Journal of Chromatography, 212 (1981) 313–322
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,834

NEW CHROMATOGRAPHIC SYSTEM FOR THE RAPID ANALYSIS AND PREPARATION OF COLOSTRUM SIALYLOLIGOSACCHARIDES*

RÜDIGER W. VEH
Institut für Anatomie, Ruhr-Universität, D-4630 Bochum (G.F.R.)
JEAN-CLAUDE MICHALSKI
Laboratoire de Chimie Biologique, Université des Sciences et Techniques de Lille I, F-59650 Villeneuve d'Ascq (France)
ANTHONY P. CORFIELD
Biochemisches Institut, Christian-Albrechts-Universität, D-2300 Kiel (G.F.R.)
MICHAEL SANDER-WEWER and DAGMAR GIES
Institut für Anatomie, Ruhr-Universität, D-4630 Bochum (G.F.R.)
and
ROLAND SCHAUER*
Biochemisches Institut, Christian-Albrechts-Universität, Olshausenstrasse 40–60, D-2300 Kiel (G.F.R.)
(Received March 30th, 1981)

SUMMARY

A new thin-layer chromatographic system on silica gel for the separation of sialyloligosaccharides is described. Calibration of the system with standard milk and colostrum sialyloligosaccharides is presented. The use of the system in monitoring different oligosaccharides is demonstrated for the purification of bovine colostrum sialyllactose isomers and a commercial sialyllactose product, and is discussed with respect to other biological fluids.

The large-scale preparation of pure sialyllactose isomers from bovine colostrum is achieved using an improved ion-exchange separation on Dowex 1-X2 (<400 mesh) employing isomolar elution at 20 m*M* for monosialyloligosaccharides and 200 m*M* for disialyllactose. The purification of four major monosialyltrisaccharides, the 2–3 and 2–6 isomers of N-acetylneuraminyllactose, N-glycolylneuraminyl2–3lactose and N-acetylneuraminyl2–6-N-acetyllactosamine, and the disialyltetrasaccharide di-N-acetylneuraminyllactose is reported. The detection and partial purification of three new minor monosialyloligosaccharides is described.

INTRODUCTION

Sialyloligosaccharides are present in large amounts in the milk and colostrum of mammals[1] and also in urine[2,3], where the excretion of particular oligosaccharides

* Glycolipids and oligosaccharides are abbreviated according to the recommendations of the IUPAC–IUB Lipid Nomenclature Document, *Lipids*, 12 (1977) 455–468.

0021-9673/81/0000–0000/$02.50

has been correlated with genetic disorders in man[4]. The isolation of the smaller oligosaccharides, in particular sialyllactose, from colostrum or milk has provided widely used substrates for the assay of sialidase activity[5].

The occurrence of the α2–3 and α2–6 derivatives of N-acetylneuraminyllactose (IINeu5AcLac) in colostrum has long been established[6], and has usually been prepared and detected using paper chromatography. However, the existence of other isomers, sialyl-N-acetyllactosamine (IINeu5AcGalβ1–4GlcNAc) and N-glycolylneuraminyllactose (IINeu5GlLac) present in bovine colostrum[7], is not usually considered although commercially available sialyllactose is obtained principally from this source.

We present a new thin-layer and column chromatographic system, where rapid identification and large-scale preparation of the different sialyllactose isomers and other sialyloligosaccharides can be achieved. Alternative systems have been reported in the literature by Maury[2] and more recently by Holmes and O'Brien[8] and Momoi and Wiegandt[9]. However, good resolution of sialyllactose isomers has not been reported previously. The preparation of sialyllactose isomers by ion-exchange chromatography has previously proved to be more difficult, and the results reported by Schneir and Rafelson[10] and Smith *et al.*[11] with Dowex and DEAE-cellulose ion exchangers give only partial separation of all isomers.

The new system will be of value in the screening for these oligosaccharides in biological fluids, especially in the preparation of highly purified oligosaccharides, and in analysis of sialyllactose cleavage in sialidase assays.

MATERIALS AND METHODS

Materials

Bovine colostrum was obtained from the Bundesanstalt für Milchforschung (Kiel, G.F.R.) and was collected within 1 h of milking and frozen at −15°C until use.

Sialyllactose was purchased from Boehringer (Mannheim, G.F.R.). Silica gel 60 and cellulose thin-layer plates (0.1 mm layer), solvents and chemicals of analytical grade were products of E. Merck (Darmstadt, G.F.R.). Dowex 1-X2, minus 400 mesh, was purchased from Bio-Rad (München, G.F.R.).

