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Separation of aldose enantiomers by capillary electrophoresis in the presence of optically active N-dodecoxycarbonylvalines

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Abstract

The 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives of aldose enantiomers were well separated from each other by capillary zone electrophoresis using a neutral phosphate buffer containing an optically active N-dodecoxycarbonylvaline (DCV). The L-enantiomers migrated first and the D-enantiomers followed, when *R*-DCV was added to electrophoretic solution, and the migration order was reversed for the addition of *S*-DCV. This separation was inherent to PMP derivatives; other derivatives such as 2-aminopyridine, 8-aminonaphthalene-1,3,6-trisulfonate and 4-nitrobenz-2-oxa-1,3-diazole-tagged glycamine derivatives were not separated at all. L-Lauroylvaline and sodium dodecyl sulfate were also not separated. Based on these results, together with the ^{1}H NMR signal change (upfield shift of the carbohydrate-proton signals in PMP–aldoses) on addition of *R*-DCV, we speculated this separation to be due to the difference in the ease of intermolecular ring formation by hydrogen bonding. © 1997 Elsevier Science B.V.

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1. Introduction

Carbohydrates in biological samples are generally accompanied by a number of isomers and/or homologues having quite similar structures, hence analysis of carbohydrates always requires separation from these compounds. Though there have been a few specific methods based on enzyme reactions, they can analyze only limited species of carbohydrates; most methods are based on simultaneous analysis of structurally resembling carbohydrates together with accompanying substances. Methods based on capillary electrophoresis (CE) are very promising, since it allows high resolution separation.

Carbohydrates can be separated as anions in strong alkali in intact state and detected by an electrochemical method based on oxidation on a copper electrode [1], like in liquid chromatography (LC) coupled with electrochemical detection [2]. Pulse amperometric detection on a gold electrode developed for LC has also been applied to detection of carbohydrates separated by CE [3]. This strong alkali method allowed separation of approximately 25 species of carbohydrates, but the separation was based on a single mode of direct zone electrophoresis. The mono- and oligosaccharides separated in alkali could also be detected by an indirect UV method [4,5]. On the other hand, carbohydrates react with the borate ion in a running buffer to give anionic complexes which can also be separated by zone electrophoresis. In this indirect zone electrophoretic system, photometric detection is possible,

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because the resultant carbohydrate–borate complexes absorb in the low-wavelength UV region [6]. However, this detection method is not sensitive.

Most of other methods for carbohydrate analysis by CE involve derivatization by reductive amination. The first paper on carbohydrate analysis by CE [7], which was based on separation as 2-aminopyridine (AP) derivatives, is a good example of this strategy. A number of other amines have hitherto been used for derivatization, including 4-aminobenzoic acid (ABA) [8] and its analogues (ethyl ester, ABEE [9] and nitrile, ABN [10]), 6-aminoquinoline (AQ) [11], 7-aminonaphthalene-1,3-disulfonic acid (ANDS) [12], 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) [13] and 1-aminopyrene-3,6,8-trisulfonic acid (APTS) [14]. A two-step procedure consisting of conversion to glycamines, followed by reaction with 3-(4-carbozenzoyl)-2-quinolinecarboxaldehyde (CBQCA) in the presence of the cyanide ion [15] also belongs to this category. The imino group introduced to carbohydrates by reductive amination with these amines is positively charged in acidic media. Derivatives formed with amphoteric amines such as ABA, ANDS, ANTS and APTS are negatively charged in alkaline solutions due to dissociation of the carboxyl and/or sulfonic acid group.

