

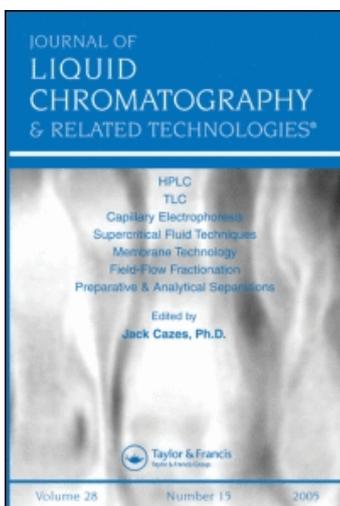
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Thin Layer Chromatography for the Separation and Analysis of Acidic Carbohydrates

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Abstract: Thin layer chromatography (TLC) is routinely used by synthetic organic chemists and natural products chemists working on a variety of types of molecules. Carbohydrate chemists and biochemists in the past largely relied on paper chromatography for rapid and inexpensive analysis. Carbohydrates are highly polar molecules and often require derivatization to be analyzed by TLC. Acidic carbohydrates pose an even more difficult challenge as they contain a formal negative charge and counterion, and can interact with commonly used stationary phases. This review describes TLC methods that have been developed for the analysis of acidic monosaccharides, disaccharides, and oligosaccharides derived from natural sources.

Keywords: Disaccharides, Monosaccharides, Oligosaccharides, Thin layer chromatography

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INTRODUCTION

Thin layer chromatography (TLC) is widely used for the analysis of synthetic organic molecules and natural products.^[1–10] One class of organic natural products that has historically seen less use of TLC is carbohydrates. This is primarily because their polar nature makes it difficult to separate these molecules on commonly used polar supports, such as silica and alumina, and also complicates the selection of eluents. Before the advent of modern chromatographic methods, neutral monosaccharides were widely separated using paper chromatography.^[11] Even more challenging than developing TLC systems for neutral monosaccharide and oligosaccharide analyses is the TLC based separation of acidic oligosaccharides. This review focuses on TLC systems for the analysis of acidic oligosaccharides and polysaccharides. This is done by briefly examining TLC methods for the separation and visualization of monosaccharides. Next, we discuss the successful application of TLC for ganglioside analysis and the application of these separations to neoglycolipids prepared from less tractable oligosaccharides. Finally, the TLC based separation of plant derived neutral and weakly acidic polysaccharides are described, which were used to develop strategies for the TLC based analysis of more strongly acidic animal polysaccharides, the glycosaminoglycans.

NEUTRAL AND ACIDIC MONOSACCHARIDES

Ten procedures for the TLC separation of simple carbohydrates in urine were compared (Table 1).^[12–20] These procedures differed in both sensitivity and resolution for the analysis of simple sugars of clinical importance. Eleven simple reference sugars, including the acidic sugar, glucuronic acid, were analyzed by these TLC systems. Based on these studies, a simple, reproducible, and quantifiable procedure was developed that permitted several sugars to be identified in normal urine samples without prior desalting or concentration. Ethyl acetate:pyridine:water (60:25:20, by vol.) was used with a Celite adsorbent and anisaldehyde visualization reagent.

Although, numerous spray reagents are available for visualizing carbohydrates on TLC plates, few are able to reveal all types of monosaccharides found in various glycoconjugates. An investigation of a modified diphenylamine–aniline–phosphoric acid (DPA) reagent,^[21] for detecting hexoses on paper chromatograms,^[22] could reveal all the above-mentioned glycoconjugates separated by TLC.^[23] Furthermore, DPA was found to be more sensitive and convenient than orcinol or resorcinol,^[24] widely used for revealing glycoconjugates on TLC plates (Figure 1).

Table 1. Procedures for TLC separation of simple carbohydrates in urine

Reference	Absorbent	Solvent (proportions by volume)	Location reagent
Reference system	Celite	Ethyl acetate:pyridine: water (60:25:20)	Anisaldehyde/ H ₂ SO ₄
Garbutt ^[13]	Celite	1-Butanol:pyridine: water (75:15:10)	Ammoniacal AgNO ₃
Eastman Kodak	Silica-gel with sodium bisulfite	Ethyl acetate:methanol:acetic acid:water (60:15:15:10)	Aniline hydrogen phthalate/ CH ₃ COOH
Eastman Kodak	Silica-gel with sodium acetate	Acetone:chloroform: methanol:water (80:10:10:5)	Aniline hydrogen phthalate/ CH ₃ COOH
Anderson and Stoddart ^[14]	Silica-gel chromatogram	Butanone:acetic acid: water (3:1:1)	Naphthoresorcinol/ H ₂ SO ₄
Hay ^[15]	Silica gel	1-Butanol:acetic acid: ethyl ether:water (9:6:3:1)	Resorcinol in phosphoric acid/H ₂ SO ₄
Lato ^[16]	Silica gel	1-Butanol:ethyl acetate: isopropanol:acetic acid: water (35:100:65:35:30)	Naphthoresorcinol/ H ₂ SO ₄
Adachi ^[17]	Silica gel	1-Propanol:water (17:3)	Thymol/H ₂ SO ₄
Paget and Coustenoble ^[18]	Silica gel	1-Butanol:methanol:water (5:3:1)	Thymol/H ₂ SO ₄
Wolfram ^[20]	Cellulose MN 300	Ethyl acetate:pyridine: water (2:1:2) (upper phase)	Aniline hydrogen phthalate/ CH ₃ COOH

