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NON-CORROSIVE DYE REAGENT FOR DETECTION OF REDUCING SUGARS IN BORATE COMPLEX ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

The 2,2′-bicinchoninate reagent described previously for sugar detection in liquid chromatography with ethanol as eluent was adapted to an automated borate complex ion-exchange chromatographic system, in which orcinol–sulphuric acid was used for detection. The new aqueous reagents are non-corrosive and stable for several weeks; a better peak resolution can be achieved than with the viscous sulphuric acid reagent. Reaction times less than 1 min and reaction coils with I.D. down to 0.3 mm were used. Owing to the high baseline stability of the detection system with the new reagents, the analyzer can be run at higher sensitivity. The colour formation is linear over a detection range of more than two orders of magnitude. In routine work amounts of 0.1–30 μg of different sugars were determined.

INTRODUCTION

In an earlier paper[1], an automated sugar analyzer was described in which sugars were separated by ion exchange of their borate complexes and detected with orcinol–sulphuric acid. The corrosiveness of this reagent caused problems in routine work and therefore non-corrosive reagents were sought. The 2,2′-bicinchoninate reagent, described by Mopper and Gindler[2] for sugar detection in ethanol–water, gave satisfactory results after modification of the original dye.

EXPERIMENTAL

Analyzer

The Biotronik sugar analyzer used in this work was as described earlier[1] except that the reagent pump and the coils were modified.

The peristaltic pump was replaced with a piston pump (Dosapro, Milton-Roy, Pont-Saint-Pierre, France). The reagent flows from a brown-coloured vessel through a debubbler down to the piston pump (minimal distance 30 cm). It passes through an

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in-line pulse damper followed by a steel capillary of I.D. 0.23 mm and length 3 m (back pressure 5–12 atm depending on flow-rate; both from Waters GmbH, Königstein, G.F.R.), before it is mixed with the column eluate in the three-way valve. Sufficiently stable flow conditions can also be obtained by inserting a simple back-pressure valve between the pump outlet and the three-way valve. In addition to the reaction coils of I.D. 0.7 mm already utilized with orcinol–sulphuric acid, coils of I.D. 0.5 mm (O.D. 2 mm, length 31.3 m; O.D. 1.5 mm, length 22 m) were used when a reaction temperature of about 100° (boiling water) was applied. At higher reaction temperatures, in addition coils of I.D. 0.5 mm (O.D. 1.5 mm, length 6 m) and 0.3 mm (O.D. 1.5 mm, length 5–14 m) were used. The reaction coil was connected to the colorimeter (cuvette 1 cm; filter 560 nm) with 0.3-mm I.D. PTFE tubing of length 1.5–4 m, so as to obtain a back pressure sufficiently high to prevent the formation of bubbles and to smooth the baseline further. This tubing was cooled with tap water[3] when the reaction temperature exceeded 100°.

In the experiments for testing the parameters of colour formation in capillary coils, the sugars (mostly xylose) did not pass through the separation column; the sample loader was attached between the column outlet and the three-way mixing valve. The flow-rate (reagent + column eluate) was constant at 70 ml/h.

Reagent with aspartic acid (ASP)

Solution A. A 4.5-g amount of disodium 2,2'-bicinchoninate (Pierce, Rockford, Ill., U.S.A.) is dissolved in about 3 l of distilled water and 215 g of sodium carbonate (anhydrous, p.a.) are added with agitation; the solution is then made up to 3.45 l with distilled water.

Solution B. A 3.7-g amount of aspartic acid (p.a.) plus 5.0 g of sodium carbonate (anhydrous, p.a.), dissolved together in about 100 ml of distilled water (foaming occurs) and 1.0 g of copper(II) sulphate (p.a.) dissolved in about 40 ml of distilled water are mixed and the volume is made up to 150 ml with distilled water. A fluffy, light blue precipitate may appear temporarily.

Solutions A and B are stable for several months.

Reagent. The final reagent is prepared by mixing 23 parts of solution A with 1 part of solution B. It has an intense blue colour and posesses a strong fluorescence. When kept in brown bottles at room temperature it turns slightly purple after several weeks; at higher temperatures it changes colour more rapidly. The coloured complexes formed with reducing sugars are constant for several months, although the purple background colour becomes increasingly pronounced after a few weeks. Freshly prepared reagent should be allowed to stand for several hours before use.

