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Journal of Chromatography A, 720 (1996) 183–199

JOURNAL OF CHROMATOGRAPHY A

Review

Postcolumn derivatization for chromatographic analysis of carbohydrates

Susumu Honda

Faculty of Pharmaceutical Sciences, Kinki University, 3-4-1 Kowakae, Higashi-osaka, Japan

Abstract

Various methods for postcolumn derivatization of carbohydrates for photometric (P), fluorimetric (F) and electrochemical (E) detection are summarized. The major methods include conversion to furfurals (with strong mineral acids, reducing carbohydrates, for P), formaldehyde (with periodate, carbohydrates in general, for P and F), glycamines (by reductive amination, reducing carbohydrates, for P and F), etc., followed by condensation with appropriate chromogenic or fluorogenic reagents; direct or indirect coloration of leuco compounds (for P) or chelating agents (for P and E), respectively, by utilizing the reducibility of reducing carbohydrates; reactions with fluorogenic reagents such as aliphatic amines (for F), 2-cyanoacetamide (for P, F and E), arginine (for F), benzamidines (for F), etc., in neutral or weakly alkaline media; and coloration of the condensates with hydrazino compounds in alkali (for P). The characteristic features of individual methods and their optimized conditions are reviewed.

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0021-9673/96/$32.00
SSDI 0021-9673(95)00212-X

1. Introduction

As pointed out elsewhere in this thematic issue, the detection of carbohydrates separated by chromatographic and electrophoretic methods presents a challenge to glycobiologists and glycotechnologists, because this group of compounds generally have neither chromophores nor fluorophores, hence direct detection using common optical detectors is generally difficult. Although the measurement of UV absorption at low wavelengths and refractive index permits carbohydrate detection, the sensitivity and selectivity are low in these methods. Therefore, much effort has been made to develop methods for derivatization to photometrically or fluorimetrically detectable forms.

There are two modes of derivatization in liquid chromatography, i.e., precolumn and postcolumn. Precolumn derivatization involves the introduction of tags detectable by either photometric or fluorimetric methods and is applicable not only to liquid chromatography but also to electrophoresis. This topic is summarized in this issue by Hase. On the other hand, postcolumn derivatization requires reaction after separation, but it should be performed before detection. Liquid chromatography meets this demand, because the appropriate reaction time at the desired temperature can be realized by controlling the dimensions of the reaction tube, flow-rate of the effluent and reaction bath temperature. For this reason, postcolumn derivatization has become familiar in liquid chromatography. In contrast, electrophoresis does not afford sufficient time for post-separation derivatization, and hence is not easily compatible with postcolumn derivatization.

In liquid chromatography, mobile phase is constantly eluted from a column containing a solid phase. Continuous mixing of a flow of a reagent solution with this constant stream, followed by reaction for an appropriate length of time at a suitable temperature by passing the mixed effluent through a reaction tube placed in a bath, converts the components of the sample into detectable derivatives, which are successively led to a detector. This interfacial process in a high-performance liquid chromatographic procedure plays an important role in monitoring separated components which are difficult to detect in the intact state. It can be regarded as a variation of flow-injection analysis. In ordinary flow-injection analysis, a sample solution is introduced into a reagent flow as short plugs at appropriate intervals and a derivatization reaction occurs in each plug during transportation to the detector, whereas in postcolumn derivatization samples are introduced in a constant stream.

Postcolumn derivatization has attracted the attention of carbohydrate analysts, because a narrow tube connecting the outlet of the separation column and the detector cell is regarded as a small reaction vessel in which a reaction mixture is moving at a constant velocity. The reactants in this small vessel are each carbohydrate components of a sample and a reagent. The carbohydrate components are homogeneous in most cases, because they have been separated and purified from other carbohydrate components and also from most of accompanying non-carbohydrate substances in the chromatographic process. Therefore, this constantly moving miniature reaction system is simple, and various kinds of reagents developed for manually operated carbohydrate analysis have been tested also with this system.

Another aspect of postcolumn derivatization in liquid chromatography is that this process is naturally automatic, since separated carbohydrates and a reagent are supplied in steady streams and the products formed after reaction in the combined stream are transported to a detector cell by a pressurized flow by a pump. Recording the detector signals implies automatic analysis. For this reason, postcolumn derivatization plays an important role in the automation of carbohydrate analysis. Several kinds of carbohydrate analysers were hitherto manufactured based on this principle and have been used in many laboratories for routine analysis of carbohydrates.

A number of methods have been developed for postcolumn derivatization of carbohydrates.

This paper focuses on the characteristic features of each drivatization method and the results of the optimization of the reaction conditions.

2. Conversion to furfurals with strong acids, followed by condensation with chromogenic reagents (for photometric detection)

Classical methods for the characterization of carbohydrates were mainly based on the colour formation observed when carbohydrate samples are treated with strong mineral acids containing a suitable chromogenic reagent, such as phenol [1], orcinol [2], anthrone [3], cysteine [4], carbazole [5], resorcinol [6] or indole [7]. In these methods, monosaccharides are generally converted by dehydration and cyclization into furfural derivatives, which are concurrently condensed with these chromogenic reagents. Some of these methods are general for reducing sugars and others are selective to particular groups of carbohydrates. Poly- and oligosaccharides are hydrolysed to monosaccharides during operation, and hence could be analysed similarly to monosaccharides. The first application of this type of colour reaction to postcolumn derivatization was in the mid-1960s [8], and in the 1970s a few analytical instrument makers marketed automated carbohydrate analysers based on this principle. Such analysers allowed the separation of mono- and oligosaccharides in several hours and detection at the subnanomole level. Fig. 1 shows the flow diagram for such an analyser and Fig. 2 shows a typical example of a separation [9]. In this example, mono- and oligosaccharides separated in the anion-exchange mode as borate complexes were derivatized with an orcinol solution in sulfuric acid. The detection limit was in the range 0.1–2.0 nmol depending on the carbohydrate species.

The appearance of this type of analyser was epoch-making for carbohydrate research and for the quality control of carbohydrate products. A number of papers were published on the improvement of carbohydrate separations using the detection systems incorporated in such

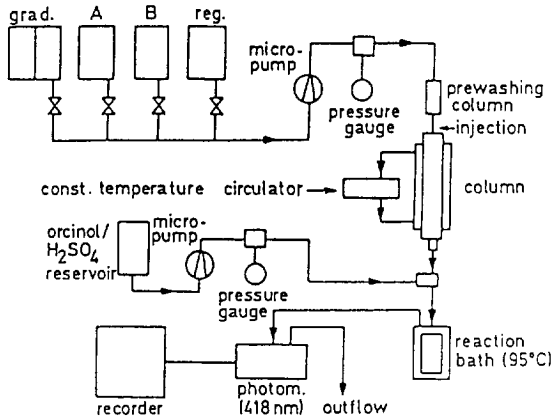


Fig. 1. Schematic diagram of the carbohydrate analyser based on postcolumn derivatization with orcinol–sulfuric acid. Grad. = gradient-generating system; A, B = buffer reservoirs; reg. = regenerating buffer reservoir. Reproduced from Ref. [9] with permission.

analysers. The direct ion-exchange mode, the anion-exchange mode as borate complexes and the gel permeation mode were successfully applied to ionic carbohydrates: uronic acids, with carbazole–sulfuric acid [10]; sialic acids, with thiobarbituric acid–sulfuric acid [11]; sialooligosaccharides, with phenol–sulfuric acid [12]); neutral mono- and oligosaccharides, with orcinol–sulfuric acid [9,13] or with thymol–sulfuric acid [14,15]; and poly- and oligosaccharides, with orcinol–sulfuric acid [16,17]. However, this type of apparatus had the serious drawback that they require the use of corrosive strong acids, which often damaged the effluent transport system and the detector. Especially when borate buffer and sulfuric acid were used as the mobile phase and the reaction medium for the postcolumn reaction, respectively, crystals of boric acid were often precipitated and clogged the tubings. As a result, the corrosive effluent sometimes blew out of connecting portions of the transport system and caused rusting of the hardware.