The following standard compounds were prepared from the sources indicated: II^{3}Neu5AcLac, II^{6}Neu5AcLac, II^3(Neu5Ac)$_2$Lac and II^{6}Neu5AcGalβ1–4GlcNAc from bovine colostrum; IV^{3}Neu5AcLcOse$_4$, IV^{6}Neu5AcLcnOse$_4$ and IV^{3}Neu5Ac-III^{6}Neu5AcLcOse$_4$ from human milk[12]; II^{3}Neu5GlLac (after ozonolysis of II^{3}Neu-5GlLacCer) from equine erythrocytes, by the method of Wiegandt and Bücking[13].

Analytical methods

Sialic acids were determined by the periodic acid–resorcinol method[14], by the periodic acid–thiobarbituric acid and orcinol–Fe^{3+} micromethods[15] and by gas–liquid chromatography (GLC)[15]. The methanolysis products of the oligosaccharides were analysed by GLC as described previously[16]. Methylation analysis was performed according to Hakomori[17].

Thin-layer chromatography (TLC) of sialic acids was performed on cellulose, developing with *n*-butanol–*n*-propanol–0.1 *M* HCl (1:2:1, v/v/v) and visualizing with the orcinol–Fe^{3+} spray reagent[15]. Oligosaccharides were separated on silica gel 60

thin-layer plates using ethanol–*n*-butanol–pyridine–water–acetic acid (100:10:10:30:3, v/v) and visualizing with orcinol–Fe^{3+} spray reagent or orcinol–H_2SO_4 (200 mg of orcinol in 100 ml of aqueous 20% H_2SO_4) sprays. To identify the nature of sialic acids in individual oligosaccharides, two-dimensional TLC was employed. The oligosaccharide sample was applied to the corner of a 20 × 20 cm cellulose thin-layer plate and run in the *n*-butanol–*n*-propanol–0.1 *M* HCl system described above. After drying, the plate was sprayed with 0.1 *M* HCl and incubated, with an overlying glass plate to prevent drying out, for 30 min at 80°C. The plate was then removed and dried, and the hydrolysis was repeated. On completion of the second hydrolysis the plate was developed in the same solvent and the components visualized with the orcinol–Fe^{3+} spray reagent. Standard Neu5Ac and Neu5Gl, as well as oligosaccharides, were run in the appropriate dimensions.

Paper chromatography was carried out on Whatman No. 3 paper in pyridine–ethyl acetate–acetic acid–water (5:5:1:3, v/v). Fowl plague virus (donated by Professor R. Rott, University of Giessen, Giessen, G.F.R.) was used as a source of sialidase; incubations were carried out as described elsewhere[5,19].

Preparative methods

Bovine colostrum (1 l) was mixed with chloroform–methanol (2:1, v/v; 5 l) and stirred vigorously for 20 min at 4°C. The suspension was centrifuged for 20 min at 4000 *g* and the upper light-yellow aqueous layer separated from the intermediate cake of precipitated protein and the lower deep-yellow organic phase. Aqueous phases were pooled (*ca.* 2 l for 1 l of colostrum) and concentrated by rotary evaporation under reduced pressure, and 300–400 ml were applied to a column (100 × 10 cm I.D.) of Sephadex G-25 fine. The column was eluted with distilled water using a peristaltic pump at a flow-rate of 3 ml/min, and the effluent was monitored for sialic acid content by the orcinol–Fe^{3+} assay and at 280 nm for glycopeptides. All procedures were conducted at 4°C. The fractions (10 ml) were combined to give three pools, as indicated in Fig. 1. This is a slight modification of the method of Ohman and Hyg-