Condensation with 1-phenyl-3-methyl-5-pyrazolone (PMP) [16] is another type of derivatization. The derivatization proceeds rapidly and quantitatively under mild conditions and does not cause release of acid- or alkali-labile substituent groups, such as the sialic acid group and the sulfate group. The PMP derivatives are negatively charged in alkaline solutions due to dissociation of the enol group in the pyrazolone ring, formed from the keto group by tautomerism. On the other hand the PMP derivatives have moderate hydrophobicity, which enables solubilization into moving sodium dodecyl sulfate (SDS) micelles. Because of these versatile chemical properties, together with their strong absorption in the UV region and their ready oxidizability on a carbon electrode, we have been undertaking studies to develop as many separation modes for PMP derivatives as possible. We have already established procedures for zone electrophoresis as borate [17] as well as divalent metal [18] complexes of PMP–mono- and oligosaccharides. Another group reported separation by SDS-micellar electrokinetic chromatography (MEKC) [19].

In this paper we describe an additional separation mode in the presence of optically active N-dodecoxycarbonylvalines.

2. Experimental

2.1. Apparatus

CE was performed with a Waters Quanta 4000 apparatus equipped with a UV monitor. A fused-silica capillary with an I.D. of 50 μm was obtained from Polymicro Technologies (Phoenix, AZ, USA). It was rinsed with 0.1 *M* sodium hydroxide before use. Every time when running buffer was changed, the capillary was equilibrated by flushing it with the new buffer to be used for at least 5 min. Capillary temperature was kept at 25°C throughout the work.

2.2. Reagents

R- and *S*-N-Dodecoxycarbonylvalines (*R*- and *S*-DCVs, respectively) were supplied by Waters Corporation. N-Lauroyl-L-valine was a gift from Ajinomoto. PMP was obtained from Kishida (Osaka, Japan) and recrystallized twice from methanol before use. Arabinose (purity, 98%), xylose (purity, 99%), galactose (purity, 98%), and glucose (purity, 98%) of L-series were obtained from Sigma–Aldrich Japan (Tokyo, Japan). All other chemicals and carbohydrate samples were of the highest grade commercially available. Deionized and double distilled water was used for preparation of running buffers.

2.3. Derivatization

Derivatization was performed by our established procedure [16]. Briefly, a sample of an aldose (10–100 nmol) or a mixture of aldoses were dissolved in 0.3 *M* sodium hydroxide (50 μl) and a 0.5 *M* methanolic solution of PMP (50 μl) was added. The resultant solution was heated for 30 min at 70°C. It was cooled, neutralized with 0.3 *M* hydrochloric acid, and evaporated to dryness under reduced pressure. The residue was redissolved in water (200 μl) and extracted three times with chloroform (200 μl). The aqueous layer was evaporated to dryness and the residue was reconstituted in a minimum

volume of water, and the solution was analyzed by CE.

2.4. ^1H NMR spectrum

An aqueous solution containing 10 μ*M* each of *R*-DCV and PMP–D-glucose was evaporated to dryness under reduced pressure, and the residue was dissolved in deuterium oxide (1 ml), and ^1H NMR spectrum was measured using a JEOL GSX-500 spectrometer operating at 500 MHz at room temperature.

3. Results and discussion

Noe and Freissmuth [20] reported the successful separation of enantiomeric aldoses as the borate complexes with *S*-1-phenylethylamine. Stefansson and Novotny [21] also described the enantioseparation of aldoses as reductive amination derivatives with AP, 5-amino-2-naphthalenesulfonic acid and 4-amino-5-hydroxy-2,7-naphthalenedisulfonic acid under carefully optimized conditions in the presence of the borate ion or cyclic dextrins. However, we could not observe any separation of PMP derivatives of enantiomeric aldoses in such solutions. Furthermore, we could not find any systems that allowed for enantioseparation without DCV, at least by direct zone electrophoresis, MEKC with SDS micelles, ion-interaction electrokinetic chromatography with Polybrene, and size-exclusion electrophoresis in agarose gel, even with careful examination of the compositions of running buffers. Enantioseparation was achieved solely by zone electrophoresis in the presence of DCVs. Therefore, we optimized conditions for separation using *R*-DCV and demonstrated enantioseparation as follows.

3.1. Optimization of enantioseparation by zone electrophoresis in the presence of R-DCV

Fig. 1a shows pH dependence of the separation of PMP–D/L-glucoses with *R*-DCV at its concentration of 50 m*M* (Fig. 1).