Postcolumn derivatization based on this principle has a historical value and is still important because it allows the simultaneous analysis of both reducing and non-reducing carbohydrates.

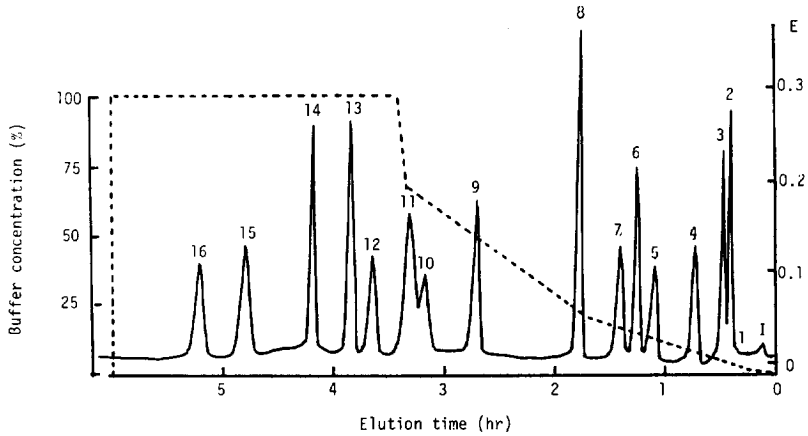


Fig. 2. Analysis of a standard mixture of mono- and disaccharides using postcolumn derivatization with orcinol–sulfuric acid. Column, Durrum DA-X4 (particle size 20 μm, 280 mm × 5 mm I.D.); column temperature, 50°C; eluent, 0.1–0.5 m*M* boric acid (pH 8.0–10.0), gradient elution; flow-rate, 1 ml/min; reagent solution for postcolumn derivatization, orcinol (1 g) in sulfuric acid (1 l); rection tube, Teflon (20 m × 0.7 mm I.D.); reaction temperature, 95°C; detection, absorption at 420 nm. Peaks: I = injection; 1 = 2-deoxyribose; 2 = sucrose; 3 = trahalose; 4 = cellobiose; 5 = maltose; 6 = rhamnose; 7 = lactose; 8 = ribose; 9 = mannose; 10 = fructose; 11 = arabinose; 12 = galactose; 13 = xylose; 14 = glucose; 15 = gentiobiose; 16 = melibiose. Sample scale, 80 nmol (monosaccharides) or 40 nmol (disaccharides). Reproduced from Ref. [9] with permission.

Recent work by Englehardt and Ohs [14] utilizing a thymol–sulfuric acid reagent permitted the detection of both types of carbohydrates in the nanogram range (picomole range as monosaccharide). In this work an improved detector system was used and a knitted Teflon capillary was employed as a reaction vessel. The knitted capillary was effective for sensitization of detection by diminishing dispersion, i.e., by preventing peak broadening.

Reactions of carbohydrates with certain kinds of reagents in strong acids gave not only coloration but also fluorescence. For example, resorcinol in hydrochloric acid [18], anthrone in sulfuric acid [19], *o*-aminothiophenol in sulfuric acid [20] and 5-hydroxytetralone in sulfuric acid [21] were reported to give fluorescence, and were used for the fluorimetric determination of carbohydrates. However, this type of fluorescence reaction has not been employed for postcolumn derivatization, because it also requires the use of corrosive strong acids. The development of much milder fluorescence reactions (see Section 5) is also a reason why this type of reaction has not been adopted for postcolumn derivatization.

3. Use of reducibility (for photometric, fluorimetric and electrochemical detection)

Reducing carbohydrates can reduce various substances. For example, some metal ions, such as iron(III) and copper(II) ions, are readily reduced to iron(II) and copper(I) ions, respectively, in the presence of reducing carbohydrates. Therefore, if compounds capable of binding to ions in the lower oxidation state [iron(II) and copper(I)] to form chelates having absorption in the UV or visible region but incapable of binding to ions in the higher oxidation state [iron(III) and copper(II)] are added to these systems, the photometric determination of reducing carbohydrates is possible. Application of this principle to postcolumn derivatization allowed the photometric detection of carbohydrates separated by liquid chromatography.

A typical example is the Cu^{2+}–2,2′-bicinchoninate method developed by Mopper and co-workers [22–25]. In this method, a reagent solution containing copper(II) sulfate, 2,2′-bicinchoninate and aspartic acid as a masking agent in ca. 5% aqueous sodium carbonate solution was used for derivatization. When reducing carbohydrates

in aqueous ethanol eluted from a column of an anion-exchange resin in the sulfate form were mixed with this reagent solution and allowed to react at an elevated temperature of ca. 100°C, the copper(II) ion in the reagent was reduced to the copper(I) ion, which bound to 2,2′-bicinchoninate to form a chelate having an absorption maximum at 562 nm. Fig. 3 shows an example of the analysis of standard carbohydrates [23]. The limit of detection was ca. 100 pmol for monosaccharides [22]. Separation by the anion-exchange mode as borate complexes was disadvantageous, because the derivatization reaction was unfavourable in aqueous media. However, careful optimization allowed detection at approximately the same level as that in the aqueous ethanolic eluate [24].

The copper(I) chelate of bis(phenanthroline) can be easily oxidized electrochemically to the copper(II) chelate. Using this electrochemical reaction, carbohydrates separated by high-performance liquid chromatography could be monitored sensitively [26]. Heating the eluate containing separated reducing sugars at ca. 90°C, after being mixed with the copper(II) bis(phenanthroline) chelate reagent, gave the copper(I) chelate efficiently, which could be monitored by electrolysis at an applied potential of 75 mV vs. Ag/AgCl on a glassy carbon electrode. The limit of detection was ca. 1 pmol and a linear range was observed between ca. 2 pmol and ca. 1 nmol.

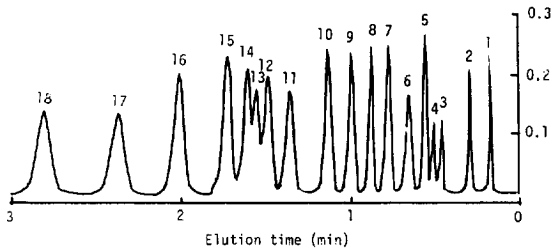


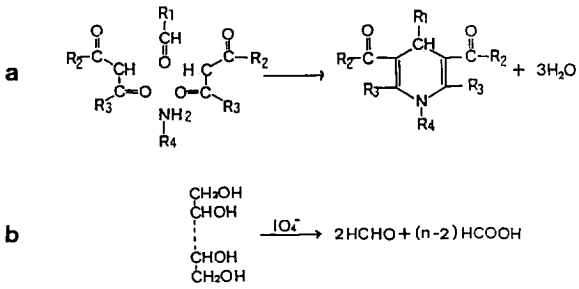
Fig. 3. Analysis of a standard mixture of monosaccharides using postcolumn derivatization with Cu^{2+}–2,2′-bicinchoninate. Column, Durrum DA × 8 (sulfate form, particle size 8–11 μm, 260 mm × 4 mm I.D.); column temperature, 88°C; eluent, 86.7% ethanol; flow-rate, 34 ml/h. The reagent solution for postcolumn derivatization was prepared by mixing a solution of $CuSO_4 \cdot 5H_2O$ (1 g) and aspartic acid (3.7 g) dissolved in water (1 l) with a solution of sodium hydrogen carbonate (38 g) and 2,2′-bicinchoninate (2 g) dissolved in water (1 l) in a 1:2 ratio. Flow-rate of the reagent solution, 18 ml/h; reaction tube, Teflon (0.3 mm I.D.); reaction temperature, 100°C; detection, absorption at 562 nm. Peaks: 1 = digitoxose; 2 = 2-deoxyribose; 3 = 2-deoxygalactose; 4 = 3-O-methylglucose; 5 = rhamnose; 6 = fucose; 7 = ribose; 8 = 6-deoxyglucose; 9 = lyxose; 10 = arabinose; 11 = xylose; 12 = fructose; 13 = tagatose; 14 = sorbose; 15 = mannose; 16 = gulose; 17 = galactose; 18 = glucose. Sample scale, 7 nmol (3-O-methylglucose and 2-deoxygalactose) or 15 nmol (others). Reproduced from Ref. [23] with permission.