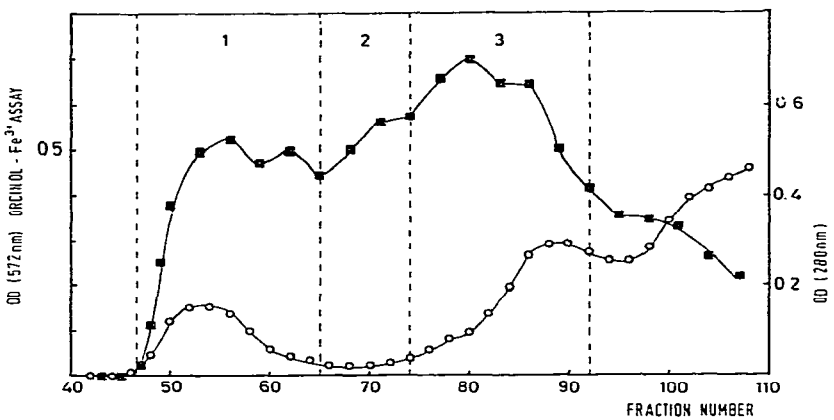

Fig. 1. Elution profile of 300 ml of concentrated aqueous phase after extraction of bovine colostrum with chloroform–methanol (2:1) on Sephadex G-25 fine eluted with water. The effluent was monitored for the absorbance at 280 nm (○) and with the orcinol–Fe^{3+} assay for sialic acids (■). Fractions were pooled as indicated to give pools 1–3.

stedt[18]. The pools were freed of lactose and neutral oligosaccharides by chromatography on Dowex 1-X8 acetate, 200–400 mesh. Sialyllactose isomers were eluted with 50 m*M* pyridinium acetate (pH 5.0), and disialyllactose with 200 m*M* pyridinium acetate (pH 5.0) to give a crude mixture of monosialyllactose isomers and disialyllactose.

Separation of sialyllactose isomers was carried out on a column (20 × 1 cm I.D.) of Dowex AG 1-X2 (minus 400 mesh, acetate form at 4°C). A sample (10 mg) of crude sialyllactose from Sephadex G-25 chromatography was dissolved in 10 ml of 2 m*M* pyridinium acetate buffer (pH 5.0) and applied to the column. The column was washed with 50 ml of the same buffer and eluted with a linear gradient (2 × 250 ml) from 10 m*M* to 50 m*M* pyridinium acetate (pH 5.0). The buffer concentration at the beginning of the sialyllactose elution was found to be 20 m*M*, and this concentration could be used for isomolar elution of the column with good separation of the different isomers. The separation of larger amounts (800 mg) of the crude mixture of sialyllactose isomers was carried out using the same technique. The sialyllactose was dissolved in 80 ml of 2 m*M* pyridinium acetate buffer (pH 5.0) and loaded on to a Dowex AG 1-X2 column (95 × 3 cm I.D.). Elution was carried out at a flow-rate of 50 ml/h using 20 m*M* buffer, and fractions of 16 ml were collected. After 8 l of buffer had been passed through the column the disialyllactose was eluted by raising the buffer concentration to 200 m*M*.

RESULTS AND DISCUSSION

The separation of sialyloligosaccharide standards by a new TLC system on silica gel is shown in Fig. 2a, which shows that a good resolution of the major sialyloligosaccharides occurring in the milk of mammals is achieved with this system. Separation of larger sialyloligosaccharides from glycoproteins[20] and gangliosides is also possible (data not shown). The new system is superior to other reported systems (*e.g.* refs. 2, 3, 8 and 9) in its resolution and application in the identification of sialyllactose isomers, and is more rapid and sensitive than paper chromatography. Thus a rapid analysis of sialyloligosaccharides is possible.

The TLC system has been used to monitor the sialyloligosaccharide components during their preparation from bovine colostrum (Fig. 2b). Fractionation of the aqueous phase of colostrum extracted with chloroform–methanol on Sephadex G-25 yielded three pools (Fig. 1). The first pool contained glycopeptides, glycolipids and small amounts of disialyllactose, and pools 2 and 3 contained the sialyllactose isomers. Disialyllactose was found chiefly in pool 2 and monosialyllactose isomers in pool 3, which was also contaminated with lactose, eluting later but still overlapping with sialyllactose owing to the high concentration in colostrum.

Removal of residual lactose by ion-exchange chromatography without resolution of individual components yielded between 0.7 and 1.2 g of sialyloligosaccharide mixture per litre of colostrum (fifteen experiments).