It can be seen that the resolution (ratio of migration time difference to average peak width, R_s) was almost unchanged in alkaline region. At pH values lower than 6 the solubility of *R*-DCV was too low to give 50 m*M* concentration. R_s increased with *R*-DCV concentration, as seen from Fig. 1b. This is plausible, because the intermolecular interaction between PMP derivatives of the glucose enantiomers and *R*-DCV will become stronger as the concentration of *R*-DCV, one of the counterparts, becomes higher.

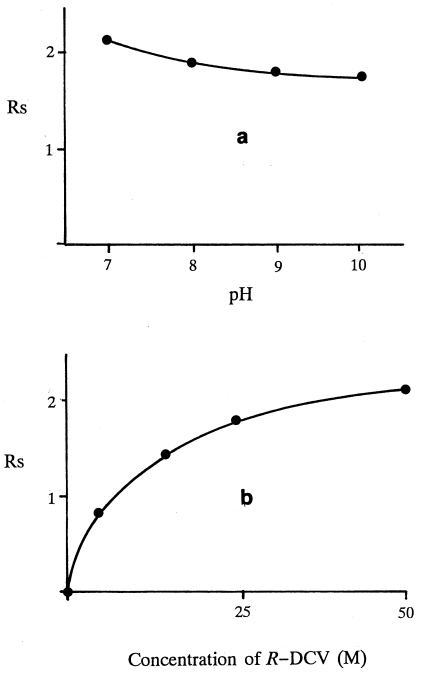

Fig. 1. pH dependence (a) and the effect of *R*-DCV concentration (b) on the resolution of PMP–D/L-glucoses. Capillary, fused-silica (54 cm×50 μm I.D.); running buffer, (a) 50 m*M* phosphate buffer containing *R*-DCV to a concentration of 50 m*M*; (b) 50 m*M* phosphate buffer (pH 7.0) containing *R*-DCV; applied voltage, 20 kV; detection, absorption at 254 nm: sample scale, $1.0 \cdot 10^{-4}$ *M*.

3.2. Separation of the PMP derivatives of aldose enantiomers by direct zone electrophoresis in the presence of DCVs

The PMP derivatives of aldose enantiomers were not separated at all in 50 m*M* phosphate buffer not containing *R*-DCV, giving a single peak, whereas baseline separation was observed under the optimized conditions (pH, 7.0; *R*-DCV concentration, 50 m*M*). Fig. 2 shows examples of such separation.

All of the PMP-derivatives of the pairs of D-/L-arabinoses, D-/L-xyloses, D-/L-galactoses and D-/L-glucoses were completely separated from each other. The values of separation factor (migration time ratio,