Carbohydrates can reduce the cerium(IV) ion to the cerium(III) ion, which fluoresces. Katz et al. [27] applied this reaction to postcolumn derivatization for fluorimetric detection. The reagent solution contained the cerium(IV) ion at a concentration of 0.5 m*M* in 1.5 *M* sulfuric acid. Addition of sodium bismuthate at a concentration of 10–30 mg/l stabilized the solution without influencing the sensitivity. Katz et al. reported an example of fluorimetric monitoring of reducing and non-reducing carbohydrates separated on an anion-exchange column. The carbohydrate concentrations were commonly 0.2 m*M*. This method required the use of sulfuric acid and the selectivity was not high.

A novel method was proposed by Mopper and Degens [28], based on the reduction of anisyltetrazolium chloride (tetrazolium blue) in alkali. This compound was changed to the coloured diformazane anion, having an absorption maximum at 520 nm, by heating with reducing carbohydrates, but the reaction medium was restricted to ethanol, otherwise the resultant diformazane was readily precipitated and clogged the tubing. The detection limit was ca. 100 pmol under the optimized conditions.

4. Periodate oxidation, followed by Hantzsch reaction leading to pyridine derivatives (for photometric and fluorimetric detection)

The reaction used for the Hantzsch pyridine synthesis involves three reactants, an aldehyde, a β-diketone or β-oxo ester and an amine



Scheme 1. (a) Hantsch reaction for pyridine synthesis. (b) Periodate oxidation of a carbohydrate giving formaldehyde.

(Scheme 1a) [29]. The resultant pyridine derivative absorbs and fluoresces in the visible region. Since this reaction involves three reactants, any of them can be determined by utilizing this reaction. The determination of formaldehyde has been well studied and a manual procedure for its photometric determination was established [30]. On the other hand, it is well known that periodate oxidation of the $-CHOH-CH_2OH$ or $-CO-CH_2OH$ group gives formaldehyde quantitatively under the optimized conditions (e.g. Scheme 1b). Subsequent reaction of the formaldehyde thus formed with 2,4-pentanedione in the presence of ammonia gives a pyridine derivative, hence this series of reactions can be used for the determination of carbohydrates. Samuelson and Stromberg [31] first applied this combined reaction to the postcolumn derivatization of carbohydrates, although in a semi-automated fashion.

The first stage of this derivatization is periodate oxidation. Classical periodate oxidation utilized an aqueous solution of periodic acid. The use of this reagent was advantageous for controlled oxidation, but the reaction rate is not high. Periodate oxidation of carbohydrates usually involves concurrent hydrolytic cleavage of the formyl ester formed and subsequent overoxidation. This occurs especially at high pH. However, rapidity and quantitative formation of formaldehyde are important in postcolumn derivatization, and the formaldehyde thus formed should be specifically converted into the pyridine derivative. Fortunately, other oxidation products do not interfere with this series of derivatizations. For this reason, periodate oxidation should be performed at a high pH, and the use of an aqueous solution of sodium metaperiodate is the most convenient.

The second state is cyclization to the pyridine derivative. The rest of the reactants (a β-diketone or a β-oxo ester and ammonia) can be added in one solution, because they do not change in the solution for a considerable length of time. There is one problem, however: the excess amount of periodate interferes with the cyclization reaction. This must be converted into a non-interfering compound. In Samuelson and Stromberg's method, sodium arsenite was added to reduce the excess amount of periodate. Thus, they consequently added three solutions, i.e., an aqueous solution of sodium metaperiodate, an aqueous solution of sodium arsenite and finally an aqueous solution containing 2,4-pentanedione and ammonium acetate, in that order. Detection was performed by monitoring the UV absorption at 410–420 nm. Several papers reported similar procedures for the analysis of mono- and oligosaccharides based on this principle (e.g., [31–34]). Fig. 4a shows an example of the analysis of

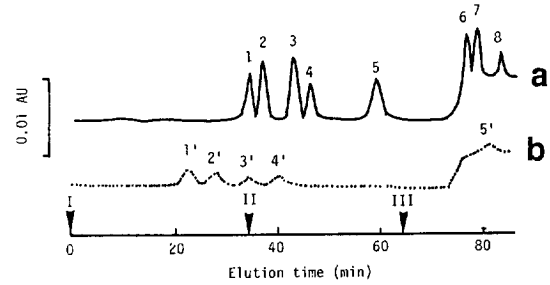


Fig. 4. Analyses of (a) alditols and (b) aldoses by separation in the anion-exchange mode as borate complexes with photometric monitoring after derivatization by the periodate oxidation–Hantzsch reaction method. Column, Hitachi 2633 (particle size of resin 11 μm, 80 mm × 8 mm I.D.); column temperature, 65°C; eluent, borate buffers (I, 0.50 *M*, pH 7.1; II, 0.30 *M*, pH 8.0; III, 0.50 *M*, pH 10.5); reaction solution for periodate oxidation, 50 m*M* sodium metaperiodate; reaction solution for Hantzsch reaction, 15% ammonium acetate, 2% 2,4-pentanedione and 0.20 *M* sodium thiosulfate in water (filtered before use); reaction temperature, 100°C; detection, absorption at 412 nm. The flow-rates of the eluent and both reagent solutions for postcolumn derivatization were commonly 0.5 ml/min. Peaks: 1 = xylitol; 2 = ribitol; 3 = arabinitol; 4 = rhamnitol; 5 = glucitol; 6 = fucitol; 7 = mannitol; 8 = galactitol; 1′ = mannose; 2′ = ribose; 3′ = arabinose; 4′ = galactose; 5′ = glucose. Sample scale, 20 nmol (alditols) or 100 nmol (aldoses). Reproduced from Ref. [34] with permission.

alditols separated in the anion-exchange mode and detected after postcolumn derivatization based on this principle [34]. In this example detection was performed by photometric monitoring, but fluorimetric monitoring gave a higher sensitivity. Under the optimized conditions, more than 0.5 nmol of alditols could be monitored with fluorescence at 503 nm and irradiation at 410 nm. Since formaldehyde was formed from the primary hydroxyl group adjacent to the secondary hydroxyl group or the keto group, alditols gave the greatest number of formaldehyde molecules (two) from each molecule. For this reason, alditols were the most sensitively detected by this method. In Fig. 4b the analysis of five-fold amounts of aldoses is also shown as a reference.

5. Reactions with fluorogenic reagents

5.1. Alkylamines (for fluorimetric and electrochemical detection)

The browning of grain flour on prolonged storage has been a problem in the food industry, and efforts have been made to ensure its prevention. This browning phenomenon is considered to be a result of a series of reactions initiated by coupling of glucose or its oligomer(s) to certain kinds of inherent amino acids or amines. The initial stage is probably the well known Amadori rearrangement in which aldosylamines are converted into aminodeoxyketoses. Further reaction pathways are very complex and have only been partially elucidated, but the formation of at least a few nitrogen-containing heterocyclic compounds has been reported. The browning reaction has not been considered from an analytical viewpoint, but coloration associated with this phenomenon could have given a hint for a new analytical procedure. If the fluorescence property of the brownish products was examined, further hints could have been brought forth.