Resolution of the individual components from the combined pools 2 and 3 was carried out on an anion-exchange column (Fig. 3). After the concentration of buffer necessary to commence elution of sialyllactose isomers had been determined, an isomolar elution was found to give a good separation of these isomers; this approach had not previously been utilized for oligosaccharide separations. The chromatogra-

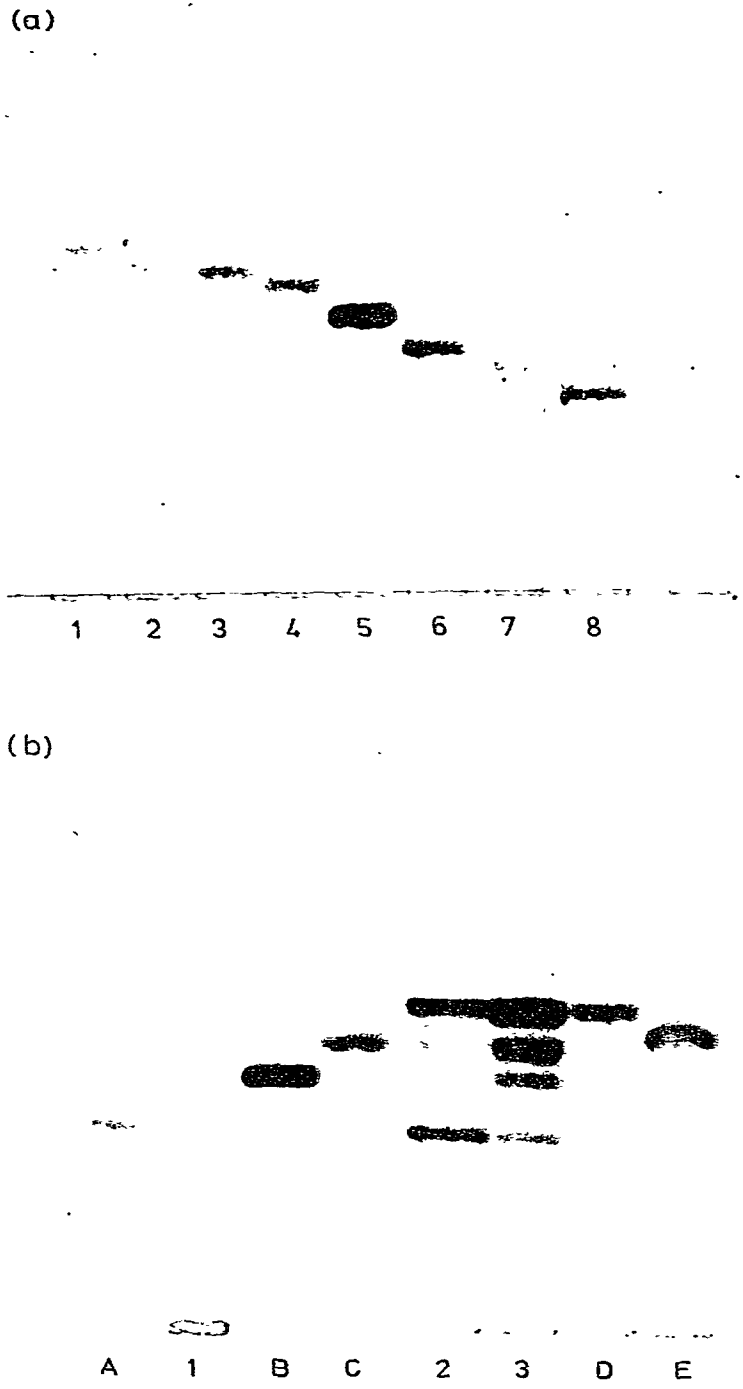

Fig. 2.

(Continued on p. 318)

