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High-pH anion-exchange chromatography with pulsed amperometric detection and molar response factors of human milk oligosaccharides

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Abstract

A method is described to separate and characterize neutral and acidic lactose-derived oligosaccharides without prior derivatization or reduction by high-pH anion-exchange chromatography and pulsed amperometric detection (HPAEC–PAD). This method has been applied to human milk oligosaccharides from donors with different blood group specificity (A, Le^a and A, Le^b). Neutral and acidic components were separated from each other by anion-exchange chromatography. A distinct separation of individual components was obtained by size-exclusion chromatography on Fractogel TSK HW 50S (acidic oligosaccharides) or Fractogel TSK HW 40S (neutral oligosaccharides containing up to 6 monomers) and Bio-Gel P-4 size exclusion (neutral oligosaccharides containing more than 6 monomers). Furthermore, the molar response factors after HPAEC–PAD have been determined for 28 components.

Keywords: Oligosaccharides; Human milk

1. Introduction

The majority of secreted proteins, of cellular membrane proteins and a large part of all surface lipids are glycoconjugates whose oligosaccharides can be released for functional or structural studies by chemical or enzymatic methods (for review, see Ref. [1]). Biological fluids such as human milk or urine from pregnant and lactating women contain neutral and acidic oligosaccharides derived from lactose [2–7]. Recently, we have shown that such oligosaccharides are present in urine of pre-term infants only if they are fed human milk whereas in formula-fed infants only a few oligosaccharide components are detectable [8].

Characterization of these oligosaccharides is important for further investigation of their biological role. Several oligosaccharide structures are known to be involved, e.g., in the interaction between leucocytes and endothelial cells, in the uptake of circulating glycoproteins by specific receptors in the liver or in cell–cell interactions [9–11]. A comprehensive review on biological functions of oligosaccharides has recently been published [12].

High-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) is a novel and sensitive method to characterize mono- and oligosaccharides without prior derivatization

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[13,14]. HPAEC–PAD has been an effective method for separating N-linked hybrid, oligomannosidic and lactosamine-type oligosaccharides from glycoproteins [15]. However, only few publications report on the identification of lactose-derived components [16].

We have applied this method to identify and characterize neutral and sialylated lactose-derived oligosaccharides in milk from two women with known blood group specificity (A, Le^b and A, Le^a status).

The purpose of our study was the separation of neutral lactose-derived oligosaccharides from sialic acid-containing components by anion-exchange chromatography followed by size exclusion on Fractogel TSK HW 40 S or Bio-Gel P-4 (neutral components) and Fractogel TSK HW 50 S (acidic components). The procedure described allows the characterization of individual components and of positional and structural isomers of fucosylated and sialylated oligosaccharides by HPAEC–PAD and fast atom bombardment-mass spectrometry (FAB-MS). Finally, the investigation of molar response factors after HPAEC–PAD of neutral and acidic oligosaccharides is a prerequisite for other studies to quantify oligosaccharides in biological fluids.

2. Experimental

2.1. Material

Human milk (1 l each) from two women with known blood group and secretor activity (A, Le^b and A, Le^a) was frozen (−20°C) until analysis. Oligosaccharides which had been isolated from human milk and characterized in earlier studies [5,17] were used as standards. Other standards were obtained from Bio Carb (Lund, Sweden) or Oxford Glycosystems (Cambridge, U.K.).

Bio-liquid chromatography was performed on a Dionex System (Sunnyvale, CA, USA) consisting of a Carbo Pac PA-1 column (250×4.6 mm I.D.) equipped with a guard column and a Model PAD 2 detector.

NaOH solution (50% (w/w); low in carbonate) was purchased from J.T. Baker (Philadelphia, PA, USA). Sodium acetate of analytical grade was from Merck (Darmstadt, Germany). Sephadex G-25 was obtained from Pharmacia (Uppsala, Sweden), Bio-Gel P-4 from Bio-Rad (Munich, Germany) and Fractogel TSK HW 40 S and 50 S from TosoHaas (Montgomeryville, PA, USA). Thin-layer plates (Silica-gel 60, 10×10 cm) were purchased from Merck. All other reagents were of analytical grade.

