection methods that required corrosive acids[5,6]. The colorimetric methods tend to suffer from higher baseline offset and noise than fluorimetric techniques, because the reagent typically has much greater absorbance than the mobile phase. Generally, the fluorescence difference between the mobile phase and the reagent is much smaller.

Derivatives of malonamide[7] or ethylenediamine[4] have proven to be very useful for the fluorimetric detection of sugars with a post-column reaction system. Cyanoacetamide has proven a most effective derivative[8] for use in the automated analysis of reducing sugars. The only drawback to this detection scheme is the relatively long reaction time (*ca.* 90 sec) and consequent band broadening. The initial work on this detection method was done at reaction temperatures of *ca.* 100°C, but tem-

0021-9673/83/$03.00

peratures in excess of 100°C were reported to accelerate greatly the reaction of ethanolamine with reducing sugars[5].

It occurred to us that perhaps the reaction of cyanoacetamide can be likewise accelerated by elevated temperatures. The relation of reaction temperature to band broadening was examined to determine if any improvements are possible in the current reaction conditions. Additionally, sugar detection with the cyanoacetamide reaction was attempted after separation on an amino column to determine whether acetonitrile mobile phases are compatible with this reaction.

EXPERIMENTAL

Apparatus

Chromatography was performed with a Varian Model 5060 ternary liquid chromatograph (Varian, Palo Alto, CA, U.S.A.). The post-column reaction system including temperature control device, was also from Varian. Varian's Fluorichrom detector was equiped with a 334-nm interference filter for excitation and a 5-58 band filter on the emission side. Additional reaction coils were purchased from Scientific Systems (State College, PA, U.S.A.). Data were collected and analyzed with the Varian Vista 402 data system. All reaction coils were 10 m in length and coiled around a 3-cm form. Coil diameters of 0.23, 0.38, 0.51 mm I.D. were used.

Columns and reagents

The 30 x 0.7 cm I.D. Aminex HPX-85C column and the 15 x 0.46 cm I.D. MicroPak-NH_2 columns were supplied by Varian. The latter column was converted to the phosphate form by conditioning with 1% phosphoric acid as specified by the manufacturer.

The 5% cyanoacetamide reagent was prepared in 0.1 *M* potassium borate (pH 10.4). Higher salt concentrations produce precipitates when mobile phases containing acetonitrile are employed. The reagent was dissolved by sonication and prepared fresh daily.

Glass-distilled water was the eluent for the Aminex column. The Aminex column was maintained at 75°C for all separations. Sugars were eluted from the amino column with 70% acetonitrile in water unless otherwise indicated. The mobile phase flow-rate for both columns was 0.8 ml/min.

Chromatography

A Rheodyne 7410 injection valve (Cotati, CA, U.S.A.) was placed between the column and the postcolumn reaction system to characterize the system and check the consistency of the reaction times by directly injecting 1 μl of a sugar solution. The postcolumn flow-rate was initially adjusted to deliver 0.4 ml/min. Later adjustments were based on the reaction time for a particular reaction coil.

A backpressure restrictor from Varian was used for operation at 120°C, and a 10-m coil of 0.23-mm (0.009-in) I.D. tubing was connected to the fluorescence detector outlet for higher temperatures.

RESULTS AND DISCUSSION

Reaction temperature

The reaction of ethanolamine borate with reducing sugars was reported to have an optimum at reaction temperatures *ca.* 150°C[3]. The reaction product is presumably analogous to that formed with cyanoacetamide. The optima in the fluorescence spectra of their reaction products with glucose were found to be similar[4]. The apparent similarity in both reactions suggests that elevated temperatures should also greatly accelerate the formation of a fluorescent product with glucose and cyanoacetamide.

