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# DETERMINATION OF CARBOHYDRATES IN HUMAN SERUM BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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Amperometric detection in high performance liquid chromatography was applied for the determination of carbohydrates in human serum. Remarkable difference of mannose content was observed for serum in disorder states compared with normal subjects. Clinical interest was suggested by an unsusceptibility of mannose to influence of meal and a significant correlation of mannose to glucose content. The selectivity involved in the method and the stability of a working electrode were excellent by virtue of a low applied potential.

Reducing carbohydrates have been detected with refractive index detector[1)2)], colorimetric[3)-5)] or fluorimetric methods[6)-8)] in high performance liquid chromatography. These techniques often suffered from drawbacks such as insufficient sensitivity, lack of selectivity, instability or hazardous corrosion of reagent. Therefore, an alternative technique, which will be easily attainable, more sensitive and highly selective, is still being pursued.

Recently, a novel detection of reducing carbohydrates by using high performance liquid chromatography with an amperometric detector has been developed[9)]. A coupling of redox reaction of metal complex as a mediator with the reducing ability of sugars provides a principle of detection as follows:

$$Cu(phen)_2^{2+} \xrightarrow[OH^-,\ \Delta\ ,\ \text{in post column}]{\text{reducing sugars}} Cu(phen)_2^{+} \xrightarrow[\text{oxidation}]{\text{electrochemical}} Cu(phen)_2^{2+}$$

where copper bis-phenanthroline (abbreviated as CBP in the following) was utilized as the mediator. The redox potential of $CBP^{2+/+}$ is ca. -50 mV vs Ag/AgCl[10)]. Hence, the hypothetical thermodynamical redox potentials of reducing sugars are assumed more negative than -50 mV, although sugars are not detectable by direct electrochemical method. As expected, the applied potential of +40 mV was enough, affording mass-transport limiting current. Such a low potential facilitated full usage of specific aspects inherent to the amperometric detection. A great deal of applications have been devoted to the oxidative uses, demanding uses of rather high positive potentials. For example, the potential more than +700 mV were needed for

the detection of catecholamines studied in an abundance of earlier publications. On high potential use, the surface of electrode is prone to irreversible change or poisoning with polymerized products, resulting in unreproducibility and/or instability [11]. In fact, the most of troubles encountered in the analyses of complex matrix containing a lot of components such as in urine or serum were rapid deteriorations in either stability or reproducibility, which were ascribable to use of high oxidation potentials. Hence, these assays inevitably involve intricate and time consuming pre-treatments of samples. In addition, use of high potential causes high and fluctuant background, raising detection limits although the net background current can be canceled out electronically. Furthermore, the high applied potential exerts drawback in selectivity, which should be the excellent advantage of the detector[12]. The new method does not suffer from these problems by virtue of low applied potential. Additional selectivity may be gained under the conditions of chemical reaction optimized for sugars[9].

Serum is well known to contain overwhelming amount of glucose compared with other carbohydrates. Minor components except glucose have not always been investigated extensively in terms of their presences, amounts and variations related to diseases [13][14]. One of the reasons is the lack of an appropriate method of analysis possessing high sensitivity, selectivity and easiness.

In this study, the new technique was applied to analyses of glucose and others in human serum in normal and disorder states. Distribution profiles of minor sugars might provide useful informations about diagnosis, treatment and metabolism. One pmol of detection limit was attained by using cation exchange column and water as eluant, previously[9]. The system was successfully adaptable to the samples containing comparable amounts of sugars such as in urine. Another separation mode should be employed for analyses of predominant content of one component such as in serum. Fortunately, glucose is the last to elute in the chromatography of anion exchange column and borate buffer.

## EXPERIMENTAL

### Materials

All chemicals were of reagent grade and commercially available. CBP was prepared according to the literature[15]. It was recrystallized from distilled water and dried at 80°C for two hours under vaccum. Reagent solution was prepared by dissolving CBP into the solution containing sodium monohydrogenphosphate as supporting electrolyte. All solutions and eluant were made from single distilled water.