2.2. Methods

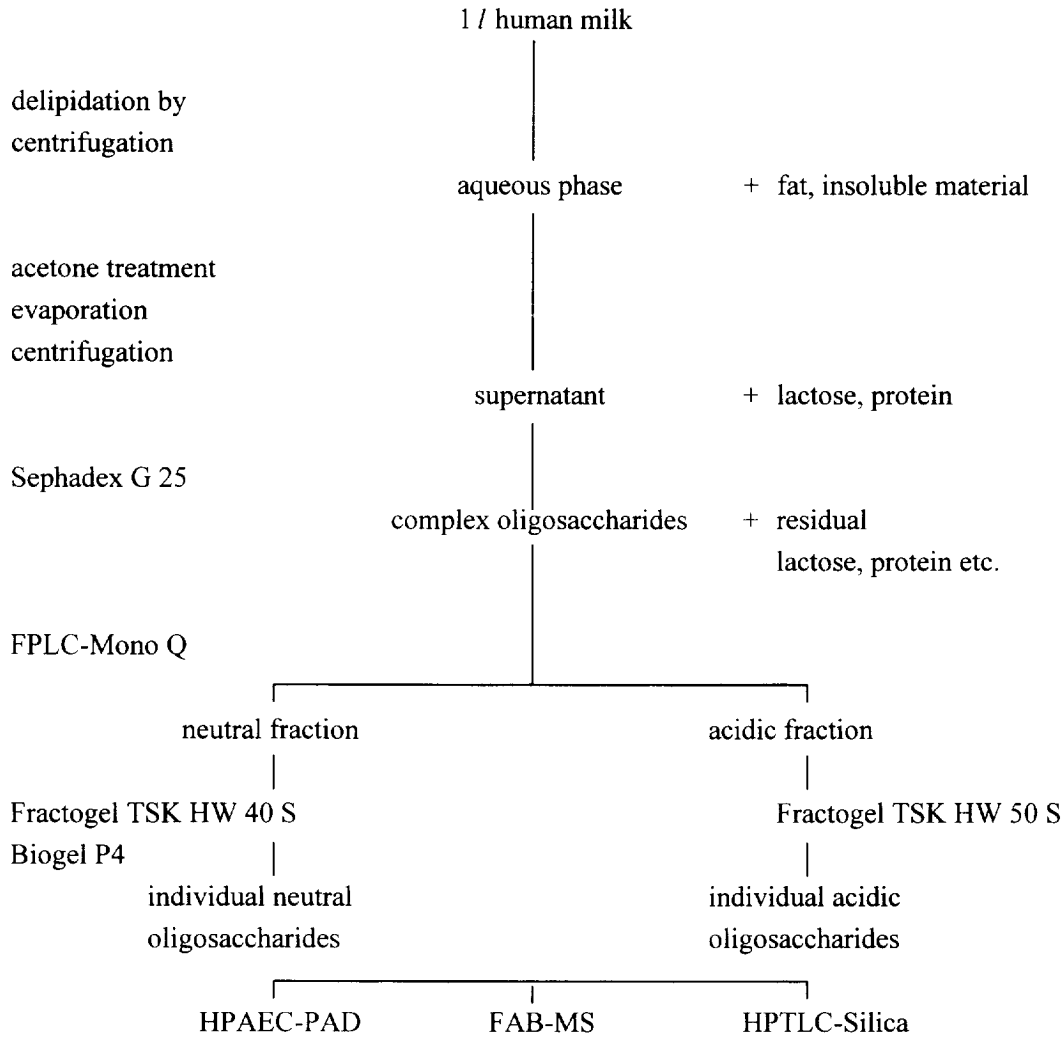
2.2.1. Isolation

The first steps for the preparation of human milk for oligosaccharide analysis were accomplished as described elsewhere [5] (Scheme 1). Briefly, milk was centrifuged at 3000 *g* for 1 h at 4°C, the lipid layer removed and the aqueous phase decanted and filtered through glass wool. Proteins were precipitated by adding an equal volume of precooled acetone. Lactose in the supernatant was removed by repeated crystallization. This preparation was applied to a Sephadex G-25 column (90×2.5 cm I.D.) and eluted with water. Carbohydrate-containing fractions were identified by the anthrone and Ehrlich methods. Fractions with residual peptides (ninhydrin-positive fractions) were excluded from the analysis. Then, salt-free but carbohydrate-positive fractions were pooled and lyophilized.

2.2.2. Anion-exchange chromatography

Neutral oligosaccharides were separated from sialic acid-containing components by anion-exchange chromatography (FPLC-Mono Q HR 5/5, 50×5 mm, particle size 10 μm, bed volume 1 ml; Pharmacia Biotech, Uppsala, Sweden). The conditions were: eluent A (H_2O) from 0–5 min followed by a gradient of up to 100% eluent B (0.6 *M* NaCl) in 37 min. The injection volume was 1 ml from 5 mg/ml or 1 mg/ml solutions, the size of the collected fractions being 1 ml. Eluting fractions were monitored at 214 nm for oligosaccharides and at 280 nm for residual proteins using a variable-wavelength UV-monitor (Pharmacia Biotech). Carbohydrate-containing fractions were desalted on a Bio-Gel P-2 column (Pharmacia Biotech).

For screening purposes carbohydrate-positive fractions were subjected to HPTLC and FAB-MS as described below.



Scheme 1. Isolation and characterization of lactose-derived oligosaccharides from human milk.

2.2.3. Size-exclusion chromatography

In order to identify neutral oligosaccharides with 2 to 6 sugar monomers, the unretarded fractions after anion-exchange chromatography were subjected to chromatography on Fractogel TSK HW 4O S (Merck) (column 160×1.5 cm I.D.) with water as the eluent according to Ref. [5]. Neutral oligosaccharides with >6 sugar monomers were separated by Bio-Gel P-4 gel permeation as described in Ref. [18]. Acidic oligosaccharides were chromatographed on Fractogel TSK HW 50 S (Merck) (column 160×1.5 cm I.D.) using 0.1% acetic acid (adjusted to pH 5.5 with ammonia) as the eluent. Fractions were monitored continuously at 214 and 280 nm or by refractive index.