A standard solution of glucose was directly injected into the postcolumn reaction system to test the effect of temperature on observed response. The peak resulting from a 20-μg injection of glucose is plotted as a function of temperature in Fig. 1. The sigmoidal shape of the reaction curve is similar to that observed with ethanolamine borate[3]. This was verified by repeating the experiment with the ethalolamine reagent. The curve was nearly identical, but displaced *ca.* 10°C towards higher temperature.

The effect of temperature was tested under chromatographic conditions by injecting sugar standards into an amino column and eluting them with 75% aceto-

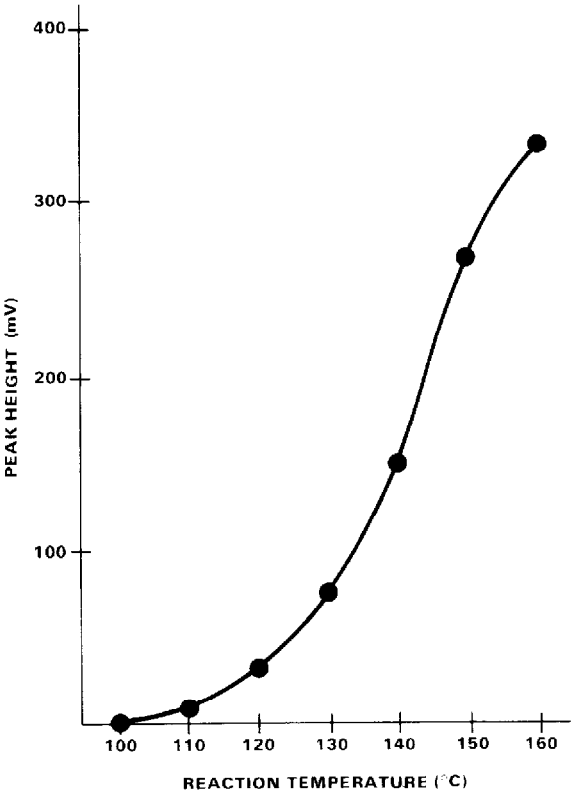


Fig. 1. Effect of temperature on the response of the cyanoacetamide post-column reaction to glucose. The reaction time was 90 sec with a 30 ft. × 0.02 in. I.D. reaction coil.

nitrile in water. The chromatograms at different reaction temperatures are shown in Fig. 2. Elevating the reaction temperature by 30°C increased the ribose response *ca.* five-fold and the maltose peak twenty-fold. The increases for fructose and glucose were *ca.* sixteen- and twelve-fold, respectively. These gains in signal were not accompanied by a comparable increase in signal noise, which was typically less than 0.1 mV, peak-to-peak. At a signal-to-noise ratio of 3 the detection limits are 45 ng and 91 ng for glucose and maltose, respectively, for a 60-sec reaction. Doubling the reaction time can also improve the detection limits by about a factor of 2. Outgassing at the higher temperature was prevented with another coil of 0.009 in. I.D. tubing. This tubing produced a backpressure that exceeded the vapor pressure of water at 135°C, which is 54.4 p.s.i. Removal of such a backpressure restrictor resulted in obvious outgassing, with much increased baseline noise.

The temperature of the reaction stream must be lowered to near ambient to maximize fluorescence output and stability. At a flow-rate of 1.2 ml/min a drop in temperature of 100°C requires dissipating only 7 W. The temperature at the cell exit was *ca.* 30°C with the lamp off when the reaction temperature was 135°C, indicating that almost all of the heat energy was effectively dissipated by the metal block at the detector inlet. With the lamp on, the cell compartment heats up to slightly above 30°C. Precooling the fluid prior to the detector inlet had no discernable effect on peak intensity, confirming that the major heat source in the cell compartment is the lamp and not the reaction fluid.