### High performance liquid chromatography

The high performance liquid chromatograph was equipped with a Constametric IIG constant flow pump ( Milton Roy, U.S.A., flow rate:0.35 ml/min ), strong anion exchange columns IEX 222 ( 12 μ, Toyo Soda, Tokyo, Japan, series connected three columns of 10 cm long × 2 mm I.D. ), sample injector with 20 μl loop volume, Model 7120 ( Rheodyne, U.S.A. ) and an amperometric detector VMD 101 ( Yanaco, Kyoto, Japan ). The reagent solution containing CBP was delivered at a flow rate of 0.80 ml/min by a constant flow pump TRI ROTAR-II ( Japan Spectroscopic, Tokyo, Japan ) and mixed with the column eluate by means of a simple stainless tee. PTFE tube ( 0.5 mm I.D., 5 m

long ) was used as a reaction coil which was immersed in a water bath (95 ± 0.1°C). The reaction mixture was passed through the cooling coil ( 30 cm long, 0.25 mm I.D.), dipped in water before reaching to the detector. Since a slight diminution of fructose was recognized at such low levels of sample concentration as several part per millions at the column temperature near or above 60°C, the columns were thermostatted at 58°C within ± 0.1°C in a water bath. Isocratic elution was employed because of the low compatibility of electrochemical detection with gradient elution. In order to shorten the injection cycle, the size of the columns were minimized as possible at the expense of separation and overloading of glucose. According to the previous study[9], pH of the mixture at the reaction coil was adjusted to be around 11.20. The low content of supporting electrolyte in reagent solution is desirable for the electrochemical detection. The low concentration of borate buffer as eluant was considered good choice. The eluant contained 0.02 M sodium tetraborate and 0.1 M boric acid and was adjusted to pH 8.98 with 2 M sodium hydroxide. The reagent solution contained 2 mM CBP in 0.2 M disodium hydrogen phosphate, adjusted to pH 11.70. The applied potential on the working electrode of glassy carbon was fixed at + 40 mV vs Ag/AgCl. Figure 1 shows the chromatograms of authentic mixture of simple carbohydrates and a 4000-fold dilution of the juice squeezed from an apple obtained under these experimental conditions.

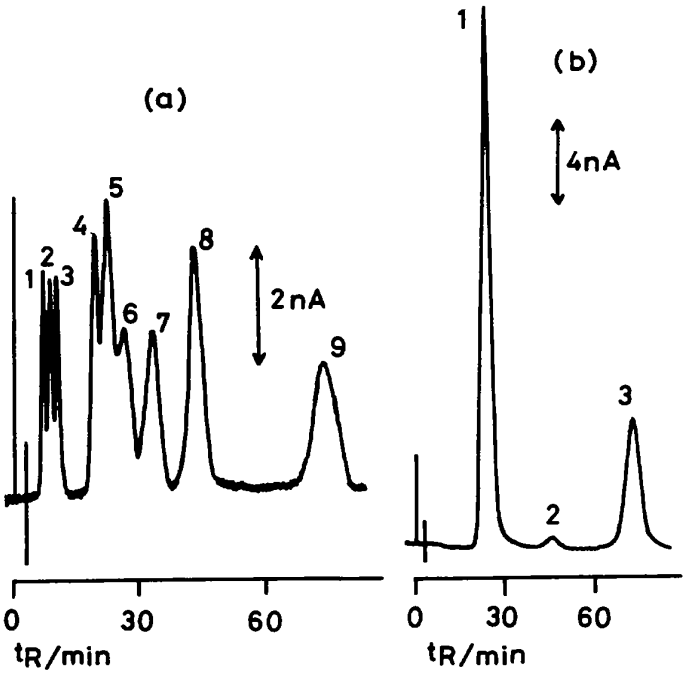


Fig. 1 Chromatograms of standard and natural product

a) authentic mixture: 1;maltose, 2; lactose, 3;ribose, 4;mannose, 5;fructose, 6;arabinose, 7;galactose, 8;xylose, 9; glucose, 1-3;each 20 ng, 4-9;each 40 ng

b) a 4000-fold dilution of juice squeezed from an apple: 1;fructose, 2;xylose, 3;glucose

Procedures

The subjects studied usually fasted overnight. Blood was collected by veinpuncture and kept for 15 min at room temperature. Serum was then separated by centrifugation for 10 min at 1500 rpm and deproteinized by mixing with equivolume of acetonitrile followed by centrifugation. The supernatant was diluted with equivolume of distilled water and 20 µl of the dilute solution was injected on HPLC.