2.2.4. High-performance thin-layer chromatography (HPTLC)

Fractions after anion-exchange, Fractogel TSK or Bio-Gel P-4 chromatography were analyzed by silica-gel HPTLC in butanol–acetic acid–H_2O (2.5:1:1, v/v) (development: twice) or butanol–ethanol–H_2O–acetic acid–pyridine (5:50:15:1.5:5, v/v). The plates were sprayed with orcinol for carbohy-

drate detection (0.1% orcinol in 20% H_2SO_4) or ninhydrin (0.3 g ninhydrin in 95 ml 2-propanol containing 5 ml 96% acetic acid).

2.2.5. High-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD)

The system used for HPAEC–PAD of neutral and sialylated oligosaccharides consisted of a Dionex Bio-LC gradient pump, Carbo Pac PA-1 column (250×4.6 mm I.D.) equipped with a guard column and a Model PAD 2 detector (Dionex, Sunnyvale, CA, USA). The response time of the PAD 2 was set to 3 s. On the gold electrode the applied potential was: E1 = +0.05 V, E2 = +0.60 V and E3 = −0.60 V; t_1 = 480 mS, t_2 = 120 mS and t_3 = 60 mS.

The Dionex eluent degas module was employed to degas and pressurize the eluents with helium. Sample injection was via a Dionex microinjection valve equipped with a 25-μl sample loop operated by a controlled helium source of 0.7 MPa. The resulting chromatographic data was integrated and plotted using a Spectra-Physics Model SP 4270 integrator.

Neutral and acidic oligosaccharides were analyzed by HPAEC–PAD using the following conditions: eluent A, 100 m*M* NaOH; eluent B, 100 m*M* NaOH and 250 m*M* Na acetate.

The elution program began with 3 ml of buffer A, followed by a gradient of up to 100% buffer B in 30 min. A re-equilibration volume of 5 ml buffer A was chosen. A flow-rate of 1.0 ml/min was used and 25 μl from 1 mg/250 μl or 1 mg/1000 μl solutions were injected.

Molar response factors were determined by injecting 3 to 6 times equimolar amounts of each oligosaccharide with a purity of >95% (standards from Oxford Glycosystems). As lacto-N-tetraose is the precursor of most complex oligosaccharides the response of this component was arbitrarily set to 1.

2.2.6. Fast atom bombardment-mass spectrometry (FAB-MS)

FAB-MS of native or peracetylated oligosaccharides was carried out using a VG analytical ZAB HF mass spectrometer (VG Instruments) as described in Ref. [19]. Oligosaccharides in Tables 1 and 2 have also been investigated by FAB-MS.

3. Results and discussion

3.1. Separation of neutral and acidic components

Due to the large variety of oligosaccharides in human milk [4,5] one of the critical steps in analyzing lactose-derived oligosaccharides in biological fluids is the separation of neutral from acidic components. This was achieved by anion-exchange chromatography.

Fig. 1 shows a FPLC-Mono Q separation of a carbohydrate-positive but ninhydrin-negative (peptide-free) fraction which was obtained by Sephadex G-25 chromatography. The unretarded fraction (I) was composed of neutral oligosaccharides and small amounts of acidic components as revealed by HPAEC–PAD analysis and which will be discussed later (see Figs. 3–5). The complex mixture of acidic oligosaccharides which were found in Mono Q

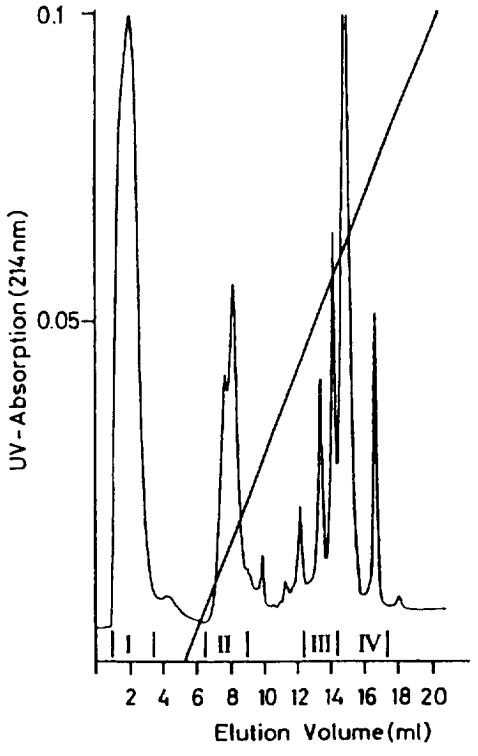


Fig. 1. FPLC-Mono Q chromatogram of carbohydrate-containing fractions after Sephadex G-25 size exclusion. Fraction I: neutral oligosaccharides (see Fig. 3a or b); fraction II–IV: acidic oligosaccharides (see Fig. 2a–c).

fractions designated II–IV after anion-exchange chromatography was demonstrated by HPAEC–PAD-analysis (Fig. 2a–c). Fraction II (Fig. 2a) was mainly composed of NeuAc-Fuc-Lac, α2-3-NeuAcLac, α2-6-NeuAcLac and NeuAc-lacto-N-tetraose a, b and c (LSTa,b,c); fraction III (Fig. 2b) of NeuAc-Fuc-LNT, $NeuAc_2$-Fuc-LNH, $NeuAc_2$-LNT, $NeuAc_2$-LNhexaoses and more complex oligosaccharides; and fraction IV (Fig. 2c) of $NeuAc_2$-LNT and multiple sialylated lacto-N-hexaoses, -octaoses etc.