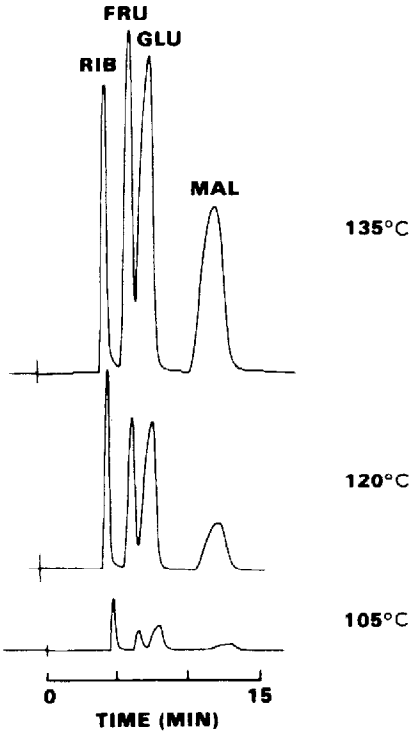


Fig. 2. Cyanoacetamide post-column detection of sugar standards, separated on the MicroPak-NH_2 column at three different reaction temperatures. For all three chromatograms, 50 μg of each sugar were applied.

Band broadening and reaction time

Clearly the cyanoacetamide reaction is not fast, especially when compared to other postcolumn reactions, such as the fluorescamine reaction with primary amines, which is complete in a matter of seconds at room temperature[9]. The half-time of the cyanocetamide reaction with glucose was shown to be *ca.* 20 min at 100°C[4]. Such long reaction times cannot be achieved with simple, unsegmented tubular reactors[10]. The longest reaction coil that could be readily accommodated by our system was 30 ft. and therefore reaction time was increased by simply changing the inner coil diameter.

The separation shown in Fig. 3 was repeated for three different coil diameters. The mobile phase was adjusted to reduce the resolution of fructose and glucose, so as to accentuate losses in efficiency due to the the extra-column variance of the reaction coils. The reaction time with the 0.02in. I.D. coil was 86 sec. The volume variance of the coil was 9700 μl^2, as determined by direct injection. The resolution between fructose and glucose was 0.975, and the peak(fructose)-to-valley separation was 58%. The intermediate reaction coil had a volume variance of 6200 μl^2 for a 65-sec reaction time, but the improvement in resolution was barely noticeable. The resolution between fructose and glucose improved slightly to 0.984, and the peak-to-valley separation was 63%. The small I.D. coil gave the best resolution but entailed a four-fold drop in signal response due to a shorter reaction time. The volume variance was only 1700 μl^2 for a 28-sec reaction time. Resolution was substantially improved to 1.15, and peak-to-valley separation increased to 79%.

The contribution of the larger reaction coils to peak broadening can be determined by comparing the chromatogram from a direct detector, such as refractive index (RI), with the fluorescence profile produced by the post-column reaction. Sugar standards were separated on a cation-exchange column with water as the eluent. The maltose peak was broadened by *ca.* 30% with the 30 x 0.015 in. I.D. coil. Glucose and galactose were broadened by *ca.* 20%. Replacing the reaction coil with the 0.009-in. I.D. coil reduced the band broadening to less than 10% for all the peaks.

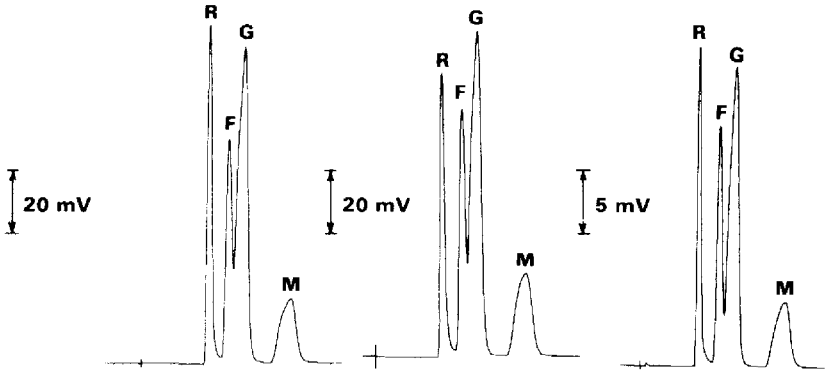
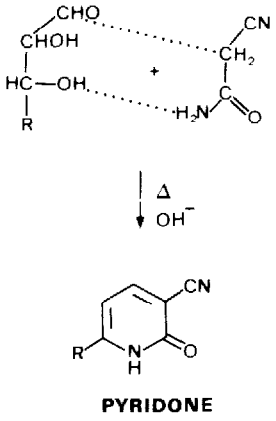