Reagent with citric acid (CIT)

CIT reagent differs from ASP reagent only in that it contains citric acid instead of aspartic acid. Citric acid dissolves easily, and therefore all of the sodium carbonate can be added to solution A. Solution B consists of 150 ml of solution containing 3.9 g of citric acid monohydrate (p.a.) and 1.0 g of copper(II) sulphate (p.a.), which can be dissolved together. Solutions A and B are mixed as for ASP reagent.

The freshly prepared reagent has a slight greenish tinge, which disappears after a few hours. It then looks like fluorescent water with a very light bluish tinge. When kept in brown bottles at room temperature it turns slightly purple after about

2 weeks. The purple background in the reaction occurs earlier and is more pronounced than with ASP reagent.

RESULTS AND DISCUSSION

Impediment of the borate buffer

The copper(I) complex of 2,2′-bicinchoninate (BCA) has been shown by Mopper and Gindler[2] to be very suitable for the detection of reducing sugars in high-performance liquid chromatography with ethanol–water as the mobile phase. This dye reagent (Mopper–Gindler reagent) is very sensitive and the reaction is first order over a wide range. In water the sensitivity is lower[2]; in addition, the linearity range of the reaction is reduced. When borate ions are present, as commonly used in borate complex ion-exchange chromatography, the Mopper–Gindler reagent produces only a very faint colour with reducing sugars. About 10 parts of reagent have to be added to 1 part of borate buffer (0.5 *M*, pH 8.8) in order to obtain satisfactory sensitivity (of the same magnitude as with orcinol–sulphuric acid).

The colour formation increased when the reagent–borate buffer mixture was adjusted to the optimal pH of the reaction (about 10.6). Therefore, the sodium carbonate concentration of the Mopper–Gindler reagent was increased to 6.1% in order to obtain the required buffering capacity; in addition the BCA concentration was doubled, which slightly increased the linear detection range (ASP reagent). Higher sodium carbonate and BCA concentrations (up to 12% sodium carbonate when the decahydrate is used for the reagent preparation, BCA up to three times and copper up to twice that in the Mopper–Gindler reagent) but also lower BCA concentrations (down to about half of the original reagent concentration) can be applied.

The formation of the coloured complex by ASP reagent with reducing sugars in borate buffer can be taken as a first-order reaction up to a peak height of about 0.5–0.8 absorbance unit, passing through the origin. This is true for a wide range of operating conditions (*e.g.*, volume ratios of reagent to column eluate of 0.7–4, reaction temperatures of 80–121° and reaction times of 0.3–20 min).

Optimal reagent volume

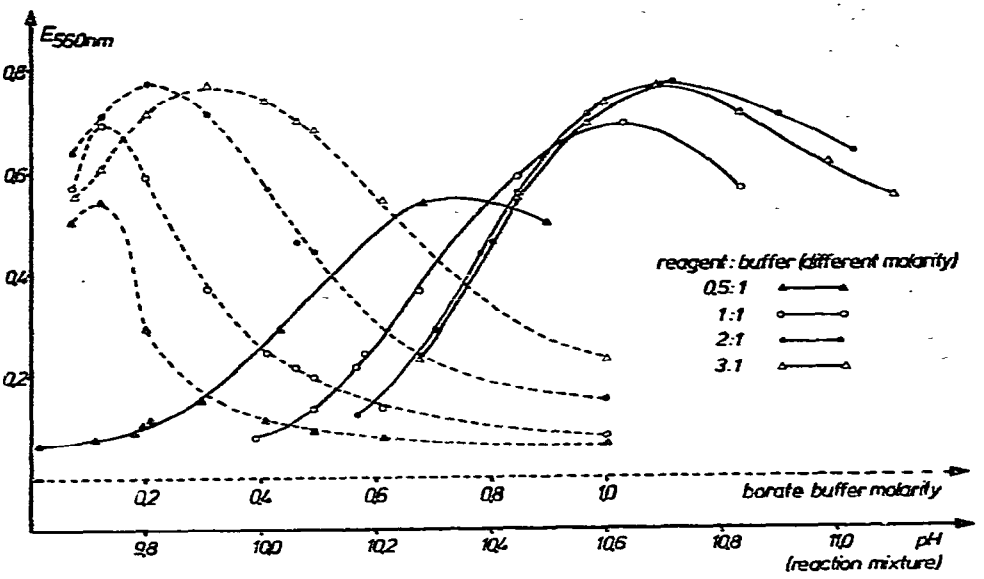
The intensity of the colour reaction increases with increase in the ratio of ASP reagent to buffer (Fig. 1). In capillary coils peak broadening is a limiting factor. Maximal sensitivity with minimal zone spreading is reached at the point of inflection of the function peak height to peak area. An example is shown in Fig. 2.