TLC analysis of the derivatized acid sugar, uronic acid, liberated from the hydrolysis of glycosaminoglycans (GAGs) has also been described.^[25] A modified procedure of methanolysis of GAG in the presence of barium acetate was used to reduce the loss of uronic acids associated with their chemical degradation and to increase the uronic acid yield from GAG. The reaction products of different GAGs were identified and analyzed quantitatively. Samples were neutralized with KOH, concentrated under vacuum, and analyzed by TLC on HPTLC plates in the solvent system: methyl acetate:chloroform:methanol:1-propanol:aqueous 0.25% KCl (25:20:20:20:17 by vol.).^[26] The plates were visualized by spraying with the orcinol-sulfuric acid reagent and followed by heating.

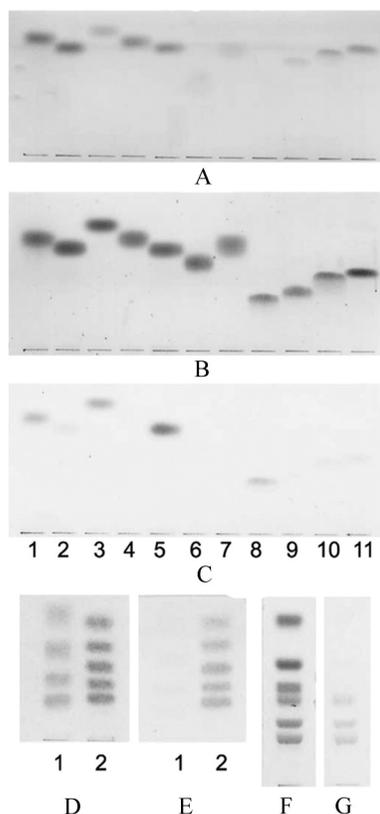


Figure 1. Detection of common sugars (A, B, and C), cello- and chito-oligosaccharides (D and E) and glycosphingolipids (F and G) on TLC plates by DPA (diphenylamine–aniline–phosphoric acid), Orcinol, and Resorcinol spray. (A, B, and C) Chromatograms sprayed with Orcinol, DPA, and Resorcinol, respectively. Positions and amounts spotted on TLC plates: (1) Ribose, 2 μ g; (2) Glucose (Glc), 2 μ g; (3) Dxyribose, 2 μ g; (4) Fucose, 2 μ g; (5) Fructose, 2 μ g; (6) Glucosamine, 8 μ g; (7) *N*-Acetylglucosamine (GlcNAc), 8 μ g; (8) Neuraminic acid (Neu5Ac), 1 μ g; (9) KDN (2-keto-3-deoxy-D-glycero-D-galactononic acid), 1 μ g; (10) KDO (3-deoxy-D-manno-octulosonic acid), 4 μ g; (11) GlcA, 1 μ g. (D and F) Sprayed with DPA. (E and G) Sprayed with Orcinol and Resorcinol, respectively. Lane 1 in D and E contains (from top) 2 μ g each of GlcNAc, *N,N'*-diacetylchitobiose, *N,N',N'*-triacetylchitotriose, and *N,N',N',N'*-tetraacetylchitotetraose. Lane 2 in D and E contains (from top) 0.5 μ g each of glucose, cellobiose, cellotriose, cellotetraose, and cellopentaose. F and G contain (from top) approximately 5 μ g each of GlcCer, LacCer, GbOse₃, Cer, GM3, GM2, and GM1. A, B, C, D and E were developed with 1-butanol/acetic acid/water (2/1/1, v/v/v). Both F and G were developed with chloroform/methanol/water (60/35/8, v/v/v). (Reprinted from Ref. [23] with permission from Elsevier).