Fig. 3. Comparison of band broadening with the cyanoacetamide reaction and three different 30-ft. reaction coils. The coils from left to right have diameters of 0.02, 0.015 and 0.009 in., respectively, and provided reaction times of 86, 65, and 28 sec, respectively. The sugars were separated on the Micro-Pak-NH_2 column, and the post-column reaction temperature was 120°C. Peaks: R = ribose; F = fructose; G = glucose; M = maltose.

The sensitivity advantage of reaction detection over RI is more than one order of magnitude for reaction times of 60 sec or longer.

Selectivity

Not only is the post-column reaction system very sensitive, but it is also selective for reducing sugars. The reaction mechanism has not yet been clearly illustrated, but ring formation is highly likely. A possible mechanism resulting in ring formation is shown below. A ketobutyraldehyde diacetal has been shown to condense with cyanoacetamide to yield 3-cyano-6-methylpyridone[11]. This mechanism requires prior oxidation of the β-hydroxy group to a ketone by an unidentified oxidant. The fluorescence optima of pyridoxal[12] and its cyanohydrin derivative[13] are similar to that found for the product of the cyanoacetamide reaction. The evidence to date suggest that this is a condensation reaction, resulting in ring closure, which may well involve several steps.



PYRIDONE

Comparison of the RI and post-column reaction detectors in Fig. 4 shows that the reaction detector responds only to the sugar components in the sample, whereas the RI detector also responds to the substantial amount of ethanol and lesser amounts of protein and high-molecular-weight compounds that are present in wine. Also the glucose peak is not readily evident, but a poorly resolved peak can be distinguished when the scale is expanded.

There are some reactive interfering compounds, such as aliphatic aldehydes, which have been reported to produce fluorescence in the cyanoacetamide reaction[14]. Non-reactive interfering compounds are compounds with similar fluorescence spectra. They can be identified by omitting the cyanoacetamide from the reagent. No interfering peaks were observed with several wine, beer, and fruit juice samples. Some soft drinks did reveal one small interfering peak that may be due to caffeine.

Linearity

Various dilutions of a sugar standard containing maltose, galactose, glucose, and fructose were automatically injected into the HPX-87C column and eluted with water. The glucose response was *ca.* 2.4 times that for fructose. The galactose response closely resembled that of glucose, and maltose was similar to fructose. The

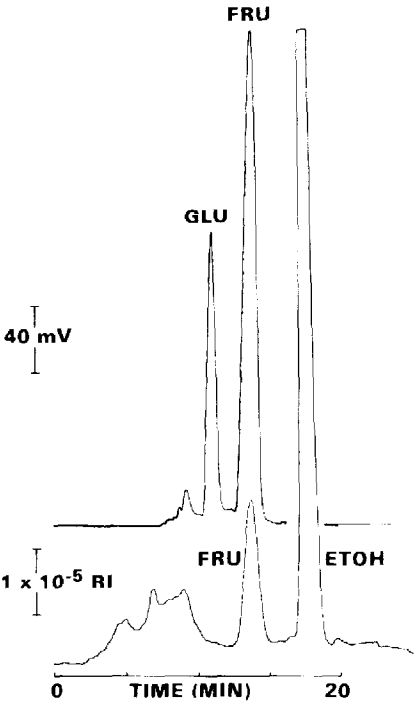


Fig. 4. Comparison of refractive index and post-column reaction detection for a sample of white wine containing 2.5% residual sugar.

linear region extends from 0.1 to 10 μg of injected sugar. The regression equation for glucose yielded a slope of 572 with an intercept of 38 and a correlation coefficient of 0.9999. The results for fructose were 241, − 2.4 and 0.9998, respectively. When more than 20 μg of reducing sugar were injected, multilevel calibration was necessary to correct for the gradual decrease in response factors.