For screening purposes, e.g. for investigations on the Lewis blood group status of the samples, a clear distinction between Le^a- and Le^b-milk can already be made at this stage by analyzing the neutral oligosaccharides (FPLC-Mono Q fraction I) (Fig. 3a and b). It is known that lacto-N-fucopentaose II (LNFP II) is a typical and major milk component in Le^a individuals (Peak 4 in Fig. 3a), whereas lacto-N-difucohexaose I is characteristic for Le^b-milk (peak 2 in Fig. 3b). Moreover, in all samples analyzed so far, the ratio of LNT to its monofucosylated derivatives seems to be dependent on the Lewis specificity: in Le^a-milk the concentration of LNT is always higher compared to that of lacto-N-fucopentaose II (compare peaks 4 and 7 in Fig. 3a) whereas in Le^b-milk, lacto-N-fucopentaose I is always the major component (compare peaks 7 and 8 in Fig. 3b). An explanation might be that in Le^a-individuals, who do not express GDP-Fuc-α1-2-galactosyltransferase in the mammary gland, lacto-N-fucopentaose II is the end product, whereas Le^b-individuals express the α1-2-fucosyltransferase, which is capable of using lacto-N-fucopentaose II as an acceptor to produce lacto-N-difuco-hexaose I, a characteristic sugar with Le^b-activity (peak 2 in Fig. 3b)

As the number of positional and structural isomers increases with increasing oligosaccharide chain length and additional fucosylation and sialylation, a further chromatographic step is required to separate individual neutral and acidic components. Therefore, we subjected fraction I (neutral oligosaccharides) after FPLC-Mono Q to chromatography on Fractogel TSK HW 40 S and collected 7 fractions designated TSK I-VII (Fig. 4).

TSK I contained residual acidic oligosaccharides (data not shown), TSK II–VII neutral components which were identified by HPAEC–PAD and FAB-MS as fucosylated tetraoses, hexaoses and octaoses.

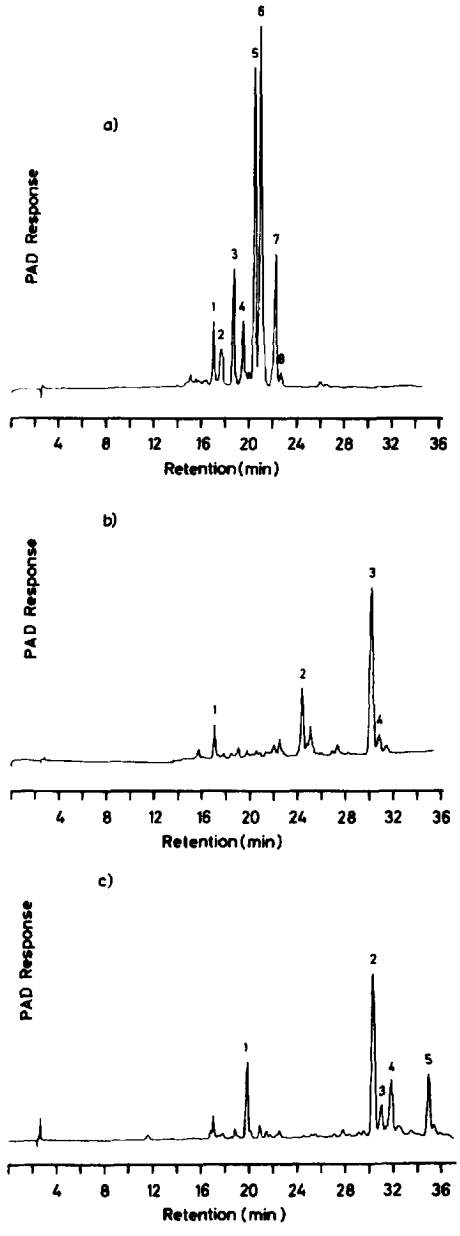


Fig. 2. HPAEC–PAD-chromatogram of fractions II (a), III (b) and IV (c) after anion-exchange chromatography (FPLC-Mono Q). (a) 1+2=isomers of NeuAc-Fuc-LNT; 3=NeuAc-Fuc-Lac; 4=NeuAc-LNT (LST c) 5=NeuAc α2-6 Lac; 6=NeuAc α2-3 Lac; 7=NeuAc-LNT (LST a); 8=NeuAc-LNT (LST b); (b) 1 NeuAc-Fuc-LNT; 2=$NeuAc_2$-Fuc-LNH; 3=$NeuAc_2$-LNT; 4=$NeuAc_2$-LNhexaoses and more complex oligosaccharides; (c) 1=isomer of NeuAc-LNT; 2=$NeuAc_2$-LNT; 3+4+5=multiple sialylated lacto-N-hexaoses, -octaoses etc. (for abbreviations and structures: see Table 2).