Honda et al. [35] first found that heating solutions containing reducing carbohydrates and ethylenediamine in slightly alkaline phosphate buffer gave strong fluorescence at 470 nm with the excitation maximum at 390 nm, and applied this fluorescence reaction to the micro-scale determination of reducing carbohydrates. They also developed a selective fluorimetric method for the determination of uronic acids [36]. Mopper et al. [37] applied this reaction for the postcolumn derivatization of mono- and oligosaccharides. Under the optimized conditions (reaction medium, 0.05–0.8 *M* borate buffer, pH 7.0–10.5; ethylenediamine concentration, 3–9 m*M*), simple mono- and oligosaccharides separated in the anion-exchange mode as borate complexes were sensitively detected. The detection limit was below 1 nmol for most saccharides. The major fluorescence was around 320 and 365 nm with emission maxima at around 410 and 460 nm. The optimum reaction temperature was 120–130°C for aldoses and 110–120°C for pentoses, but higher temperatures (140–150°C) were necessary for non-reducing di- and trisaccharides. Mopper et al. considered the fluorescent entity in this derivatization to be the condensation product(s) between the osone or the enediol derivative formed by the Lobry de Bruyn–Alberda van Ekenstein reaction and this aliphatic amine. Increased sensitivity of oligosaccharides at elevated temperature was probably due to concurrent breakdown yielding aldoses or aldose-like substances. Some aldoses, especially aldopentoses, showed decreased sensitivity on elevation of the reaction temperature, obviously due to partial decomposition. Fig. 5 shows an example of the analysis of apple juice with this system [37].

The reaction of carbohydrates and ethylenediamine gave not only fluorescent products but also electrochemically oxidizable derivatives. Honda et al. [38] established conditions for the detection of reducing monosaccharides based on electrolysis on a glassy carbon electrode. They used a 0.1 *M* ethylenediamine sulfate solution in 0.7 *M* borate buffer (pH 9.0) as the reagent solution to be added to the eluate. This system was effective for various modes of separation including ligand exchange (eluent, water), hydrophilic interaction [eluent, acetonitrile–water (3:1)] and anion exchange as borate complexes (eluent, 0.7 *M* borate buffer, pH 8.5). An applied voltage of 350 mV vs. Ag/AgCl was appropriate and the reaction temperature may

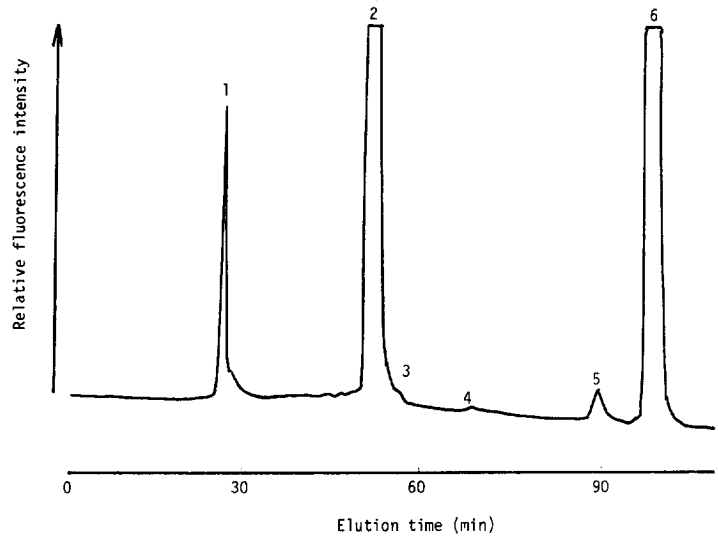


Fig. 5. Analysis of the carbohydrates in apple juice using postcolumn derivatization with ethylenediamine. Column, Durum DA × 4 (particle size 20 μm, 250 mm × 6 mm I.D.); column temperature, 78°C; eluent, 0.7 *M* borate buffer (pH 8.6); flow-rate, 0.7 ml/min; reagent for postcolumn derivatization, 7.5 m*M* (as the final concentration in the reaction mixture) in water; reaction tube, Teflon (50 m × 0.5 mm I.D.); reaction temperature, 145°C; detection, fluorescence at 455 nm with irradiation at 360 nm; sample scale, 20 μl. Peaks: 1 = sucrose; 2 = fructose; 3 = mannose; 4 = galactose; 5 = sorbitol; 6 = glucose. Reproduced from Ref. [37] with permission.

be slightly varied in the range 140–150°C. Addition of ethylenediaminetetraacetic acid to the eluent at a concentration of 0.01% effectively stabilized the baseline. The limit of detection was 1 pmol, but it should be taken into account that the limit may vary among chromatographic systems.

Other aliphatic amines could also be used as fluorogenic reagents for reducing carbohydrates. Kato and Kinoshita employed ethanolamine [39] and 2-aminopropionitrile [40] instead of ethylenediamine. In the ethanolamine method the reagent solution contained ethanolamine and boric acid commonly at a 2% (w/w) concentration, and simple mono- and oligosaccharides separated by the reversed-phase partition mode [eluent, acetonitrile–water (3:1)] and the anion-exchange mode as borate complexes (eluent, 0.5 *M* borate buffer, pH 8.7) were derivatized at 150°C and detected by measuring the fluorescence intensity at 436 nm with irradiation at 357 nm. Linearity was observed in the range 0.3–5.4 nmol for both separation modes. In the 2-aminopropionitrile method the reagent solution contained 6% 2-aminopropionitrile fumarate in 50 m*M* sodium tetraborate (pH 7.5). When reducing carbohydrates separated in the anion-exchange mode as borate complexes were derivatized at 160°C and monitored at the excitation and emission maxima (335 and 428 nm, respectively), the linear range was 0.533–5.33 nmol for glucose and 0.27–5.33 nmol for xylose. The reversed-phase partition mode gave approximately the same linear range. Kato et al. [41] also reported the use of taurine. Use of a reagent solution of 8% taurine in 50 m*M* borate buffer (pH 8.7) at a reaction temperature of 150°C gave similar results to those obtained with the ethanolamine and the 2-aminopropionitrile methods. The excitation and emission maxima were at 368 and 446 nm, respectively. The linear range for glucose in the partition mode [eluent, acetonitrile–water (4:1)] was 0.27–5.3 nmol and that in anion-exchange mode as borate complexes (0.5 *M* borate buffer, pH 8.7) was 2.7–27 nmol. All the methods for fluorimetric detection mentioned in this paragraph are presumably based on the same kinds of reactions as with

ethylenediamine, and no fundamental improvement was obtained with these aliphatic amine analogues. Recently, Del Nozal et al. [42] re-examined the experimental parameters in the ethanolamine method and proposed slightly modified conditions (wavelengths, 400 nm for excitation and 445 nm for emission; reagent concentration, 20%; boric acid concentration, 20%; reaction temperature, 140°C), but the sensitivity was not significantly increased. Prior hydrolysis of the separated carbohydrates by introducing 1.5 *M* *p*-toluenesulfonic acid into the eluate before mixed with the reagent solution increased the sensitivity of oligosaccharides. For example, prior hydrolysis of maltose gave a peak response more than twice the response observed without introduction of the *p*-toluenesulfonic acid solution.