The response curves obtained with the amino column were similar over the range 1–20 μg of reducing sugar. The response at lower levels was somewhat erratic. Apparently, the amino column binds a small amount of sugar irreversibly[15]. This results in a rapid decline in response for dilutions below the 1-μg level. The decrease in response factors above 20 μg was very similar to that observed with the resin column and is probably a consequence of decreased conversion at higher sugar concentrations, because the reagent is still in excess.

Reproducibility

Over short times (2 h or less), the within-analysis reproducibility was found to be 2–3% for five injections on the amino column. The standard contained 5 mg/ml of ribose, fructose, glucose and maltose. Reproducibility on the resin column was a comparable 3–4% for a standard containing maltose, glucose, galactose and fructose. The reproducibility of this analysis with the RI detector was 0.5–1.2% for the same sample. The increased variation with the reaction system was probably due to small changes in reaction time.

Reproducibility over longer time periods may be somewhat worse, especially

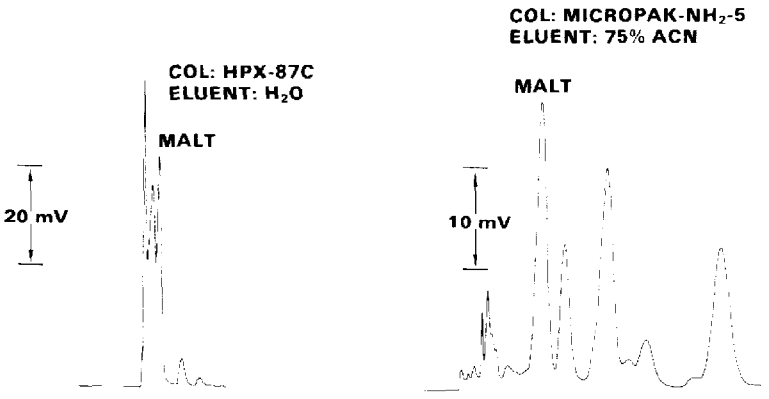


Fig. 5. Comparison of selectivity on the HPX-87C and Micropak-NH_2 columns. A 10-μl sample of beer was injected into both columns, and sugars were detected with the cyanoacetamide reaction at 120°C by using the 0.015 in. I.D. reaction coil. ACN = acetonitrile.

if the reagent flow-rate gradually decreases. To minimize such effects, control standards should be included after every five or six samples. This can compensate for gradual changes in reaction time in the course of a day.

Applications

Beer contains a number of monomeric and oligomeric saccharides. The sugar profiles shown in Fig. 5 reveal large selectivity differences between the amino and resin columns. Larger sugars are eluted early on the resin column, but are more strongly retained on the amino column. The three peaks that were poorly resolved on the resin column were readily resolved into four major peaks on the amino column.

Gradient separations are also possible on the amino column. The gradient proceeded from 90 to 60% acetonitrile over 20 minutes. The effect of acetonitrile on the reaction response was tested by direct injection of 1 μl below the column. Solutions of glucose and maltose, 5 mg/ml each, were injected in the presence of 90 and 60% acetonitrile in the column effluent. The additional water reduced the response to the monosaccharide by 30% and maltose by 45%. Clearly, gradient separations require representative control samples for accurate quantitation. The gradient separation of the beer carbohydrates shown in Fig. 6 ressembles the isocratic separation in the previous figure, except that peaks eluted later are compressed and earlier peaks are more widely separated.