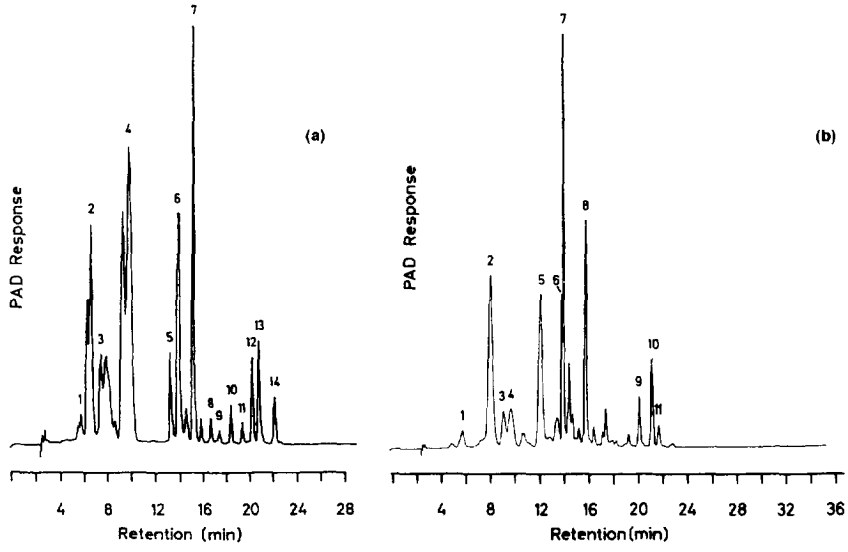


Fig. 3. (a) HPAEC–PAD-chromatogram of fraction I after anion-exchange chromatography (FPLC-Mono Q) (neutral oligosaccharides from Le^a-milk). 1=di- and trifucosylated hexaoses and more complex structures; 2=Fuc_3-LNT; 3=Fuc_2-LNhexaose; 4=Fuc-LNT (LNFP II=Le^a); 5=Fuc-LNoctaose; 6=Fuc-LNhexaose; 7=LNT; 8=NeuAc-Fuc-LNT; 9=structural isomer to 8? 10=NeuAc-Fuc-Lac; 11=NeuAc-LNT (LST c); 12=NeuAc α2-6 Lac; 13 NeuAc α2-3 Lac; 14 NeuAc-LNT (LST a).
(b) HPAEC–PAD-chromatogram of fraction I after anion-exchange chromatography (FPLC-Mono Q) (neutral oligosaccharides from Le^b-milk). 1=difucosylated hexaoses, octaoses etc.; 2=Fuc_3-LNT; 3+4=unknown; 5=Lac; 6=2′-Fuc-Lac; 7=Fuc-LNT (LNFP I); 8=LNT; 9=NeuAc-LNT (LST c); 10=NeuAc α2-6 Lac; 11=NeuAc α2-3 Lac (separating conditions: see Fig. 2 and Section 2.2; for abbreviations and structures: see Table 2).

The composition of fraction II–VII, the retention time of individual components after HPAEC–PAD and FAB-MS data of the peracetylated components are shown in Table 1.

An even better separation of higher oligosaccharides in TSK HW 40 S fractions II and III was obtained by Bio-Gel P-4 size exclusion. Fig. 5 shows examples of the distinct separation of mono- to trifucosylated lacto-N-octaoses (Fig. 5a), fucosylated decaoses (Fig. 5b) and dodecaoses (Fig. 5c), respectively. Besides HPAEC–PAD-analysis the identity of the components has been verified by FAB-MS.

3.2. Characterization of oligosaccharides by HPAEC–PAD

As can be seen in Fig. 2 and Fig. 3 the HPAE–PAD method is a useful tool for screening fractions which still contain a mixture of different components. Under the conditions used in this study the following retention times have been obtained: (a) for non-fucosylated as well as mono-, di- and trifucosylated oligosaccharides (lacto-N-tetraoses to dodecaoses), <16 min; (b) for fucosylated as well as monosialylated oligosaccharides, 16–19.5 min; and (c) for sialyl-lactose, sialylated lacto-N-tetraoses a, b, c (LST a, b and c), $NeuAc_2$lacto-N-tetraoses and fucosylated as well as sialylated complex oligosaccharides, >19.5–35 min.

Neutral and acidic oligosaccharides in HM with blood group A, Le^a- or Le^b-activity that have been isolated and characterized by HPAEC–PAD and FAB-MS in this study are listed in Table 2.

Under the HPAEC–PAD conditions used, several positional isomers like lacto-N-fucopentaose I and II or sialyl-lacto-N-tetraose a and b, and structural isomers such as lacto-N-tetraose and lacto-N-neotetraose or lacto-N-fucopentaose II and III can easily be separated in a single step by HPAEC–PAD (see retention times in Table 3).