## RESULTS AND DISCUSSION

Pre-treatment of serum

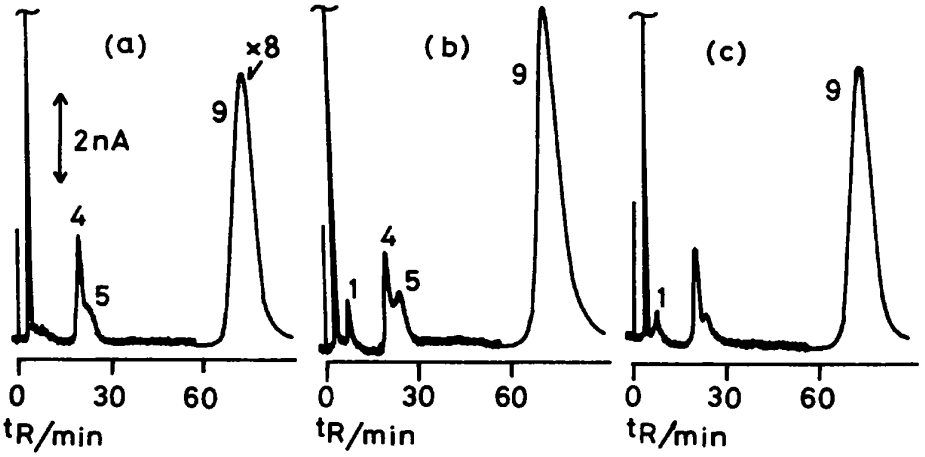


Fig.2 Effect of meal (a) before, (b) 1 h later, (c) 2 h later. Numbers labelled on each peak are referred to those in Fig.1(a), as is the case in following figures.

The time until deproteinization affected the persistence of sugars. About 20 % diminution of mannose was observed in serum deproteinized after 72 h, compared with that immediately deproteinized. Slight decrease was also recognized in the serum separated at 2 h after collection of blood. However, the change of sugars was not found in the deproteinized serum stored in a refrigerator. Thus, serum was separated and deproteinized soon after collection and stored in the refrigerator until injection on HPLC. It was found that sugars did not degrade even if stored in the refrigerator as long as two weeks.

## Effect of meal

Sera were collected from healthy subjects immediately before, and 1 and 2 h after a meal. Chromatograms of the sera are shown in Fig.2. Increase of glucose and appearance of maltose were notable 1 h after the meal. Glucose gradually restored to an initial level and maltose decreased gradually 2 h after the meal. Fructose seemed to depend on the kind of foods taken. It is well known that glucose content in serum is severely affected by meal and it provides the problem often suspected in glucose assay. It was noticeable that mannose was unsusceptible to perturbation by the meal. The chromatograms shown in Fig.2 depicted typical ones commonly observed for healthy subjects. That is, maltose, lactose and ribose were not found in the serum before meal and mannose content is ca. 500 μg/100 ml serum, which was almost constant as shown later.

## Chromatograms of sera from patients

Patients with a variety of diseases showed markedly different chromatograms in contrast to normal subjects, of which chromatograms were almost the same. Diseases studied in this work involved liver cirrhosis, hepato cellular carcinoma, acute and chronic hepatitis, cholestasis, myocardial infarction, diabetes mellitus,

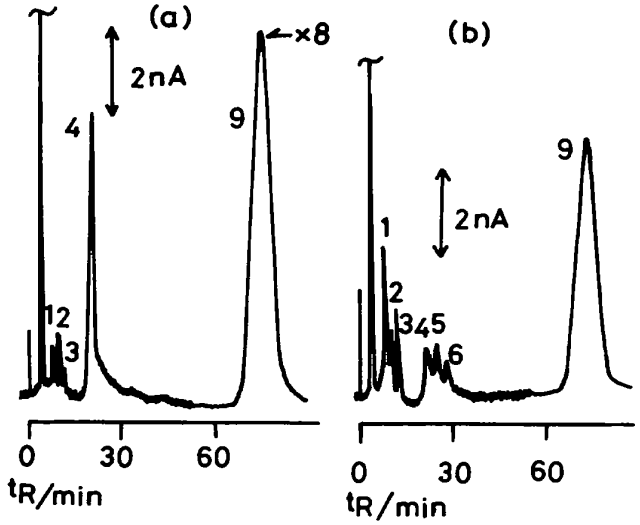


Fig.3 Chromatograms for an esophageal cancer(a) and a stomach cancer(b)