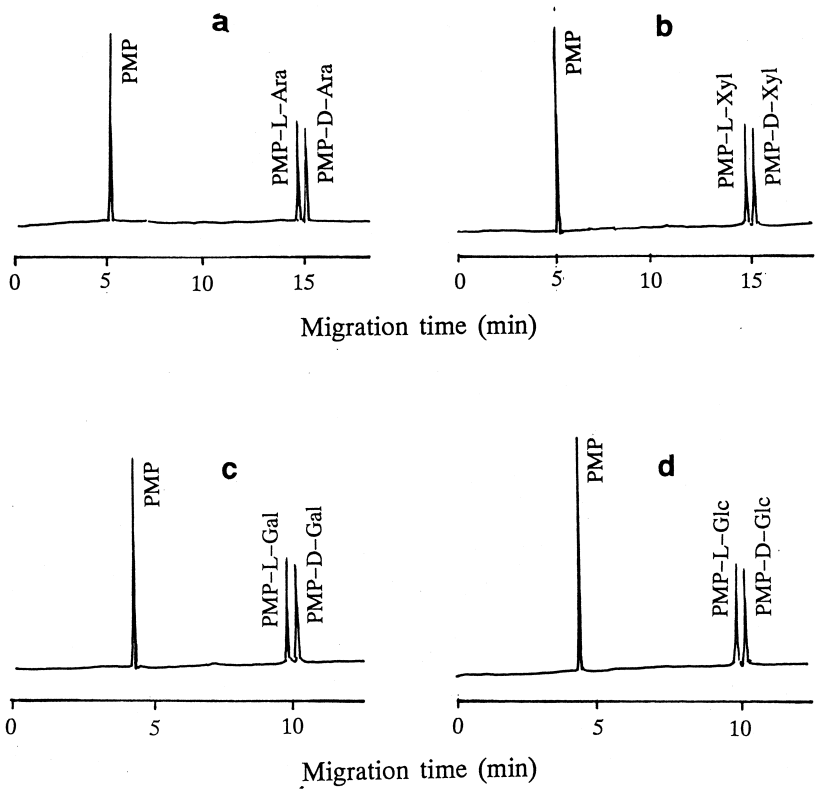

Fig. 2. Separation of the PMP derivatives of the enantiomers of arabinose (a), xylose (b), galactose (c) and glucose (d). Running buffer, 50 m*M* phosphate buffer (pH 7.0) containing *R*-DCV to a concentration of 50 m*M*. Other analytical conditions as in Fig. 1.

α) and resolution (R_s) for these pairs (in the above order) were as follows. α: 1.022, 1.020, 1.026, 1.036; R_s: 1.84, 1.81, 2.04, 2.11. In all these electropherograms migration was in the order of L-enantiomer, followed by D-enantiomer. Similar separation was observed with *S*-DCV (electropherograms not shown), but the migration order was reversed.

3.3. Speculation of the mechanism of enantioseparation

The enantioseparation achieved above with DCV, $C_{12}H_{23}$–O–CO–NH–CR–COOH [R: $CH(CH_3)_2$] poses an interesting problem from the viewpoint of separation mechanism. In order to speculate it, several basic experiments were done using other types of derivatives of these aldoses and other candidates for enantioseparation under various conditions. A tentative ^{1}H NMR study was also performed.

3.3.1. Separation of other types of derivatives

The ANTS derivatives did not give any sign of separation under the same conditions as those optimized for the PMP derivatives. The AP derivatives also showed no separation. In this case acidic conditions were used, because otherwise the derivatives were not ionized and did not migrate by electrophoresis. The glycamine derivatives labeled by 4-nitrobenz-2-oxa-1,3-diazole (NBD) [22] gave no separation as well under any conditions examined. All these experiments suggest that the presence of the PMP group is essential for the enantioseparation.

3.3.2. Invalidity of lauroylvaline (LV) and SDS

Fig. 3a shows separation of PMP–D-/L-glucoses

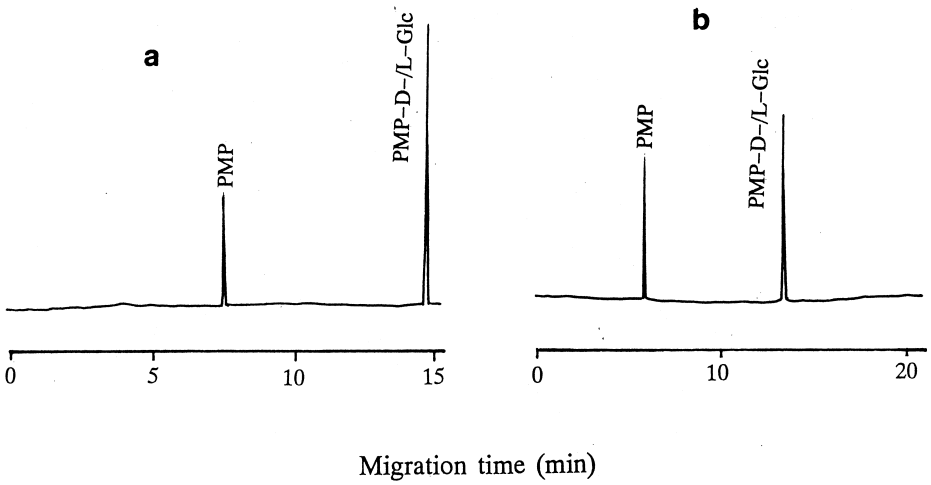

Fig. 3. Failure in separation of PMP–D-/L-glucoses in 50 m*M* phosphate buffer containing LV (a) and SDS (b) to a concentration of 50 m*M*. Other analytical conditions as in Fig. 1.