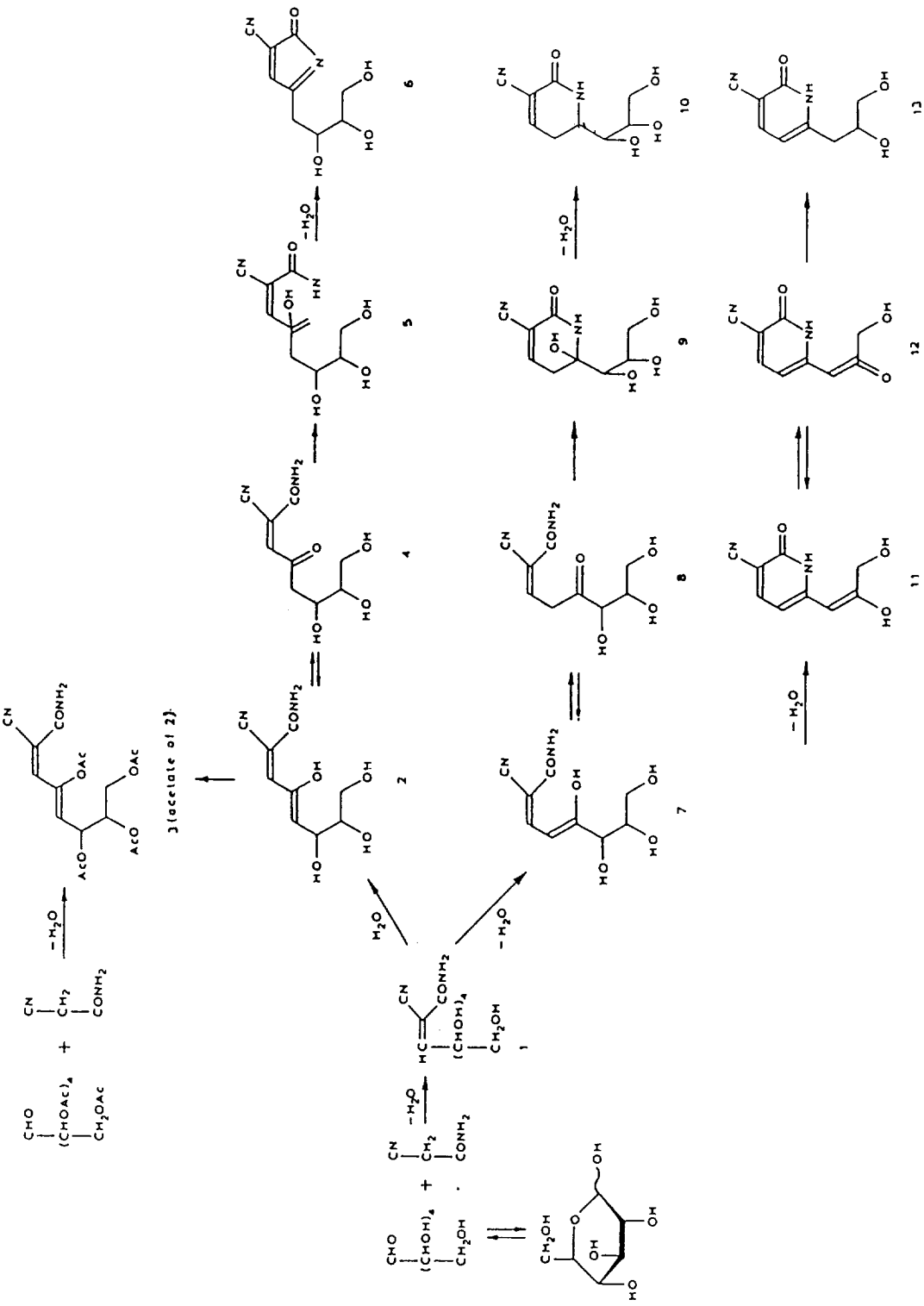
Umegae et al. [43] examined the reactivities of 28 species of 1,2-diarylethylenediamines with simple sugars and found that some of them gave fluorescence; the *p*-methoxyphenyl derivative gave the most intense fluorescence. In 0.1 *M* sodium hydroxide solution it gave fluorescence with excitation and emission maxima at 330 and 460 nm, respectively. They applied this reaction to the postcolumn derivatization of carbohydrates [44], using 15–20 m*M* 1,2-bis(*p*-methoxyphenyl)ethylenediamine solution in 0.7–0.75 *M* sodium hydroxide at a reaction temperature of 150°C. Under these conditions, various mono- and oligosaccharides separated in the anion-exchange mode with a borate buffer could be derivatized and monitored. The linear range was 0.1–10 nmol. Despite the introduction of the aromatic substituent group, this ethylenediamine derivative gave fluorescence at nearly the same wavelength as the native compound (ethylenediamine).

In the postcolumn derivatization with aliphatic amines for fluorimetric detection, reaction tubes of either Teflon or stainless steel could be used, but only Teflon tubes were utilized in the derivatization for electrochemical detection, because dissolution of minute amounts of metals from the stainless-steel tubing into the eluates caused baseline instability. The tube size, together with the flow-rates of the eluate and the reagent solution, was important, because it changes the reaction time. The standard inner diameter of the tube was 0.2–0.5 mm; the tube length varied depending on the reactivity of the reagent used and the flow-rates of both the eluate and the reagent solution.

5.2. 2-Cyanoacetamide (for photometric, fluorimetric and electrochemical detection)

Following the finding of fluorescence formation from reducing carbohydrates with ethylenediamine [35], Honda et al. extended the search for fluorogenic compounds. 2-Cyanoacetamide was one of the promising compounds found in this series of investigations [45]. This reagent forms intense fluorescence at ca. 380 nm when heated with aldoses in weakly alkaline solutions. The wavelength for maximum excitation was ca. 330 nm. Since this reaction was well suited for postcolumn derivatization, careful optimization was performed using eluents in various separation modes as reaction media. During the course of application studies, it was also found that the reaction products absorb 280-nm light intensely. Commercial mercury lamps emit abundantly at this wavelength. Fortunately the products were further detectable by electrolysis on a glassy carbon electrode. Thus, the postcolumn derivatization with 2-cyanoacetamide is associated with three detection methods, i.e., photometry, fluorimetry and amperometry.

The mechanism of reaction of glucose with this reagent is complicated, as shown in Scheme 2 [46]. The first step is the Knovenagel-type condensation of glucose in the aldehyde form with the methylene group in 2-cyanoacetamide to give compound **1**, which is subsequently dehydrated and cyclized via two routes. One route leads to a 2-pyrrolidone derivative (**6**) and the other route gives a 2-pyridone derivative (**13**). These heterocyclic derivatives are considered to be the fluorescence-generating entities, because both gave fluorescence spectra similar to that of the reaction mixture. The UV absorptivity and electrochemical oxidizability are considered to arise from the intermediate compound (**1**), since iso-



Scheme 2. Mechanism of reaction of glucose with 2-cyanoacetamide.

lated **1** gave an absorption maximum at approximately the same wavelength as that of the reaction mixture and this compound also showed high oxidizability under the conditions used for electrochemical detection. Heating of the isolated compound **1** in the same medium as that used for the reaction yielded fluorescence at the same wavelength as that for the glucose–2-acetamide reaction mixture. This provided further evidence for this reaction mechanism. Anyhow, this reaction is complex, and a number of intermediates co-existed in the reaction mixture. The yields of compounds giving fluorescence and UV absorptivity as well as electrochemical oxidizability might not be as high as in general reactions for precolumn derivatization, but the sensitivity in either detection method was sufficiently high, because the total derivatives from the applied sample were led into the detector cell. This situation is comparable to that in precolumn derivatization, where generally part of the derivative is introduced into an analytical column.

The 2-cyanoacetamide method was applied for the postcolumn derivatization of aldoses separated by anion-exchange chromatography as borate complexes. Since the reaction conditions for the photometric and fluorometric detection were different, the conditions were optimized for each detection method (Ref. [47] for UV absorption and [48] for fluorimetric detection). Fig. 6 shows an example of the chromatograms detected by fluorescence. All naturally occurring aldoses were analysed in ca. 70 min with a detection limit of ca. 0.1 nmol, although the sensitivity was varied with aldose.