esophageal cancer, acute and chronic myelocytic leukemia, stomach cancer, chronic renal failure, gall bladder cancer, diabetes insipidus, malignant lymphoma, collagen diseases, gastric ulcer, ovarial cyst, nephrotic syndrome, hyperthyroidism, hyperlipidemia etc. Two notable aspects were derived from inspection of chromatograms obtained for these diseases. It may be pointed out as an evidence of some kind of disease that all of maltose, lactose and ribose are involved. Other characteristics of disorders reflect in alteration of mannose content. Figure 3 shows chromatograms obtained for esophageal cancer and stomach cancer. It was noticeable that mannose and glucose increased in Fig.3(a), while decrease of mannose in Fig.3(b). Figure 4 shows chromatograms for the patients with diabetes mellitus. Increases of mannose as well as of glucose in both of them were remarkable. Figure 5(a) was obtained for the patient of acute leukemia at admission. Another 4-fold dilution of serum compared with usual procedure was needed to meet a dramatically increased peak. The largest peak was identified as fructose, not as mannose. Mannose was recognized only small leading shoulder of fructose. Figure 5(b) shows the chromatogram for the same subject after hospitalization of about one month, when the therapy induced clinical improvement. Fructose disappeared and both of mannose and glucose were approximately normal. It was only slight symptom of disorder that there was an unknown peak marked u.

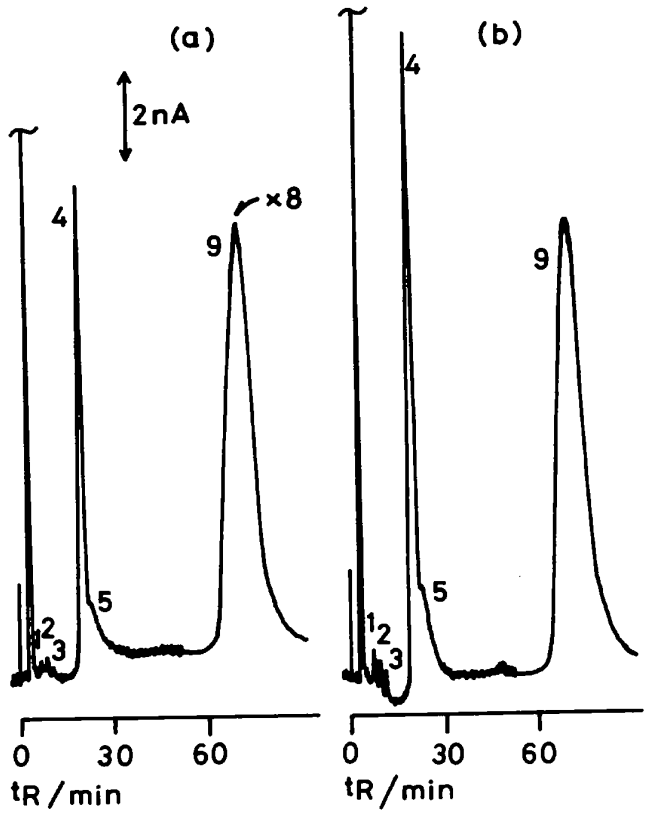


Fig.4 Chromatograms for diabetes mellitus(DM)
(a) DM with alcoholism
(b) DM with ischemic heart disease

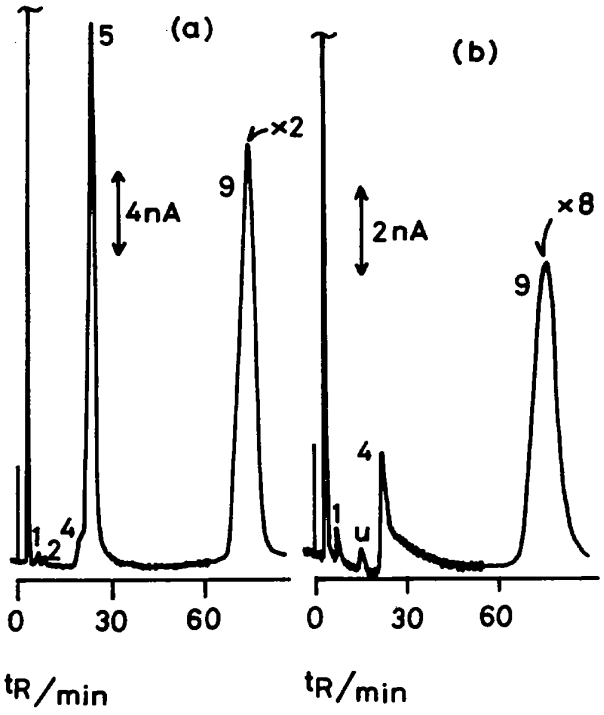


Fig.5 Chromatograms obtained for an acute leukemia
(a) at admission
(b) after one month of hospitalization

Mannose

It was proved that mannose was always involved in human serum. The distribution of its concentration gathered at narrow range for healthy persons ( 496 ± 36 μg/100 ml serum, n=16 ) but scattered over wider for disorder states ( 948 ± 462 μg/100 ml serum, n=83, maximum = 2960, minimum = 240 μg/100 ml ), as compared in Fig.6. The reason is ambiguous at present.