Optimal temperature

The optimal temperature of the reaction in test-tubes was found to be 95° (10-min incubation). Above this temperature the colour intensity and the reproducibility of the results decreased. In capillary coils the optimal temperature of the reaction bath was always above 100°. The value depended on the reaction time, heat exchange velocity and volume ratio of reagent to borate buffer.

Optimal reaction time

For constant flow-rates and coil diameters, peak areas and peak heights increased first with reaction time (increasing coil length) but subsequently decreased.



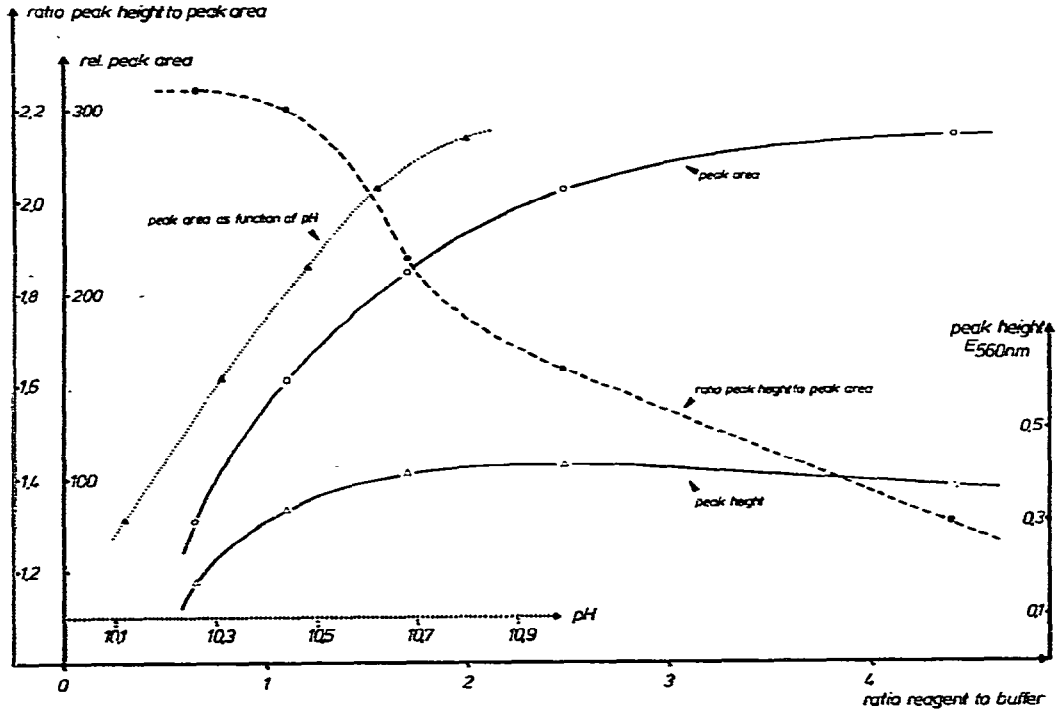


Fig. 1. Colour formation as function of the pH of the reaction mixture ASP reagent–sodium borate buffer (solid line) and of the molarity of the buffer (broken line). Reaction in test-tubes: 10 μg of xylose, 100°, 10 min. Reagent to buffer ratios: ▲, 0.5:1; ○, 1:1; ●, 2:1, △, 3:1.