Since the uronic acid and the amino sugar are the major monosaccharide groups in proteoglycans, the 2-cyanoacetamide method was also applied to these groups of monosaccharides. Uronic acids were separated and derivatized postcolumn in a similar manner as for aldoses [49]. Amino sugars were separated in the direct cation-exchange mode and derivatized under slightly different conditions [50]. Both groups of monosaccharides were detected both photometrically and fluorimetically. Reducing carbohydrates are anomerized in aqueous solutions

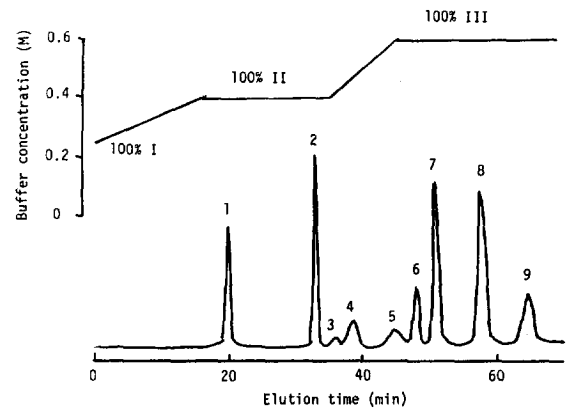


Fig. 6. Analysis of aldoses by anion-exchange chromatography with fluorimetric monitoring after postcolumn derivatization with 2-cyanoacetamide. Column, Hitachi 2633 (particle size of resin 11 μm, 80 mm × 8 mm I.D.); column temperature, 65°C; eluent, gradient elution with borate buffers (I, 0.25 *M*, pH 8.2; II, 0.40 *M*, pH 7.4; III, 0.60 *M*, pH 9.3); flow-rate, 1.0 ml/min; reaction solution for postcolumn derivatization, 5% 2-cyanoacetamide in borate buffer prepared by mixing 10% aqueous solution of 2-cyanoacetamide and 0.60 *M* borate buffer (pH 9.3) at the same flow rate, 0.25 ml/min; reaction tube, Teflon (10 m × 0.5 mm I.D.); reaction temperature, 100°C; detection, fluorescence at 383 nm with irradiation at 331 nm. Peaks; 1 = arabinose; 2 = lyxose; 3 = ribose; 4 = mannose; 5 = arabinose; 6 = fucose; 7 = galactose; 8 = xylose; 9 = glucose. Sample scale, 10 nmol each. Reproduced from Ref. [48] with permission.

but generally one cannot see anomer peaks in liquid chromatography performed at room temperature or above, owing to rapid interconversion. One can see a single peak for each reducing carbohydrates as a time-averaged peak. At low temperature, however, the equilibrium is slow and anomer peaks are often separated from each other. Honda et al. [51] established conditions that could separate anomers of almost all aldoses in the ligand-exchange mode on a cation-exchange resin in the calcium form, and monitored the separated anomers after postcolumn derivatization with 2-cyanoacetamide. In this case only UV monitoring was possible, because the eluate contained a high concentration of acetonitrile. The postcolumn derivatization for electrochemical detection required care to avoid fluctuations of the baseline due to temperature variations and also required the elimination of trace amounts of metals that interfered with the

electrochemical reaction. An established procedure was also reported by Honda et al. [52]. In all these procedures, temperatures below 100°C were used in order to avoid problems resulting from pressure rise. Under such conditions ketoses could also be monitored like aldoses. Schlabach and Robinson [53] re-examined the reaction with 2-cyanoacetamide and proposed conditions for the more sensitive detection of both groups of monosaccharides. Use of a narrower tube for the derivatization reaction increased the theoretical plate number by suppressing dispersion during reaction.

5.3. Arginine and benzamidines (for fluorimetric detection)

Mikami and Ishida [54] reported a method for postcolumn derivatization with arginine. Although the reaction mechanism has not been elucidated, the reaction conditions were mild, similarly to those in the alkylamine and 2-cyanoacetamide methods. The excitation and emission maxima were at 320 and 430 nm, respectively. The reagent solution contained arginine and boric acid. The optimum concentrations of these compounds varied with carbohydrate species, but 1–5% concentrations were generally appropriate. At a reaction temperature as high as 160°C, glucose and fructose gave approximately the same fluorescence intensity, but sucrose and raffinose gave only a 4% intensity relative to glucose. Alditols and 2-deoxy sugars were also negative or almost negative to this reaction. The detection limit of glucose was ca. 25 pmol at a signal-to-noise ratio of 5.

Amidines are structurally similar to arginine in that they all have the $NH_2-\overset{|}{C}=NH$ group. Benzamidine and its *p*-methoxy derivative were selected as fluorogenic compounds by Kai et al. [55], and its application to postcolumn derivatization was studied by the same group [56] and also by Coquet et al. [57]. The benzamidine and *p*-methoxybenzamide methods were different from the arginine method, because the former requires strong alkali as the reaction medium. A typical procedure involved the use of 1.0 *M* potassium hydroxide as a reagent solution at a flow-rate of 0.5 ml/min and reaction at 100°C in a short (5 m) tube. The reagent solution (40 m*M* *p*-methoxybenzamidine in water) had to be introduced into the effluent after the potassium hydroxide solution had been added because an alkaline solution of the reagent was unstable. Under these conditions, the detection limit for glucose separated on an amine-bonded column with aqueous acetonitrile was 15 pmol at a signal-to-noise ratio of 3. Higher concentrations of acetonitrile gave stronger fluorescence but polyamines added to the eluent as a column life-extending agent influenced the fluorescence intensity. Therefore, lower concentrations were desirable.

6. Coloration of condensates with hydrazino compounds (for photometric detection)

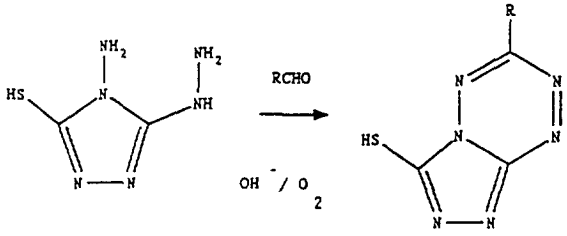
Lever [58] found that the reaction of reducing carbohydrates with certain kinds of aromatic acid hydrazides in strong alkali leads to a yellow colour. Although the exact reaction mechanism based on the structure elucidation of isolated products has not been reported, this coloration is considered to be due to the formation of the anionic species of carbohydrate hydrazones.

The application of this colour formation to the postcolumn derivatization of carbohydrates was reported by Vrátný et al. [59]. They prepared the reagent solution by mixing 10 parts of a 5% solution of *p*-hydroxybenzoic acid hydrazide in 0.5 *M* hydrochloric acid with 75 parts of 0.75 *M* sodium hydroxide. A yellow colour developed on mixing the chromatographic eluate and the reagent solution in a 1:2 ratio and the mixed effluent was heated at ca. 100°C and monitored at 410 nm. This system allowed the detection of glucose and fructose separated in the ligand-exchange mode (eluent, water) with approximately the same sensitivity. However, non-reducing carbohydrates could not be detected with this system. Passing the eluate through a hydrolysis column containing a cation-exchange

resin in the H^+ form prior to derivatization permitted the detection of non-reducing carbohydrates.

Vrátný et al. [60] improved the efficiency of derivatization by converting *p*-hydroxybenzoic acid hydrazide into its amino analogue (*p*-aminobenzoic acid hydrazide). After careful optimization they could detect 4 pmol of sucrose with prior catalytic hydrolysis. Kramer and Engelhardt [61] compared the sensitivities of glucose among various postcolumn derivatization methods. According to their report, the *p*-aminobenzoic acid hydrazide method gave the highest sensitivity (sensitivity relative to the *p*-aminobenzoic acid hydrazide method: thymol–sulfuric acid method, 0.95; 2-cyanoacetamide method with UV detection, 0.75).

Del Nozal et al. [62] reported a novel method for the photometric detection of reducing carbohydrates based on condensation with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald). This reagent was initially developed for aldehyde detection in thin-layer chromatography. The reaction mechanism of the condensation with aldehyde is shown in Scheme 3. The formation of the tetrazine–triazole fused ring condensate requires the hydroxide ion and an oxidant. Under the conditions established, the reagent solution contained Purpald and hydrogen peroxide at concentrations of 0.4% (w/w) and 80 m*M*, respectively, in 2 *M* sodium hydroxide. Postcolumn derivatization of several aldoses, separated in the ligand-exchange mode with water as eluent, at 90°C and monitoring the products by absorption at 550 nm allowed detection down to at least the 0.1 m*M* level.

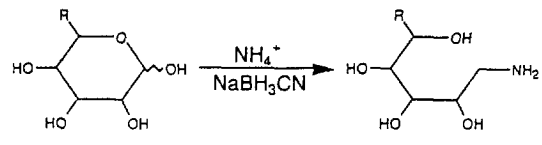


Scheme 3. Reaction of an aldehyde with Purpald.