in the presence of N-lauroyl-L-valine, $C_{12}H_{23}$–CO–NH–CHR–COOH [R: $CH(CH_3)_2$] (LV).

This compound has the amide (–CO–NH–) group but lacks the oxygen atom which is present in the carbamate (–O–CO–NH–) group in DCV. It is important that LV gave a single peak in contrast to DCV. This means that the presence of the carbamate group is required for the enantioseparation; the amide group is insufficient to fulfill the structural requirement.

SDS also has a similar structure to DCV in that it has both the alkyl group and the sulfate group, which are hydrophobic and hydrophilic, respectively. However, this compound was not useful for enantioseparation as mentioned earlier, naturally because it has no reactive group to PMP–aldoses (Fig. 3b).

3.3.3. Tentative ^1H NMR study

Fig. 4b shows the ^1H NMR spectrum of an equimolar mixture of *R*-DCV and PMP-D-glucose in phosphate buffer (pH 7.0) prepared in deuterium oxide.

Under these conditions where the buffer composition was same as that of the running buffer, except that water was replaced by deuterium oxide, the signals of the sugar ring protons showed upfield shift as compared to those of PMP–D-glucose (Fig. 4a), suggesting participation of these protons in the interaction of *R*-DCV with PMP–D-glucose. The signals marked with an asterisk in Fig. 4b is those in *R*-DCV, and the large peak with a large blank asterisk is due to DO*H*. Though we have not yet succeeded in complete elucidation of the spectrum, this evidence apparently indicates the interaction between these two compounds. The most probable mechanism will be the formation of hydrogen bonds, such as >C=O···H–O– and >O···H–O–, together with >C=O···H–N< between the two molecules.

3.3.4. Proposed mechanism

DCV is considered to exist as micelles in which the hydrophobic portion forms the core structure and the hydrophilic portion, composed of the carbamate group and the amino acid moiety, exists outside the core.

On the basis of the results mentioned above the formation of intermolecular rings on the surface of the micelles can be postulated, as shown in Fig. 5.

Based on the structural requirement that the carbamate group is essential but the amide group alone does not cause enantioseparation, all of the –O–, –CO– and –NH– groups in DCV are considered to be involved in the ring formation with PMP–aldose. The scheme in Fig. 5 indicates two large-membered rings (rings A and B) between these compounds. Ring A (nine-membered) is formed through the following two hydrogen bonds; one between –O– (in DCV) and HO– (at C-3 in the