In contrast to galactose or fructose, mannose is known to have metabolic effects similar to those of glucose. For example, mannose significantly evokes insulin secretory response of the human pancreas, as elucidated from the intravenous infusion studies[16]. Significant correlation between amounts of mannose and glucose for patients was verified as shown in Fig.7 ( r = 0.72, n = 83 ). Concentration of mannose seems not to be under the influence of intake of foods, as mentioned pre-

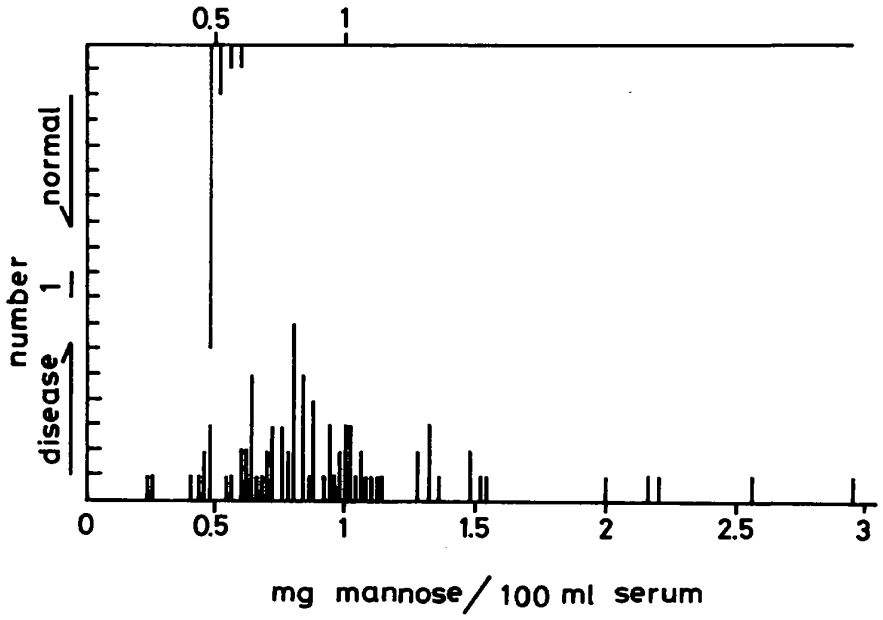


Fig.6 Population histogram of mannose contents for normal and diseased persons

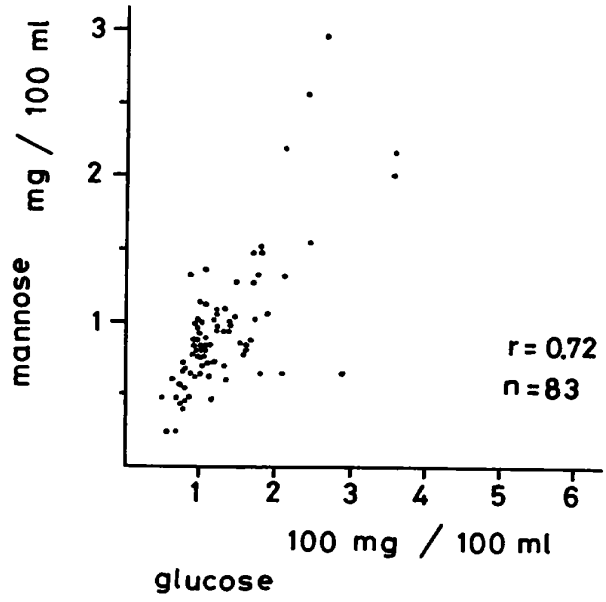


Fig.7 Distribution of mannose content vs glucose content observed for patients with diseases

viously. It was anticipated in some extent since there is no mechanism of active transport of mannose across gut. No influence of meal and correlation to glucose suggest clinical interest of mannose in complement to glucose.

In order to confirm the presence of mannose, isolated chromatographic zone supposed to contain mannose was analysed by a modified quadrupole mass spectrometer ( ANELVA TE 600 GC/MS ) in API mode. Mass spectra definitely clarified single component of molecular weight 180, consistent with mono-hexose. Capacity factors of mono-saccharides except mannose did not coincide with that of chromatographic peak thought as mannose. It is unlikely that it could be identified to other substances, from the consideration of specificities involved in the method ( separation by anion exchange chromatography, limited condition of chemical reaction and electrochemical detection with extremely low applied potential ).