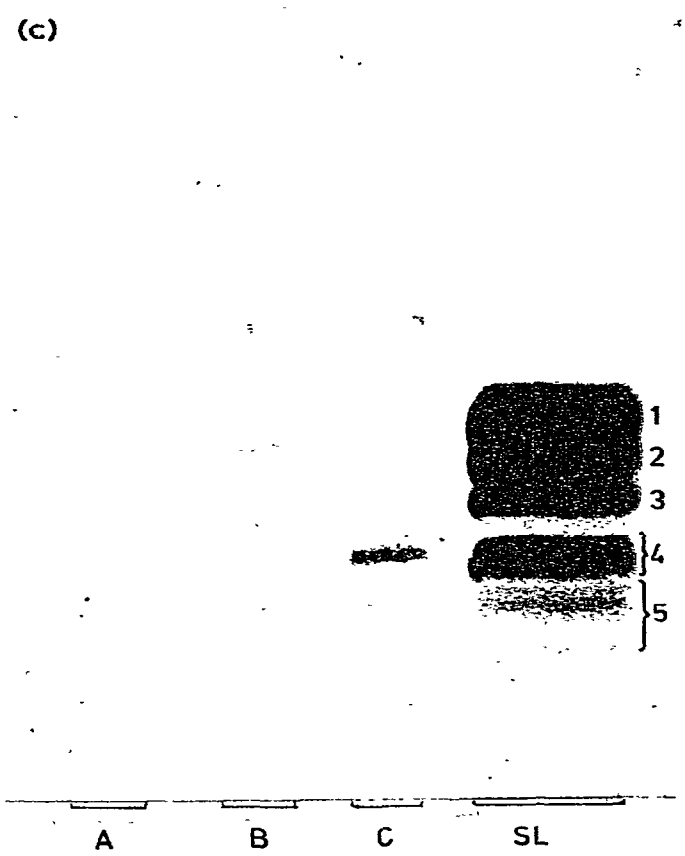

Fig. 2. (a) Chromatography of standard sialyloligosaccharides on silica gel thin-layer plates in ethanol–*n*-butanol–pyridine–water–acetic acid (100:10:10:30:3, v/v). 1 = II^{3}Neu5AcLac (1.0); 2 = IV^{3}Neu5AcLcOse$_4$ (0.94); 3 = II^{3}Neu5GlLac (0.93); 4 = II^{6}Neu5AcGalβ1–4GlcNAc (0.89); 5 = II^{6}Neu5AcLac (0.80); 6 = IV^{6}Neu5AcLcnOse$_4$ (0.70); 7 = II^3(Neu5Ac)$_2$Lac (0.65); 8 = IV^{3}Neu5AcIII^{6}Neu5AcLcOse$_4$ (0.57). The figures in brackets are the $R_{II^3Neu5AcLac}$ values for each component. (b) TLC as in (a) of the pools 1–3 from Sephadex G-25 chromatography of bovine colostrum sialyloligosaccharides. The pools 1–3 and standards II^3(Neu5Ac)$_2$Lac (A), II^{6}Neu5AcLac (B), II^{6}Neu5AcGalβ1–4GlcNAc (C), II^{3}Neu5AcLac (D) and II^{3}Neu5GlLac (E) were run on silica gel plates. (c) TLC as in (a) of a commercial sample of sialyllactose (SL) and the standards II^{3}Neu5AcLac (A), II^{3}Neu5GlLac (B) and II^3(Neu5Ac)$_2$Lac (C). The commercial sample shows bands corresponding to II^{3}Neu5AcLac and II^{3}Neu5GlLac (1), II^{6}Neu5AcGalβ1–4GlcNAc (2), II^{6}Neu5AcLac (3), II^3(Neu5Ac)$_2$Lac (4) and higher molecular weight oligosaccharides (5).

phy revealed four main trisaccharide components, identified as II^{3}Neu5AcLac, II^{6}Neu5AcLac, II^{6}Neu5AcGalβ1–4GlcNAc and II^{3}Neu5GlLac. These compounds were analysed by GLC and compared with reference oligosaccharides on TLC and paper chromatography (Table I). The structure of II^{6}Neu5AcGalβ1–4GlcNAc was also confirmed by methylation analysis. Cross-contamination between these fractions was less than 0.5%, as calculated from GLC and TLC analyses.

Disialyllactose was also separated by ion-exchange chromatography, and its structure was confirmed by GLC after methanolysis. The isolated product gave only one band on TLC (Fig. 2a and b), although several bands eluted with 200 m*M*