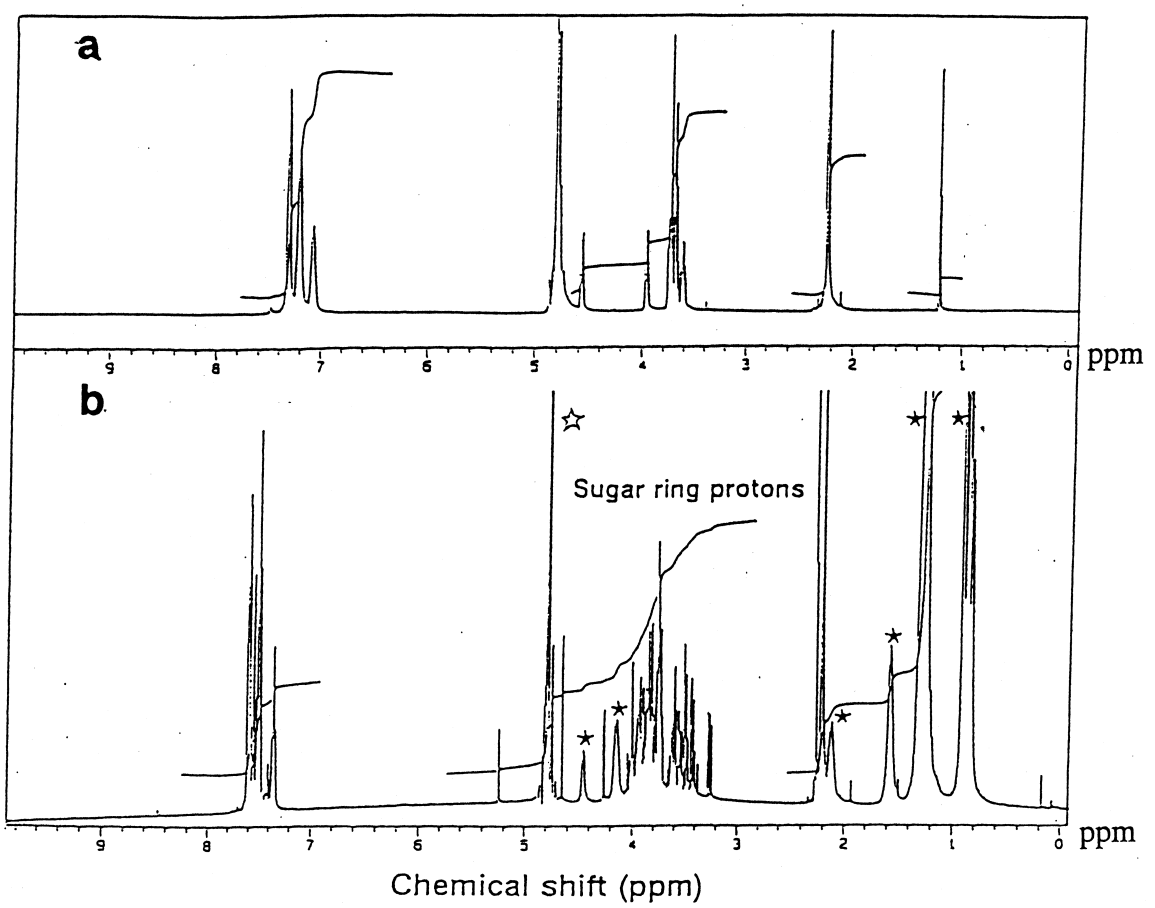

Fig. 4. 500 MHz ^{1}H NMR spectra of PMP–D-glucose (a) and an equimolar mixture of *R*-DCV and PMP–D-glucose (b) in deuterium oxide. The asterisked peaks in (b) are those of *R*-DCV and a large signal with a blank asterisk at ca. 4.9 ppm is that of HO*D*.

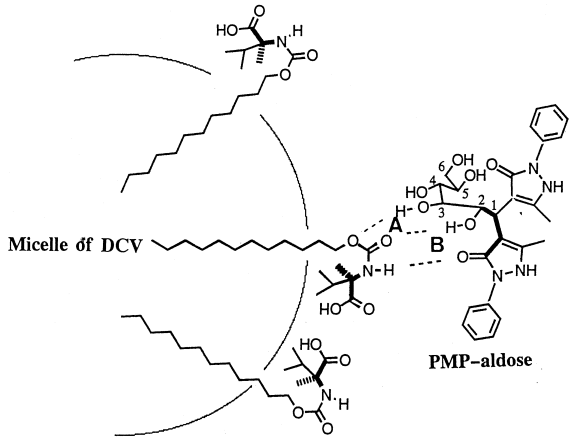

Fig. 5. Proposed mechanism of the interaction between DCV and a PMP–aldose.

aldose moiety of PMP–aldose) [hydrogen bond α] and the other between –CO– (in DCV) and HO– (at C-2 in the aldose moiety of PMP–aldose) [hydrogen bond β]. Ring B (eleven-membered) is formed through hydrogen bond β and the third hydrogen bond between –NH– (in DCV) and –CO– (in one of the pyrazolone rings of PMP–aldose) [hydrogen bond γ]. Both of the hydroxyl groups at C-2 and C-3 in all aldoses exemplified in Fig. 1 are *trans*-oriented, and this disposition will make the rings much more stable.