Quantitation

The very simple chromatograms were obtained for serum. No interference from other naturally occuring serum constituents were encountered. Each peak was identified with no ambiguity on the basis of exactly matching retention with standard. The contents of galactose and xylose were below detectable range of the method. Arabinose was rarely observed.

Amount of glucose in serum overloaded the columns and the detection system under the experimental condition, resulting in non-linear relation between the detector response and glucose content. However, glucose could be quantitated by comparison with calibration curve. Reasonable values were obtained for healthy subjects ( 81.5 ± 16.5 mg/100 ml serum, n = 16 ). The coefficient of variation for repeated injections ( n = 4 ) of a glucose solution ( 100 mg/100 ml ) was ± 6 % but getting worse at higher concentration. The coefficient of variation for mannose was far better since the concentration of mannose was within the linear range of response. Within-day and day-to-day variations were evaluated ± 2.1 % ( n = 4 ) and ± 4.1 %, respectively. Long term variation of the detector and HPLC was checked with standard authentic mixture from time to time, with satisfactory results.

Detection limit of mannose was estimated as 5 pmol even under a moderate setting of the sensitivity, which depended on CBP content in the reagent solution[9]. An additional improvement of the detection limit may be expected at least by two fold.

The background current of the detector was oxidative and about 30 nA. $CBP^{+}$ is easily re-oxidized by dissolved oxygen. This was exploited by Anson et al., who used polymer coated electrode impregnated with $CBP^{2+/+}$ to catalyze the electroreduction of dissolved oxygen in water[10]. But this property was undesirable for our purpose and the elimination of dissolved oxygen was indispensable. Reductive current of dissolved oxygen at + 40 mV contributed a certain extent to the background current, when oxygen was not excluded. However, the same elimination procedure as carried out in the previous work[9] made it minimal and improved reproducibility as well as sensitivity. The origin of the background current was not clear. Since the background current increased with increasing CBP content in the reagent and with raising temperature of the reactor, CBP itself may participate in causing of the background current. Elucidation of the detail might lead to further enhancement of

the detector performance.

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REFERENCES

1) D.L. Hendrix, R.E. Lee, J.G. Baust, H. James : J. Chromatogr., 210, 45 (1981).
2) J. Schmidt, M. John, C.J. Wandrey : J. Chromatogr., 213, 151 (1981).
3) M.H. Simatupang : J. Chromatogr., 178, 588 (1979).
4) M. D'Amboise, T. Hanai, D. Noel : Clin. Chem., 26, 1348 (1980).
5) M. Sinner, J. Puls : J. Chromatogr., 156, 197 (1978).
6) S. Katz, W.W. Pitt, J.E. Mrochek, S. Dinsmore : J. Chromatogr., 101, 193 (1974).
7) S. Honda, Y. Matsuda, M. Takahashi, K. Kakehi, S. Ganno : Anal. Chem., 52, 1079 (1980).
8) K. Mopper, R. Dawson, G. Liebezeit, H.P. Hansen : Anal. Chem., 52, 2018 (1980).
9) N. Watanabe, M. Inoue : Anal. Chem., 55, 1016 (1983).
10) K. Shigehara, F.C. Anson : J. Electroanal. Chem., 132, 107 (1982).
11) G. Sittampalam, G.S. Wilson : Anal. Chem., 55, 1608 (1983).
12) P.T. Kissinger : " Liquid Chromatography Detectors ", Edited by T.M. Vickrey, p. 125 (1983), (Marcel Dekker, Inc., New York).
13) J.E. Mrochek, S.R. Dinsmore, T.P. Waalkes : Clin. Chem., 21, 1314 (1975).
14) T.P. Monson, K.P. Wilkinson : Clin. Chem., 25, 1384 (1979).
15) B.J. Hathaway, I.M. Procter, R.C. Slade, A.A.G. Tomlinson : J. Chem. Soc., A, 2219 (1969).
16) O.P. Ganda, J.S. Soeldner, R.E. Gleason, I.G.M. Cleator, C. Reynolds : J. Clin. Endocrinol. Metab., 49, 616 (1979).

Keyword phrases

carbohydrates in serum; amperometric detector; electrochemical detection of sugars; high performance liquid chromatography; post column reaction.

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