Fig. 2. Peak area (○—○) and peak height (△—△) as functions of the volume ratio of ASP reagent to sodium borate buffer (0.49 *M*, pH 8.8) and peak area (△—△) as a function of the pH of the reaction mixture. Reaction coil: 31.3 m × 0.5 mm I.D. in boiling water (connection with colorimeter: 4 m × 0.3 mm I.D.). Sample: 2.7 μg of xylose in 10 μl of sodium borate buffer (0.49 *M*, pH 8.8).

This was more pronounced for the peak heights than for the peak areas, and maximal peak heights were reached earlier than maximal peak areas. The point of inflection of the function peak height to peak area indicates the optimal coil length for maximal colour formation with minimal zone spreading for a given reaction temperature (Fig. 3).

Optimal conditions

On the basis of experiments in which one parameter was varied, it can be deduced that high colour formation with low peak broadening and short reaction times is obtained by applying volume ratios of reagent to buffer in the range 1–2, high reaction temperatures (about 120°) and fine capillary coils. For example, a greater

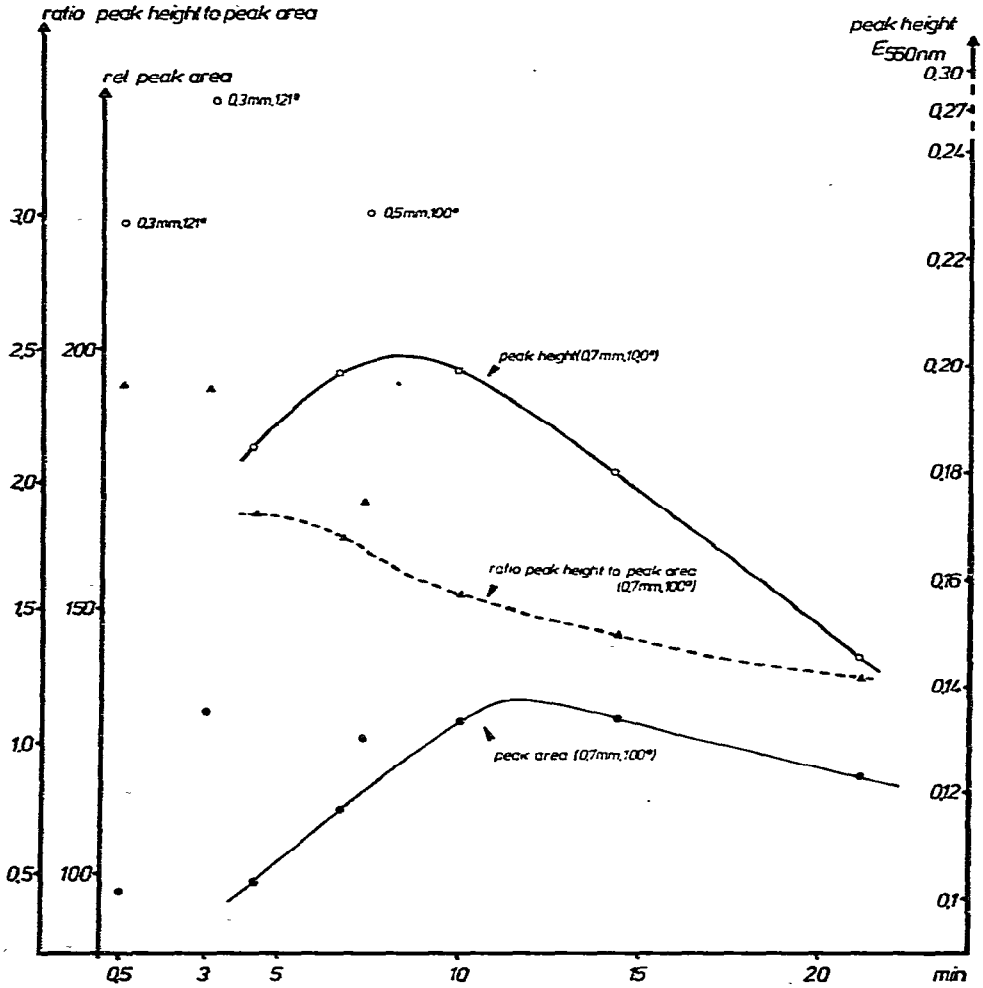


Fig. 3. Peak area and peak height as functions of the reaction time. Volume ratio of ASP reagent to sodium borate buffer (0.49 *M*, pH 8.8), 2.2; sample, 1.3 μg of xylose in 10 μl of sodium borate buffer (0.49 *M*, pH 8.8); reaction coils, 10–56 m × 0.7 mm I.D. in boiling water (solid and broken lines), 31.3 m × 0.5 mm I.D. in boiling water (values at 3 min) and 5.5 m × 0.3 mm I.D. at 121° (values at 0.5 min).