Under the optimized conditions electroosmotic flow is toward the cathode and the PMP derivatives of aldoses are drawn back to the anode by electrostatic force with the magnitude depending on their negativity. If there is difference in the stability of

ring A between the enantiomers, the PMP derivative of the enantiomer having more stable ring A will give weaker negativity and result in faster migration. When *R*-DCV was used, the faster moving enantiomers were of the L-series. When *S*-DCV was used, the velocity was reversed and the enantiomers of the D-series gave more stable ring A and migrated faster.

The thus-proposed three-point recognition mechanism will be plausible to explain the enantioseparation described in this paper, but further evidence, especially complete elucidation of the ^{1}H NMR spectrum, will be necessary for confirmation of this postulation.

References

[1] L.A. Colón, R. Dadoo, R.N. Zare, Anal. Chem. 65 (1993) 476.
[2] R.D. Rocklin, C.A. Pole, J. Liq. Chromatogr. 6 (1983) 1577.
[3] T.J. O'Shea, S.M. Lunte, W.R. LaCourse, Anal. Chem. 65 (1993) 948.
[4] A.E. Vorndran, P.J. Oefner, H. Scherz, G.K. Bonn, Chromatographia 33 (1992) 163.
[5] A. Klockow, A. Paulus, V. Figueredo, R. Amado, H.M. Widmers, J. Chromatogr. A 680 (1994) 187.
[6] S. Hoffstetter-Kuhn, A. Paulus, E. Grossman, H.M. Widmer, Anal. Chem. 63 (1991) 1541.
[7] S. Honda, S. Iwase, A. Makino, S. Fujiwara, Anal. Biochem. 176 (1989) 72.
[8] E. Grill, C. Huber, P. Oefner, A. Vorndran, G. Bonn, Electrophoresis 14 (1993) 1004.
[9] A.E. Vorndran, E. Grill, C. Huber, P.J. Oefner, G. Bonn, Chromatographia 34 (1993) 109.
[10] H. Schwaiger, P.J. Oefner, C. Huber, E. Grill, G. Bonn, Electrophoresis 15 (1992) 941.
[11] W. Nashabeh, Z. El Rassi, J. Chromatogr. 600 (1992) 279.
[12] K.B. Lee, A. Al-Hakim, D. Logananthan, R.J. Linhardt, Carbohydr. Res. 214 (1991) 155.
[13] C. Chiesa, C. Horváth, J. Chromatogr. 645 (1993) 337.
[14] A. Guttman, Fu-Tai, A. Chen, R.A. Evangelista, N. Cooke, Anal. Biochem. 233 (1996) 234.
[15] J. Liu, O. Shirota, D. Wiesler, M. Novotny, Proc. Nat. Acad. Sci. USA 88 (1991) 2302.
[16] S. Honda, E. Akao, S. Suzuki, M. Okuda, K. Kakehi, J. Nakamura, Anal. Biochem. 180 (1989) 351.
[17] S. Honda, S. Suzuki, A. Nose, K. Yamamoto, K. Kakehi, Carbohydr. Res. 215 (1991) 193.
[18] S. Honda, K. Yamamoto, S. Suzuki, M. Ueda, K. Kakehi, J. Chromatogr. 558 (1991) 327.
[19] C. Chiesa, P. Oefner, L.R. Zieske, A. O'Neil, J. Cap. Electrophoresis 2 (1995) 175.
[20] C.R. Noe, J. Freissmuth, J. Chromatogr. A 704 (1995) 503.
[21] M. Stefansson, M. Novotny, J. Am. Chem. Soc. 115 (1993) 11573.
[22] S. Honda and S. Kawakami, unpublished results.