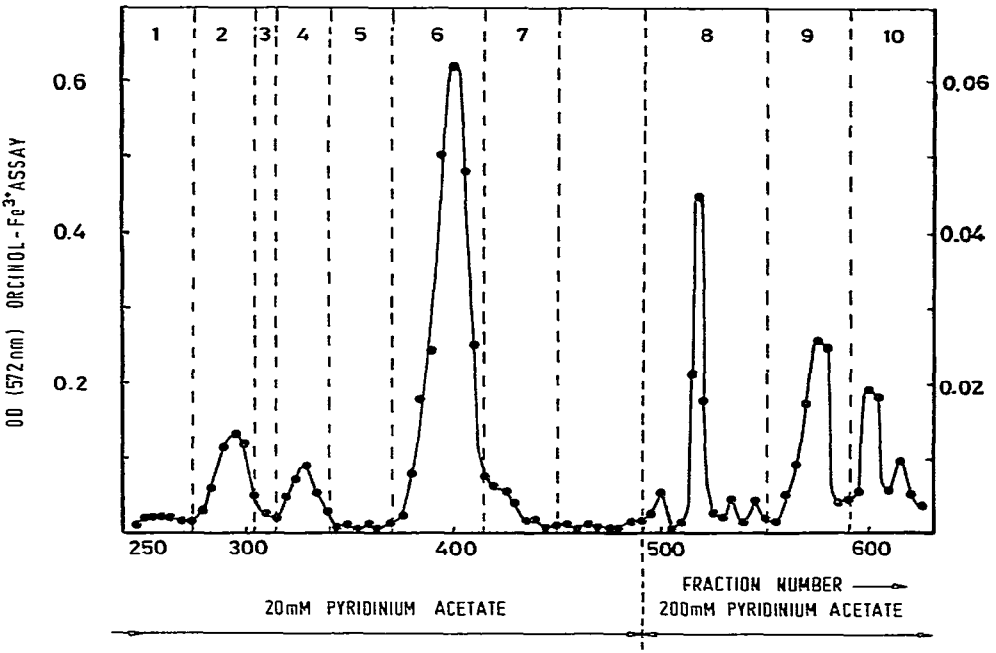

Fig. 3. The elution profile of crude sialyllactose from Sephadex G-25 chromatography (pools 2 and 3), on Dowex AG 1-X2 eluted with 20 m*M* and 200 m*M* pyridinium acetate (pH 5.0) as indicated. Samples were measured using the orcinol–Fe^{3+} assay. From fraction 490 onwards the scale is reduced by a factor of 10 as indicated on the right-hand axis. The fractions were pooled to give samples 1–10 as indicated; fractions 450–490 were discarded. Further details are given in the text.

pyridinium acetate (Fig. 3). The reason for this behaviour of disialyllactose is unclear at present. The structure of the disialyllactose was further analysed by incubation with fowl plague virus sialidase, and separation of the products on the TLC system. The formation of lactose was detected at different times of incubation and with different enzyme concentrations. No formation of II^{6}Neu5AcLac was detected. The virus sialidase cleaves 2–3 and 2–8 glycosidic linkages to Neu5Ac but 2–6 linkages are very slowly cleaved[5,19]. Thus incubation with the sialidase should result in an enrichment of II^{6}Neu5AcLac if this linkage exists in disialyllactose. The results showed only lactose and very small amounts of II^{3}Neu5AcLac, the other expected product, but no II^{6}Neu5AcLac. This result was further supported by 360 MHz nuclear magnetic resonance spectroscopy of the disialyllactose isolated, because only evidence for a 2–3 linkage was found, and so the presence of a 2–6 linkage could be ruled out[21].

In addition to these major oligosaccharide components, three minor oligosaccharides were detected and analysed (Table I) for the molar ratios of the component monosaccharides by GLC and for the type of sialic acid by a new method combining two-dimensional TLC with intermediate mild acid hydrolysis (Fig. 4). On the basis of these results and their elution positions on anion-exchange chromatography (Fig. 3), it is tentatively concluded that the components 3 and 5 in Table I are II^{6}Neu5GlGalβ-1–4GlcNAc and II^{6}Neu5GlLac, respectively. Component 1 contained 2 mol of galactose per mole of glucose and Neu5Ac. The structure of this component has not yet

TABLE I

ANALYSIS OF SIALYLOLIGOSACCHARIDES SEPARATED BY DOWEX CHROMATOGRAPHY

Crude sialyllactose (800 mg) was fractionated on Dowex 1-X2 as detailed in the text, and fractions 1–10 were collected as indicated in Fig. 3. Lactose was washed through the column and constituted the weight not recovered as sialyloligosaccharides 1–10.