CONCLUSIONS

Detection of reducing sugars by the cyanoacetamide reaction is compatible with both ion-exchange resins and silica packings, derivatized with aminopropylsilane. The reaction is somewhat enhanced by the presence of up to at least 90% by volume acetonitrile in water. Low baseline noise, excellent sensitivity and compatibility with the major column types in sugar chromatography are key advantages of post-column reaction detection with cyanoacetamide.

Relatively long reaction times are the only major drawback of this method.

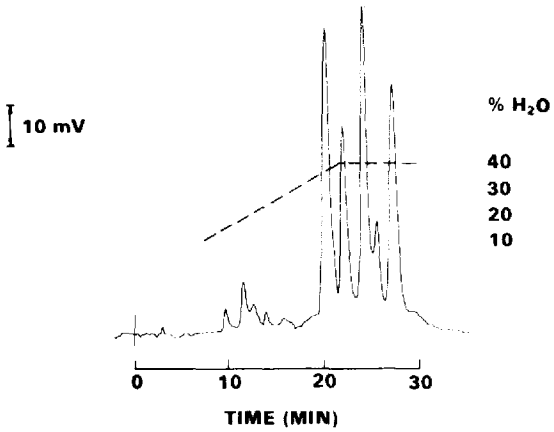


Fig. 6. Gradient separation of carbohydrates in beer on the MicroPak-NH_2 column. The reaction conditions were the same as in Fig.5.

Band broadening can be substantial, when 100-sec reaction times or longer are required. However, the reaction time and concomitant band broadening can be reduced, by increasing the reaction temperature. Temperatures of 135°C or higher can be used to accelerate the reaction. At higher reaction temperatures, the reaction time can be reduced to *ca.* 30 sec, and reaction coils with a 0.01-in. I.D. can be used in place of those with larger diameters. The narrow-bore reaction coil contributes less than 10% to peak variance for most sugars, and degrades resolution by less than 5%. It is most appropriate when sugar concentrations range from 0.1 to 10%, as is common in many food and beverage samples.

REFERENCES

1 M. D'Amboise, T. Hanai and D. Noel, *Clin. Chem.*, 26 (1980) 1348.
2 M. Sinner and J. Puls, *J. Chromatogr.*, 156 (1978) 197.
3 T. Kato and T. Kinoshita, *Anal. Biochem.*, 106 (1980) 238.
4 S. Honda, Y. Matsuda, M. Takahashi, K. Kakehi and S. Ganno, *Anal. Chem.*, 52 (1980) 1079.
5 J.I. Ohms, J. Zec, J.V. Benson and J.A. Patterson, *Anal. Biochem.*, 20 (1967) 51.
6 P.B. Kesler, *Anal. Chem.*, 39 (1967) 1416.
7 S. Honda, K. Kahimoto, K. Sudo, K. Kakehi and K. Takiura, *Anal. Chim. Acta*, 70 (1974) 133.
8 S. Honda, M. Takahashi, K. Kahehi and S. Ganno, *Anal. Biochem.*, 113 (1981) 130.
9 S. Stein, P. Bohlen, J. Stone, W. Dairman and S. Udenfriend, *Arch. Biochem. Biophys.*, 155 (1973) 202.
10 A.H.M.T. Scholten, U.A.Th. Brinkman and R.W. Frei, *Anal. Chem.*, 54 (1982) 1932.
11 L. J. Binovi and H. G. Arlt, Jr., *J. Org. Chem.*, 26 (1961) 1656.
12 D. Coursin and V. Brown, *Proc. Soc. Exptl. Biol. Med.*, 98 (1958) 315.
13 V. Bonavita, *Arch. Biochem. Biophys.*, 88 (1960) 366.
14 S. Honda, M. Takahashi, Y. Nishimura, K. Kakehi and S. Ganno, *Anal. Biochem.*, 118 (1981) 162.
15 C. Brons and C. Olieman, *J. Chromatogr.*, 259 (1983) 79.