peak height than that obtained in 8 min at 100° in a 0.7-mm I.D. coil (length 18 m) is reached in 0.4 min at 121° in a 0.3-mm I.D. coil (length 5.5 m; see Fig. 3). In this instance the zone spreading is very low. The peak width ($w = 4\sigma$) was 58 sec when the 0.3-mm I.D. coil, a volume ratio of reagent to buffer of 1 and a reaction temperature of 121° were used (peak height for 0.14 mg of xylose, $E = 0.17$; ratio of peak height to peak area, 3.3; compare with the values in Fig. 3).

Detection range and reproducibility

About 50 reducing sugars (common hexoses and pentoses, amino sugars, methyl ethers of sugars, di- and oligosaccharides, glucuronic acids), fructose and furfural were tested with ASP reagent in capillary coils, all with positive results. Amounts of sugars down to 0.01 μg were readily detectable (range of absorbance 0–0.1, recorder 0–10 mV). The coefficient of variation of the peak area for repeated injections ($n = 9$) of a sugar solution was less than $\pm$ 1%. This is in the range of the reproducibility of the injection volume.

CIT reagent

The colour formation with reducing sugars is about as constant as that for ASP reagent, but after a few weeks the background colour is more intense. CIT reagent showed the same sensitivity and very similar characteristics to ASP reagent in the coil detection system.

Application

The BCA reagent, forming coloured complexes with copper(I), can be applied only to reducing sugars and similar compounds (*e.g.*, fructose and furfural). In spite of the increased buffering capacity of the new reagents, the colour formation is influenced by the molarity and the pH of the borate buffer. These factors therefore have to be kept constant, or buffer changes in stepwise elution have to be executed in an exactly reproducible manner.

The following contaminating substances, usually present in wood carbohydrate chemistry, did not interfere in the quantitative evaluation of reducing sugars in the sample: buffering salts, proteins, sulphuric acid, hydrochloric acid, acetic acid, sodium hydroxide and wood extractives.

Very constant and reproducible flow characteristics were achieved with the micro-piston pump. Series of analyses lasting several days were run without baseline deviation (absorbance range 0–0.2).

Clogging problems encountered formerly with sulphuric acid did not occur. With the new reagents the detection system was rinsed with water about once a week and from time to time additionally with 5% orthophosphoric acid. No clogging occurred even when the reaction mixture was left in the coil at 100° for 2 days. When higher reaction temperatures are used, rinsing with water is necessary.

The new reagents mix well with the aqueous column eluate, being also in an aqueous state. They cause less peak broadening in the reaction coil than the viscous sulphuric acid reagent and therefore better resolution of neighbouring peaks is obtained. Owing to the high baseline stability and the improved resolution, the detection system of the analyzer can be run at increased sensitivity. Therefore, the detection limit for reducing sugars is even lower than with orcinol–sulphuric acid and quantitative sugar detection can be achieved over a range of two to three orders of magnitude.