7. Analysis of amino sugars and reducing carbohydrates (as glycamines) by use of amino acid analysers (for photometric and fluorimetric detection)

Amino sugars are basic carbohydrates widely distributed in glycoconjugates and usually exist in oligo- or polysaccharide chains as N-acylated forms. Acid hydrolysis of glycoconjugates for monosaccharide composition analysis, however, removes the acyl group to give free amino sugars. On the other hand, neutral reducing carbohydrates can be converted into glycamines by reductive amination with an ammonium salt in the presence of borohydride or its analogues (Scheme 4).

Since free amino sugars and glycamines are kinds of aliphatic primary amines, they react with ninhydrin-like amino acids to give purple pigments. Hence these amines can be analysed using an amino acid analyser. Hara et al. [63] optimized the separation of glycamines derived from neutral monosaccharides by ion-exchange chromatography and succeeded in the simultaneous analysis of glucose, galactose, mannose, fucose, rhamnose, xylose, arabinose and ribose in 3 h with photometric detection after postcolumn derivatization with ninhydrin. Calibration graphs for these monosaccharides showed linearity over a range of 0.5–2 μmol. They applied this method to the analysis of the monosaccharides in a few glycolipids and obtained reasonable data. Perini and Peters [64] modified this method by postcolumn derivatization with *o*-phthalaldehyde. In this case detection was carried out fluorimetrically and the sensitivity was much increased (limit of detection 0.05–5 nmol), but separation was not satisfactory. Therefore, the composition analysis of glycopro-



Scheme 4. Conversion of an aldose into a glycamine by reductive amination.

teins reported in the literature seems problematic in terms of accuracy and reproducibility.

A few papers are available concerning the analysis of amino sugars [65–67] and reduced amino sugars [66] using conventional amino acid analyzers. They all used postcolumn derivatization with ninhydrin.

8. Conclusion

Characteristic features of postcolumn derivatization methods have been reviewed. However, it is not easy to select the best method for a particular research project. As an aid for method search, compatible detection methods and applicable groups of carbohydrates for individual derivatization methods are summarized in Table 1, together with the reaction media employed.

There is a general tendency for carbohydrates that electrochemical detection is more sensitive than fluorimetric detection, which in turn is more sensitive than photometry, analogously to other groups of compounds. Which method to choose for detection depends on the amount of sample available and the kinds and amounts of accompanying substances. If samples contain only minute amounts of carbohydrate components,

Table 1
Summary of various postcolumn derivatization methods

Method	Detection[a]	Applicability	Reaction medium[b]
Conversion to furfurals followed by condensation with chromogenic reagents			
Phenol–H_2SO_4	P	Aldoses	S Ac
Orcinol–H_2SO_4	P	Ketoses	S Ac
Thymol–H_2SO_4	P	Oligosaccharides	S Ac
Carbazole–H_2SO_4	P	Uronic acids	S Ac
Thiobarbituric acid–H_2SO_4	P	Amino sugars	S Ac
Use of reducibility			
Cu^{2+}–2,2′-bicinchoninate	P	Reducing carbohydrates	S Al
Cu^{2+}–bis(phenanthroline)	E		S Al
Cerium(IV) ion	F		S Ac
Anisyltetrazolium chloride	P		S Al
IO_4^- oxidation–Hantzsch reaction	P,F	Carbohydrates having a a–CHOH–CH_2OH or –CO–CH_2OH group	N
Heating with fluorogenic reagents			
Aliphatic amines	F	Reducing mono- and oligosaccharides	W Al
2-Cyanoacetamide	P,F,E		W Al
Arginine	F		W Al
Benzamidines	F		S Al
Coloration of the condensates with hydrazino compounds			
p-Aminobenzoic acid hydrazide	P	Reducing oligosaccharides	S Al
Purpald	P		S Al
Use of amino acid analysis systems	P,F	Amino sugars Aldoses (as glycamines)	N

[a] P = photometric detection; F = fluorimetric detection; E = electrochemical detection.
[b] S Ac = strongly acidic solution; N = neutral solution; W Al = weakly alkaline solution; S Al = strongly alkaline solution.

electrochemical detection will be the best, provided that interference by accompanying substances can be eliminated. In cases where ample amounts of samples can be used, as in food and wood pulp samples, photometric detection will suffice.

In general, reactions with chromogenic reagents such as phenol, orcinol and thymol in strong acids have wide applicability to most reducing carbohydrates including aldoses, ketoses and oligo- and polysaccharides, but are not valid for non-reducing carbohydrates such as alditols, aldonic acid and cyclitols. Certain kinds of chromogenic reagents, such as carbazole and thiobarbituric acids, are selective to the corresponding groups (uronic acids and sialic acids) when reacted in strong acids. A number of methods based on heating with fluorogenic reagents (alkylamines, 2-cyanoacetamide, arginine and benzamidines) are effective with most reducing mono- and oligosaccharides, but are not applicable to non-reducing carbohydrates. The methods utilizing reducibility are naturally oriented to reducing carbohydrates. The methods based on coloration of the condensates with hydrazino components have the same limitation. In contrast, the periodate oxidation–Hantzsch reaction method allows the detection of the carbohydrates having the primary hydroxyl group adjacent to the secondary hydroxyl or the keto group, as exemplified by alditols and ketoses, respectively. Direct detection of amino sugars and indirect detection of aldoses as glycamines by amino acid analysis systems are practical methods, especially in laboratories dealing with amino acids and related substances.

On the other hand, the reaction media employed in some of these derivatizations present a technological problem, because the reaction products have to be introduced directly into the detector cell. It is a matter of course that the use of strong acid and alkali should be avoided as far as possible in order to protect hardware. In this respect, the methods based on reducibility, periodate oxidation–Hantzsch reaction and reactions with fluorogenic reagents can be recommended.

Although a number of studies have been published on the postcolumn derivatization of carbohydrates, there have been no papers devoted to detailed observations of peak broadening. If dispersion due to the postcolumn derivatization process were small compared with that in a chromatographic process, there would be no problem of peak broadening due to derivatization. However, it is sometimes crucial and efforts to improve separation are spoiled by this phenomenon. The main cause of dispersion of the derivative(s) band occurs at the stage of mixing the sample solution with reagent solution(s). Scholten et al. [68] discussed the suitability of reactors in postcolumn derivatization using a model system composed of 3- and 4-chloroanilines and fluorescamine. According to their report, an air-segmented reactor gave the least dispersion, a tubular non-segmented reactor followed and a glass bed-packed reactor gave the greatest dispersion, but the difference between the air-segmented and tubular non-segmented reactors was slight. Three tee pieces having different angles (60°, 120° and 180°) between the sample and reagent streams were also compared using a water–aqueous sodium nitrate dilution system. The results indicated only slight difference among angles, but unbalanced velocities of the water and sodium nitrate streams caused enhanced dispersion. The length of the reaction tube also is an important factor for dispersion. Although the average length is several metres, this length is the shorter limit considering the allowable reaction temperature. The optimum reaction temperature varies with the derivatization reaction, but in most cases is close to the boiling point of the reaction solution. Owing to pressure rise, the boiling point is usually elevated to a higher level than that at atmospheric pressure, sometimes as high as 150°C for aqueous reaction solutions. However, it should be taken into account that higher temperatures cause bubbling, which increases dispersion and baseline turbulence.

Mixing of eluates emerging from a column with reagent solution(s) causes dispersion of separated zones of carbohydrate components, but the reproducibility of elution time is generally so high (relative standard deviation $<1\%$, provided that eluent and reagent delivery systems of high capability are used) that comparison

of the capacity factor with that of an authentic specimen ensures reliable identification. Needless to say, measurement of the void volume for the calculation of capacity factor requires a reference value obtained by direct connection of the injection valve to the detector. The relative standard deviation of the peak response is slightly lower than that of the capacity factor, especially for highly sensitivity detection (fluorimetric and electrochemical detection), but it is within allowable values in practical carbohydrate analysis.

Recent progress in glycobiology has involved the analysis of oligo- and polysaccharides in glycoconjugates, and precolumn labelling has played a more important role than postcolumn derivatization because recovering derivatives after separation and quantification are necessary for structure elucidation. However, the importance of postcolumn derivatization in association with the automated analysis of such carbohydrates should be stressed.