Both fucosylation and sialylation have a strong influence on the retention of oligosaccharides. In general, the attachment of fucose leads to decreased retention times, whereas N-acetylneuraminic acid prolongs the retention. Other factors influencing the interaction of oligosaccharides with the column

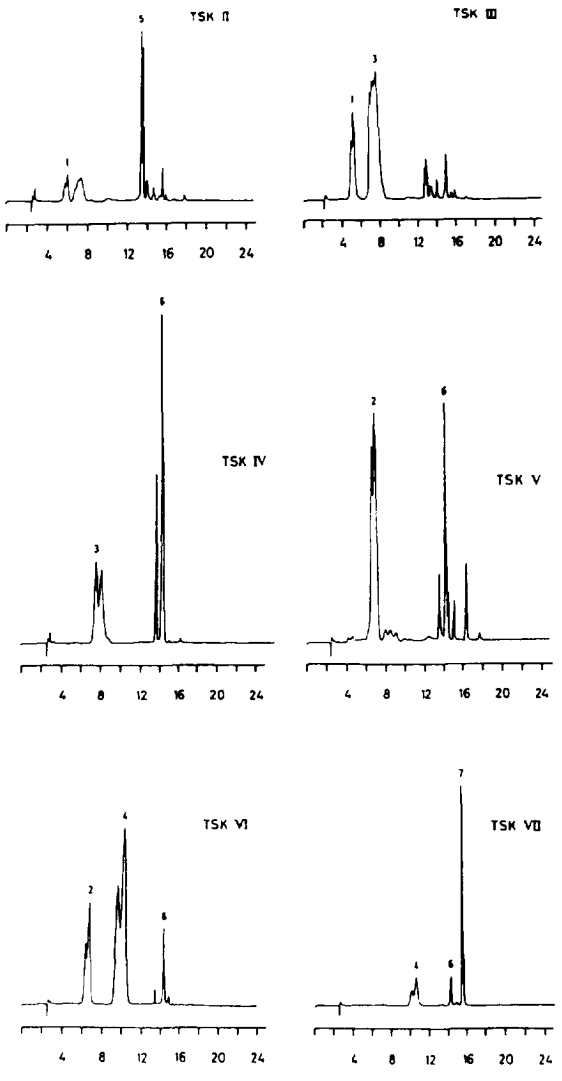


Fig. 4. HPAEC–PAD chromatogram of neutral oligosaccharides after Fractogel TSK HW 40S size exclusion. Peak numbers are the same as in Fig. 3a. (separating conditions: see Fig. 2 and Section 2.2; for abbreviations and structures: see Table 2).

matrix have been discussed previously in detail [14,15,20–22].

3.3. Investigation of molar response factors of oligosaccharides after HPAEC–PAD-analysis

We further focused on molar response factors of lactose-derived oligosaccharides after pulsed amperometric detection. These factors are a prerequisite for studies which focus on quantitative aspects of lactose-derived oligosaccharides in biological fluids. As lacto-N-tetraose is the precursor of most complex oligosaccharides the response of this component was arbitrarily set to 1. The relative response factors determined for 28 free oligosaccharides and for 5 blood group active components are shown in Table 3.

The position of attachment of fucose has a strong influence on both the detector response and the retention behaviour. This is exemplified by 3-fucosyl-lactose and 2′-fucosyl-lactose whose detector responses were 2.18 and 0.84, respectively (Table 3). The responses of the four monofucosylated derivatives of lacto-N-tetraose, lacto-N-fucopentaose I, II, III and V are quite different, ranging from 0.86 to 1.84 (Table 3). Besides fucose, the linkage of N-acetylneuraminic acid strongly influences the detector signal as well. This is demonstrated for both sialyl-lactoses (NeuAcα2-3Lac and NeuAcα2-6Lac) and for NeuAc-lacto-N-tetraose (LST a, b and c) and for other sialylated components (Table 3).

As there is an increasing interest in looking at the metabolism of oligosaccharides, e.g, in certain diseases, in pregnant and lactating women or in newborn infants fed breast milk or an infant formula [23], suitable methods are needed to be able to perform such studies.

The advantage of the conditions we used for HPAEC–PAD compared to methods described by other authors is the separation of neutral and acidic components in a single run using a gradient system. Thus, short running times (less than 40 min) can be achieved even for fractions which still contain a mixture of complex oligosaccharides. Although 1 l milk has been used in the present study, only 5–10 ml are necessary for an analytical investigation of the oligosaccharide composition of biological fluids such as milk or urine.

In conclusion, the separation of neutral from acidic oligosaccharides which is best achieved by anion-exchange chromatography is necessary for investigations of the HM oligosaccharide pattern. The greatest advantage has been the use of high pH anion-exchange chromatography with pulsed amperometric detection. This system allows the separation of isomeric components and the detection of even small differences in the monosaccharide com-

Table 1
Retention on HPAEC–PAD and molecular ions ($M^+ + Na^+$) of components in TSK-Fractions II–VII

	Retention time (min)	FAB-MS of peracetylated components $M^+ + Na^+$ (*m/z*)
TSK-Fraction II		
Peak 1: complex components	<6.0	
Peak 3: Fuc_2LNH	8.0	2311
Peak 5: Fuc LNO	13.1	2656
TSK-Fraction III		
Peak 1: complex components	<6.0	
Peak 3: Fuc_2LNH	8.0	2311
TSK-Fraction IV		
Peak 3: Fuc_2LNH	8.0	2311
Peak 6: Fuc LNH	14.1	2082
TSK-Fraction V		
Peak 2: Fuc_2LNT	6.4	1736
Peak 6: Fuc LNH	14.1	2082
TSK-Fraction VI		
Peak 2: Fuc_2LNT	6.4	1736
Peak 4: Fuc LNT (LNFP I)	10.2	1506
Peak 6: Fuc LNH	14.1	2081
TSK-Fraction VII		
Peak 4: Fuc LNT (LNFP I)	10.2	1506
Peak 6: Fuc LNH	14.1	2081
Peak 7: LNT	15.7	1276