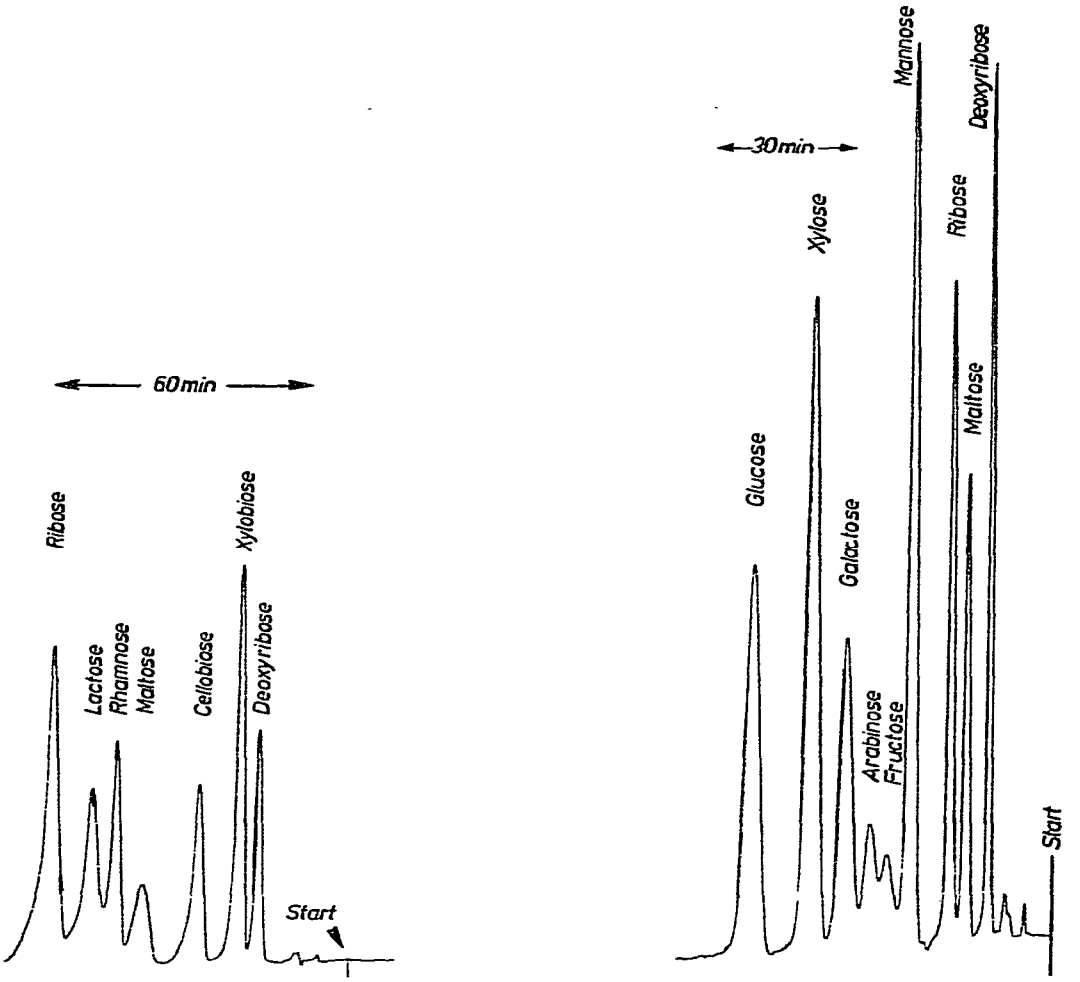


Fig. 4. Separation of 0.7 μg of deoxyribose, 1.5 μg of xylobiose, 1.0 μg of cellobiose, 1.1 μg of maltose, 1.0 μg of rhamnose, 1.5 μg of lactose and 1.9 μg of ribose. Column: Durrum DAX4, 30 cm × 0.4 cm I.D., 60°. Buffer: 0.20 *M* sodium borate, pH 8.2, 33 ml/h, 26 atm. ASP reagent: 37 ml/h. Reaction coil: 31.3 m × 0.5 mm I.D. in boiling water. Detector response: full scale = 0.2 *E*.

Fig. 5. Separation of 1.6 μg of deoxyribose, 1.6 μg of maltose, 2.9 μg of ribose, 4 μg of mannose, 0.5 μg of fructose, 1.3 μg of arabinose, 2.5 μg of galactose, 4.0 μg of xylose and 4.1 μg of glucose. Column: see Fig. 4. Buffer: 0.49 *M* sodium borate, pH 8.8, 37 ml/h, 29 atm. ASP reagent: 43 ml/h. Reaction coil: 31.3 m × 0.5 mm I.D. in boiling water. Detector response : full scale = 0.1 *E*.

In routine work, the reaction coils were kept in boiling water and the absorbance range was set at 0–0.2 (recorder range 0–50 mV with extension up to 150 mV); 0.1–30 μg of individual sugars were determined by automatic peak-area integration (Fig. 4). The detection system can be run at higher sensitivity (Fig. 5).

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