Pool	*Fraction No.*	*Amount (mg)*	*Sialic acid*	*GLC (a) or TLC (b)*	*Monosaccharides molar ratio-GLC*				*Silica gel* $R_{II^3Neu5AcLac}$	*Comment*
					Glc	*Gal*	*GlcNAc*	*Neu5Ac*		
1	251–275	7	Neu5Ac	b	1	2	–	1	0.97	Unknown
2	276–305	70	Neu5Ac	b, a	–	1	1	1	0.89	Neu5Ac2–6N-acetyllactosamine
3	306–315	6	Neu5Gl	b	–	1	1	1	0.83	Neu5Gl2–6N-acetyllactosamine
4	316–340	37	Neu5Ac	b, a	1	1	–	1	0.79	Neu5Ac2–6lactose
5	341–370	6	Neu5Gl	b	1	1	–	1	0.80	Neu5Gl2–6lactose
6	371–415	332	Neu5Ac	b, a	1	1	–	1	1.00	Neu5Ac2–3lactose
7	416–450	19	Neu5Gl	b, a	1	1	–	1	0.93	Neu5Gl2–3lactose
8	490–550	–	Neu5Ac	b, a	–	–	–	1	0.66	free Neu5Ac
9	551–590	8	Neu5Ac	b, a	1	1	–	2	0.65	disialyllactose
10	591–630	7	Neu5Ac	b, a	1	1	–	2	0.65	disialyllactose

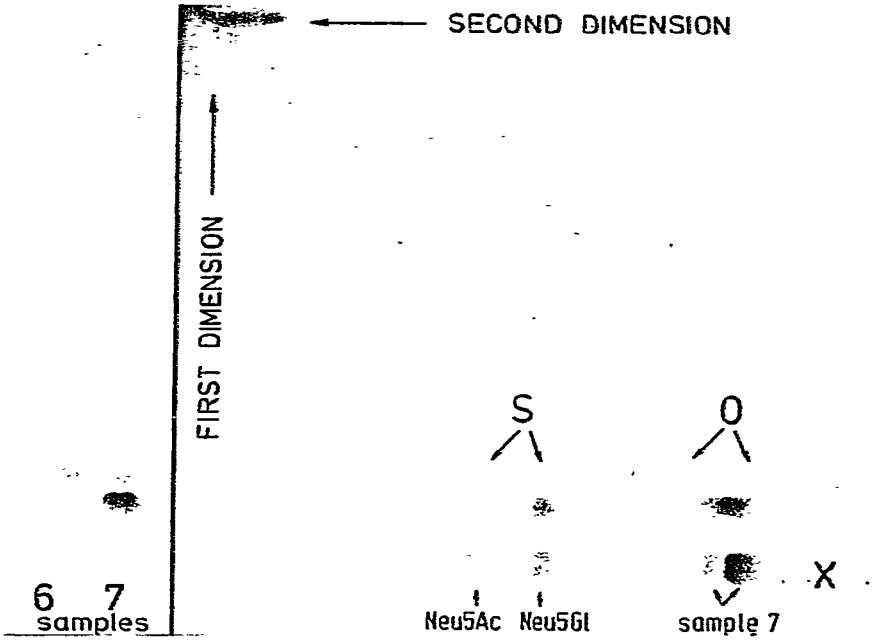

Fig. 4. Two-dimensional chromatography of sample 7 (see Table I) from ion-exchange chromatography (Fig. 3) on cellulose thin-layer plates. Development in both directions was with *n*-butanol–*n*-propanol–0.1 *M* HCl (1:2:1, v/v/v). After application of the sample at × and development of the plate in the first dimension, the plate was dried, sprayed with 0.1 *M* HCl and incubated at 80°C for 60 min as described in the text. After drying, a mixture of sample 7 with Neu5Ac and Neu5Gl standards was applied to position × and development in the second dimension carried out. In addition, samples 6 and 7 (see Table I) were run only in the first dimension for comparison (left-hand side of the chromatogram). The products of two-dimensional chromatography, visualized with the orcinol–Fe^{3+} spray reagent, are indicated as sialic acid (S) or oligosaccharide (O) products.

been determined. These minor components were difficult to analyse as they still contained oligosaccharides from neighbouring fractions (Figs. 3 and 4).

Using this method it is possible to process gram amounts of crude sialyllactose isomers and to prepare homogenous products in amounts suitable for routine analysis of sialidases, for example. The yields of the major sialyloligosaccharides in a typical experiment are noted in Table I for a crude mixture containing smaller amounts of disialyllactose. Typical yields for 1 l of colostrum were: II^3Neu5AcLac, 530 mg; II^6Neu5AcLac, 65 mg; II^3Neu5GlLac, 32 mg; II^6Neu5AcGalβ1–4GlcNAc, 110 mg; $II^3(Neu5Ac)_2$Lac, 285 mg.

Thus, in contrast to previous methods, the isomolar elution technique employed here combines good resolution of individual components with the scaling-up of the applied sample size and represents a significant improvement[10,11,18].