References

[1] M. Dubois, K.A. Gilles, J.K. Hamilton, R.A. Rebers and F. Smith, Anal. Chem., 28 (1956) 350.
[2] J. Bruckner, Biochem. J., 60 (1955) 200.
[3] F. Schutz, Papier-Fabr., 36 (1938) 55.
[4] Z. Dische, Proc. Soc. Exp. Biol. Med., 55 (1944) 217.
[5] Z. Dische, Z. Biochem., 189 (1927) 77.
[6] V.H. Roe, J. Biol. Chem., 107 (1934) 15.
[7] G. Ceriotti, J. Biol. Chem., 198 (1952) 297.
[8] J. Havlicek and O. Samuelson, Anal. Chem., 47 (1975) 1854.
[9] W. Voelter and H. Bauer, J. Chromatogr., 126 (1976) 693.
[10] S. Johnson and O. Samuelson, Anal. Chim. Acta, 36 (1966) 1.
[11] M.J. Krantz and Y.C. Lee, Anal. Biochem., 63 (1975) 464.
[12] D.F. Smith, D.A. Zopf and V. Ginsburg, Anal. Biochem., 85 (1978) 602.
[13] M.H. Simatsupang, J. Chromatogr., 180 (1979) 177.
[14] H. Engelhardt and P. Ohs, Chromatographia, 23 (1987) 657.
[15] M. Kramer and H. Engelhardt, J. High Resolut. Chromatogr., 15 (1992) 24.
[16] K.J. Kramer, R.D. Speirs and C.N. Childs, Anal. Biochem., 86 (1978) 692.
[17] M. John, J. Schmidt, C. Wandrey and H. Sahm, J. Chromatogr., 247 (1982) 281.
[18] C.J. Rogers, C.W. Chambers and N.A. Clarke, Anal. Chem., 38 (1966) 1851.
[19] H. Hirayama, K. Hiraki and Y. Nishikawa, Bunseki Kagaku, 20 (1991) 1435.
[20] S. Nakano, H. Taniguchi, T. Furuhashi and K. Mikoshiba, Yakugaku Zasshi, 93 (1993) 350.
[21] T. Momose and Y. Ohkura, Chem. Pharm. Bull., 3 (1956) 209.
[22] K. Mopper and E.M. Gindler, Anal. Biochem., 56 (1973) 440.
[23] K. Mopper, Anal. Biochem., 85 (1978) 528.
[24] K. Mopper, Anal. Biochem., 87 (1978) 162.
[25] M. Sinner and J. Puls, J. Chromatogr., 156 (1978) 197.
[26] N. Watanabe and M. Inoue, Anal. Chem., 55 (1983) 1016.
[27] S. Katz, W.W. Pitt, Jr., J.E. Mrochok and S. Dinsmore, J. Chromatogr., 101 (1974) 193.
[28] K. Mopper and E.T. Degens, Anal. Biochem., 45 (1972) 147.
[29] A. Hantzsch, Liebigs Ann. Chem., 215 (1882) 72.
[30] T. Nash, Biochem. J., 55 (1953) 416.
[31] O. Samuelson and H. Stromberg, Carbohydr. Res., 3 (1966) 89.
[32] B. Carlsson, T. Isaksson and O. Samuelson, Anal. Chim. Acta, 43 (1968) 47.
[33] K. Larsson and O. Samuelson, Carbohydr. Res., 50 (1976) 1.
[34] S. Honda, M. Takahashi, S. Shimada, K. Kakehi and S. Ganno, Anal. Biochem., 128 (1983) 429.
[35] S. Honda, K. Kakimoto, K. Sudo, K. Kakehi and K. Takiura, Anal. Chim. Acta, 70 (1974) 133.
[36] S. Honda, K. Sudo, K. Kakimoto, K. Kakehi, H. Yuki and K. Takiura, Anal. Chim. Acta, 77 (1975) 199.
[37] K. Mopper, R. Dawson, G. Liebezeit and H.-P. Hansen, Anal. Chem., 52 (1980) 2018.
[38] S. Honda, K. Enami, T. Konishi, S. Suzuki and K. Kakehi, J. Chromatogr., 361 (1986) 321.
[39] T. Kato and T. Kinoshita, Anal. Biochem., 106 (1980) 238.
[40] T. Kato and T. Kinoshita, Bunseki Kagaku, 31 (1982) 615.
[41] T. Kato, F. Iinuma and T. Kinoshita, Nihonkagakukaishi, (1982) 1603.
[42] M.J. Del Nozal, J.L. Bernal, F.J. Gomez, A. Antolin and L. Tobibio, J. Chromatogr., 607 (1992) 191.
[43] Y. Umegae, H. Nohta and Y. Ohkura, Anal. Chim. Acta, 217 (1989) 263.
[44] Y. Umegae, H. Nohta and Y. Ohkura, Anal. Sci., 5 (1989) 675.
[45] S. Honda, Y. Matsuda, M. Takahashi, K. Kakehi and S. Ganno, Anal. Chem., 52 (1980) 1079.
[46] S. Honda, K. Kakehi, K. Fujikawa, Y. Oka and M. Takahashi, Carbohydr. Res., 183 (1988) 59.
[47] S. Honda, M. Takahashi, Y. Nishimura, K. Kakehi and S. Ganno, Anal. Biochem., 118 (1981) 162.

[48] S. Honda, M. Takahashi, K. Kakehi and S. Ganno, Anal. Biochem., 113 (1981) 130.
[49] S. Honda, S. Suzuki, M. Takahashi, K. Kakehi and S. Ganno, Anal. Biochem., 134 (1983) 34.
[50] S. Honda, T. Konishi, S. Suzuki, M. Takahashi, K. Kakehi and S. Ganno, Anal. Biochem., 134 (1983) 483.
[51] S. Honda, S. Suzuki and K. Kakehi, J. Chromatogr., 291 (1984) 317.
[52] S. Honda, T. Konishi and S. Suzuki, J. Chromatogr., 299 (1984) 245.
[53] T.D. Schlabach and J. Robinson, J. Chromatogr., 282 (1983) 169.
[54] H. Mikami and Y. Ishida, Bunseki Kagaku, 32 (1983) E207.
[55] M. Kai, K. Tamura, M. Yamaguchi and Y. Ohkura, Anal. Sci., 1 (1985) 59.
[56] M. Kai, K. Tamura, H. Watanabe and Y. Ohkura, Bunseki Kagaku, 38 (1989) 568.
[57] A. Coquet, J.-L. Venthey and W. Haerdi, J. Chromatogr., 553 (1991) 255.
[58] M. Lever, Anal. Biochem., 47 (1972) 273.
[59] A. Vrátný, J. Ouhrabkova and J. Copikova, J. Chromatogr., 191 (1980) 313.
[60] A. Vrátný, U.A.Th. Brinkman and R.W. Frei, Anal. Chem., 57 (1985) 224.
[61] M. Kramer and H. Engelhardt, J. High Resolut. Chromatogr., 15 (1992) 24.
[62] M.J. Del Nozal, J.L. Bernal, V. Hernandez, L. Toribio and R. Mendez, J. Liq. Chromatogr., 16 (1993) 1105.
[63] S. Hara, H. Ikegami, A. Shono, T. Mega, T. Ikenaka and Y. Matsushima, Anal. Biochem., 97 (1979) 166.
[64] F. Perini and B.P. Peters, Anal. Biochem., 123 (1982) 357.
[65] K. Murayama and N. Shindo, J. Chromatogr., 143 (1977) 137.
[66] K. Murayama, N. Shindo and H. Koide, Anal. Biochem., 70 (1976) 537.
[67] P.-W. Cheng and T.F. Boat, Anal. Biochem., 85 (1978) 276.
[68] A.H.M. Scholten, U.A.Th. Brinkman and R.W. Frei, J. Chromatogr., 218 (1981) 3.