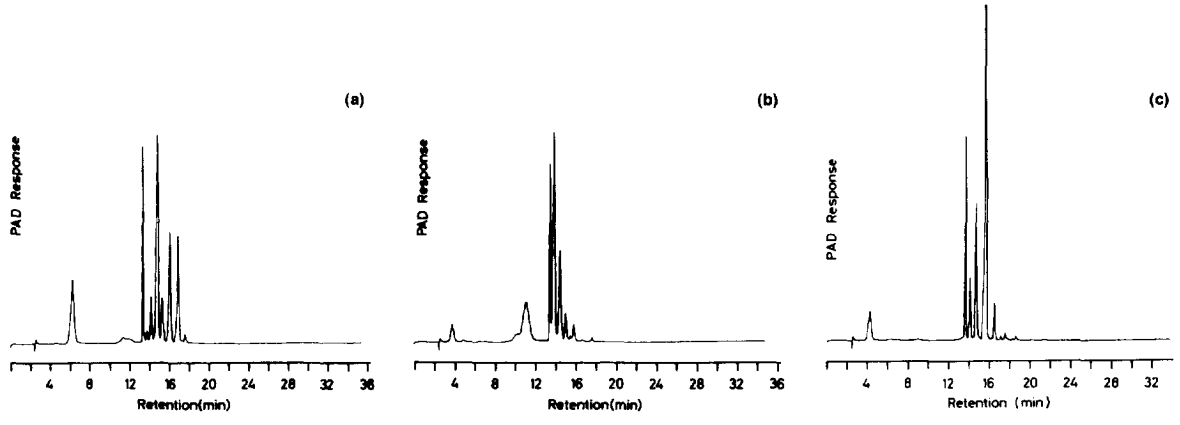


Fig. 5. HPAEC–PAD chromatogram of oligosaccharides after Bio-Gel P-4 size exclusion; (a) mono- to trifucosylated LNOctaoses; (b) fucosylated decaoses; (c) dodecaoses; (separating conditions: see Fig. 2 and Section 2.2).

Table 2
Oligosaccharides in milk of mothers with blood group A, Le[a]- or A, Le[b]-activity

Trivial name	Abbreviation	Structure
Lactose	Lac	Galβ1-4Glc
2′-Fucosyl-lactose[a]	2′-Fuc-Lac	Fucα1-2Galβ1-4Glc
3-Fucosyl-lactose	3-Fuc-Lac	Galβ1-4Glc 3 | 1 Fucα
3′-Sialyl-lactose-3-fucosyl-lactose	3′NeuAc-3-Fuc-Lac	NeuAcα2-3Galβ1-4Glc 3 | 1 Fucα
Difucosyl-lactose[a]	Fuc_2-Lac	Fucα1-2Galβ1-4Glc 3 | 1 Fucα
Lacto-N-tetraose (type 1)	LNT	Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Lacto-N-neo-tetraose (type 2)	neo-LNT	Galβ1-4GlcNAcβ1-3Galβ1-4Glc
Lacto-N-fucopentaose I[a]	LNFP I	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Lacto-N-fucopentaose II	LNFP II	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 | 1 Fucα
Lacto-N-fucopentaose III	LNFP III	Galβ1-4GlcNAcβ1-3Galβ1-4Glc 3 | 1 Fucα
Lacto-N-fucopentaose V	LNFP V	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 3 | 1 Fucα
Lacto-N-difuco-hexaose I[a]	LNDFH I	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 | 1 Fucα
Lacto-N-difuco-hexaose II	LNDFH II	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 3 | | 1 1 Fucα Fucα
Sialylα2-3lactose	NeuAcα2-3Lac	NeuAcα2-3Galβ1-4Glc
Sialylα2-6lactose	NeuAcα2-6Lac	NeuAcα2-6Galβ1-4Glc
Sialyl-lacto-N-tetraose a (LST a)	NeuAc-LNT	NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Sialyl-lacto-N-tetraose b (LST b)	NeuAc-LNT	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 6 | 2 NeuAcα
Sialyl-lacto-N-tetraose c (LST c)	NeuAc-LNT	NeuAcα2-6Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Sialyl-fucosyl-lacto-N-tetraose I	NeuAc-Fuc-LNT I	–[b]
Sialyl-fucosyl-lacto-N-tetraose II	NeuAc-Fuc-LNT II	–[b]
Disialyl-lacto-N-tetraose	$NeuAc_2$LNT	NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc 6 | 2 NeuAcα

[a] Le[b]-active components: 2′-fucosyl-lactose, difucosyl-lactose, lacto-N-fucopentaose I, lacto-N-difuco-hexaose I.
[b] Not determined yet.