The availability of relatively large amounts of sialyllactose from colostrum and its suitability as a substrate for sialidases has led to its widespread commercial availability. Although the occurrence of the four major trisaccharides has been described[7], subsequent workers have reported only the 2–3 and 2–6 isomers of sialyllactose, or noted other minor and unidentified oligosaccharides[10,18]. Disialyllactose occurs as a

major component in bovine colostrum, as has been reported earlier[1,7,18], and is also present in the commercial sample of sialyllactose in amounts similar to those found in the present work (Fig. 2c). The commercial sample shows the presence of two bands in the region of disialyllactose and other slower migrating sialyloligosaccharides not found in the present work. A major band migrates as $II^6Neu5AcGal\beta1-4GlcNAc$, and $II^3Neu5GlLac$ is probably included in the large $II^3Neu5AcLac$ band (Fig. 2c). Use of these samples for sialidase or other assays must take into account the different behaviour of each isomer in such experiments[5,19].

Using the TLC system presented here, a rapid analysis is possible of sialyloligosaccharides from colostrum, milk, urine and other biological fluids, as well as commercial samples, and the ion-exchange method provides a new system for the large-scale preparation of pure sialyllactose isomers.

ACKNOWLEDGEMENTS

Jean-Claude Michalski was recipient of an EMBO fellowship during this work. The technical assistance of Marzog El Madani (Kiel) is gratefully acknowledged. The authors thank Dr. W. Kaufmann, Bundesanstalt für Milchforschung, Kiel, for donating bovine colostrum. This work was supported by the Deutsche Forschungsgemeinschaft (Grant Scha 202/9) and the Fonds der Chemischen Industrie.

REFERENCES

1 A. Kobata, in M. I. Horowitz and W. Pigman (Editors), *The Glycoconjugates*, Academic Press, New York, Vol. I, 1977, pp. 423–440.
2 P. Maury, *Biochim. Biophys. Acta*, 252 (1971) 48–57.
3 P. Maury, *Biochim. Biophys. Acta*, 252 (1971) 472–480.
4 G. Strecker and J. Montreuil, *Biochimie*, 61 (1979) 1199–1246.
5 A. P. Corfield, J.-C. Michalski and R. Schauer, in G. Tettamanti (Editor), *Proc. Int. Symp. on Sialidases and Sialidoses, Genova*, Edi Ermes Publ., Milan, 1981, in press.
6 R. Kuhn, *Naturwissenschaften*, 46 (1959) 43–50.
7 R. Kuhn and A. Gauhe, *Chem. Ber.*, 98 (1965) 395–413.
8 E. W. Holmes and J. S. O'Brien, *Anal. Biochem.*, 93 (1979) 167–170.
9 T. Momoi and H. Wiegandt, *Hoppe-Seyler's Z. Physiol. Chem.*, 361 (1980) 1201–1210.
10 M. L. Schneir and M. E. Rafelson, Jr., *Biochim. Biophys. Acta*, 130 (1966) 1–11.
11 D. F. Smith, D. A. Zopf and V. Ginsburg, *Anal. Biochem.*, 83 (1976) 602–608.
12 L. Grimmonprez, S. Bouquelet, B. Bayard, G. Spik, M. Monsigny and J. Montreuil, *Eur. J. Biochem.*, 13 (1970) 484–492.
13 H. Wiegandt and H. W. Bücking, *Eur. J. Biochem.*, 15 (1970) 287–292.
14 G. W. Jourdian, L. Dean and S. Roseman, *J. Biol. Chem.*, 246 (1971) 430–435.
15 R. Schauer, *Methods Enzymol.*, 50C (1978) 64–89.
16 J. P. Kamerling, G. J. Gerwig, J. F. G. Vliegenthart and J. R. Clamp, *Biochem. J.*, 151 (1975) 491–495.
17 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
18 R. Ohman and O. Hygstedt, *Anal. Biochem.*, 23 (1968) 391–402.
19 R. Drzeniek, *Curr. Top. Microbiol. Immunol.*, 59 (1972) 35–74.
20 B. Bayard, E. P. Kerckaert, D. Roux and G. Strecker, *Protides Biol. Fluids, Proc. Colloq.*, 27 (1979) 153–156.
21 J. F. G. Vliegenthart, Bio-Organic Chemistry Department, University of Utrecht, Utrecht, The Netherlands, personal communication.