Table 3
Retention, molecular mass and response factors of neutral and sialylated human milk oligosaccharides using HPAEC–PAD

Component	Retention time (min)	M_r	Molar Response Factor (LNT =1)
Neutral oligosaccharides			
2'-Fucosyl-lactose	13.24	488	0.84
3-Fucosyl-lactose	7.73	488	2.18
Difucosyl-lactose	9.81	634	3.54
Lacto-N-tetraose	15.50	708	1.00
Lacto-N-neo-tetraose	14.24	708	0.68
Lacto-N-fucopentaose I (LNFP I)	13.76	854	0.86
Lacto-N-fucopentaose II	10.15	854	1.53
Lacto-N-fucopentaose III	8.90	854	1.84
Lacto-N-fucopentaose V	14.84	854	0.94
Lacto-N-difucohexaose I	8.00	1000	1.54
Lacto-N-difucohexaose II	5.11	1000	1.90
Monofucosyl-lacto-N-hexaose I	14.51	1219	1.20
Monofucosyl-lacto-N-hexaose III	14.17	1219	1.10
Difucosyl-lacto-N-hexaose a	12.25	1365	2.69
Difucosyl-lacto-N-hexaose b	6.43	1365	2.26
Trifucosyl-lacto-N-hexaose	5.16	1511	2.10
Acidic oligosaccharides			
N-Acetylneuraminic acid	17.70	300	0.80
6'-Sialyl-lactose	20.72	634	1.19
3'-Sialyl-lactose	21.32	634	0.73
3'-Sialyl-3-fucosyl-lactose	18.95	780	1.36
6'-Sialyl-N-acetyllactosamin	19.94	675	0.53
3'-Sialyl-N-acetyllactosamin	20.53	675	0.40
Sialyl-lacto-N-tetraose c	19.66	999	1.48
Sialyl-lacto-N-tetraose a	22.62	999	0.88
Sialyl-lacto-N-tetraose b	22.84	999	1.05
Disialyl-lacto-N-tetraose	30.12	1290	1.33
Disialyl-monofucosyl-lacto-N-hexaose	25.45	1802	1.09
Blood group active oligosaccharides			
B-Trisaccharide	7.91	488	1.19
H-Disaccharide	8.22	326	1.62
A-Trisaccharide	5.59	530	1.77
A-Tetrasaccharide	8.26	692	1.94
A-Heptasaccharide	6.53	120	2.33

position and linkages. Quantification of such components is possible by considering their PAD-response factors, which have been determined for 28 components.

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References

[1] W.J. Lennarz and G.W. Hart, Methods Enzymol., 230 (1994).
[2] G. Strecker and J. Montreuil, C. R. Acad. Sci. Paris, 277 (1973) 1393–1396.
[3] A. Lundblad, in M. Horowitz and W. Pigman (Editors), Mammalian Glycoproteins, Academic Press, New York, 1976, pp. 441–458.
[4] A. Kobata, in M.I. Horowitz and W. Pigman (Editors), The Glycoconjugates, Vol. 1, Academic Press, New York, 1977, pp. 423–440.
[5] H. Egge, A. Dell and H. v. Nicolai, Arch. Biochem. Biophys., 224 (1983) 235–253.

[6] B. Stahl, S. Thurl, J. Zeng, M. Karas, F. Hillenkamp, M. Steup and G. Sawatzki, Anal. Biochem., 223 (1994) 218–226.
[7] G.P. Reddy and C.A. Bush, Anal. Biochem., 198 (1991) 278–284.
[8] S. Rudloff, G. Pohlentz, L. Diekmann, H. Egge and C. Kunz, Acta Paediatr., (1996) in press.
[9] M.T. Carlos and J.M. Harlan, Blood, 84 (1994) 2068–2101.
[10] G. Ashwell and J. Harford, Ann. Rev. Biochem., 51 (1982) 531–554.
[11] N. Hogg and R.C. Landis, Curr. Opin. Immunol., 5 (1993) 383–390.
[12] A. Varki, Glycobiology, 3 (1993) 97–130.
[13] R.R. Townsend, M.R. Hardy, O. Hindsgaul and Y.C. Lee, Anal. Biochem., 174 (1988) 459–470.
[14] M.R. Hardy, R.R. Townsend and Y.C. Lee, Anal. Biochem., 170 (1988) 54–62.
[15] M.R. Hardy and R.R. Townsend, Methods Enzymol., 230 (1994) 208–209.
[16] W.T. Wang, K. Erlansson, F. Lindh, T. Lundgren and D. Zopf, Anal. Biochem., 190 (1990) 182–187.
[17] C. Kunz, Oligosaccharide, Proteine und Glykoproteine in Frauenmilch und im Urin von Frühgeborenen, Thieme, Stuttgart, 1994.
[18] A. Kobata, K. Yamashita and Y. Tachibana, Methods Enzymol., 50 (1978) 216–220.
[19] H. Egge and J. Peter-Katalinic, Mass Spectrometry Reviews, 6 (1987) 331–393.
[20] M.W. Spellman, Anal. Chem., 62 (1990) 1714–1722.
[21] L.M. Hernandez, L. Ballou and C.E. Ballou, Carbohydr. Res., 203 (1990) 1–11.
[22] L.M. Chen, M.G. Yet and M.C. Shao, FASEB J., 2 (1988) 2819–2824.
[23] C. Kunz and S. Rudloff, Acta Paediatr., 82 (1993) 903–912.