GANGLIOSIDES

Gangliosides, glycosphingolipids containing one or more sialic acid residues, are characteristic constituents of the outer surface of animal cells. Gangliosides are located with the ceramide portion embedded in the lipid layer and the sialooligosaccharide residue facing outwards into the extracellular environment. The isolation of gangliosides from granulocytes relied on standard procedures.^[27] An overlay approach was used to assess virus binding to thin layer chromatograms on which the gangliosides were separated.^[28] Glass backed silica gel 60 precoated HPTLC plates (size 10 cm × 10 cm, thickness 0.24 mm, E. Merck, Darmstadt, Germany) were developed in chloroform:methanol:aq. 2 mM CaCl₂ (20:85:20, by vol.) and visualized by resorcinol.^[24] The plates were thoroughly dried for 0.5 h over P₂O₅ under vacuum in a desiccator and the silica gel was then fixed by chromatography in hexane saturated polyisobutylmethacrylate. After soaking for 15 min in 1% bovine serum albumin (BSA) in PBS to block non-specific binding, the solution was thoroughly withdrawn by suction and radiolabelled virus suspension was applied to each lane and visualized by autoradiography. The mixture used for TLC overlaying, contained eight major gangliosides (Figure 2). The structures have previously been shown to be GM3(Neu5Ac), IV3Neu5Ac-nLcOse4Cer, IV6Neu5Ac-nLcOse4Cer, and VI3Neu5Ac-nLcOse6Cer.^[27]

The glycosphingolipid compositions of rat mammary tumor cell lines with different metastatic potentials for the lung (parental cell line (MTC) and its subclones MTLn2 (non-metastatic) and MTLn3 (metastatic)) were studied using a TLC blotting/secondary ion mass spectrometry system.^[29] Glycosphingolipids were separated by TLC on pre-coated HPTLC plates (silica gel 60, E. Merck). The solvent systems were chloroform:methanol:aq. 0.2% CaCl₂ (60:35:8, by vol.) and chloroform:methanol:0.2% aq. CaCl₂ (55:45:10, by vol.). Gangliosides were visualized by spraying the plate with resorcinol/HCl reagent and neutral glycosphingolipids by spraying the plates with the orcinol/H₂SO₄ reagent. Glycosphingolipids were separated on the HPTLC and transferred to a PVDF membrane by TLC blotting by immersing. The developed HPTLC plate was immersed in a blotting solvent mixture of 2-propanol:aq. 0.2% CaCl₂:methanol (40:20:7, by vol.) for 20 s, and placing it on a glass plate and covering it with a PVDF membrane and a glass microfiber filter, and pressing for 30 s with a 180°C iron, after which the PVDF membrane was separated from the plate and dried. TLC blotting-SIMS was accomplished by developing glycosphingolipids on the HPTLC plate, which were sprayed with primuline reagent and detected under ultraviolet light (365 nm). The visible bands were marked with a colored pencil and the glycosphingolipids were then blotted on a PVDF membrane with the colored markings also being transferred. Each

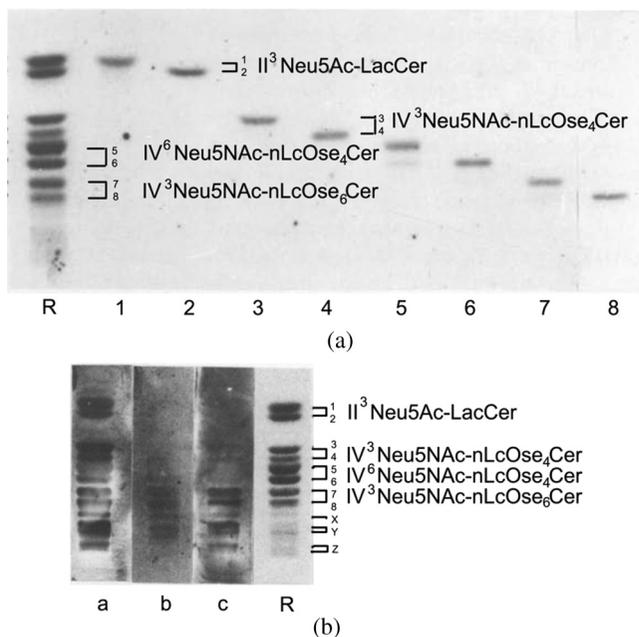


Figure 2. Gangliosides analysis by TLC. (a) Resorcinol stain of individual gangliosides isolated from human granulocytes by preparative HPTLC. R, 20 μ g of total granulocytes gangliosides. Gangliosides (1 μ g of 1 to 8) were chromatographed. (b) Autoradiographies of TLC overlay assays of gangliosides from human granulocytes with ³⁵S labeled influenza A/PR/8/34 (lane a), influenza A/X-31 (lane b) and Sendai virus (lane c), exposure time 20 d. Lane R shows the resorcinol stained ganglioside mixture. In each lane 20 μ g of gangliosides were applied. (Reprinted from Ref. [28] with permission from Springer).

marked glycosphingolipid was punched out and placed on a SIMS target tip of a mass spectrometer triethanolamine matrix. Negative secondary ion mass spectrometry (SIMS) spectra were obtained with a TSQ 70 triple quadrupole mass spectrometer. The marked area on the membrane could also be excised and immersed in distilled water to remove the primuline reagent. The excised membrane was transferred methylated, and the product released into the methylation solvent, extracted, then hydrolyzed and converted to partially methylated alditol acetates for analysis by GC-MS. TLC immunostaining of the glycosphingolipids was also performed.^[30]

Total lipid fractions extracted from rat placenta were subjected to glycolipid analysis using silica gel TLC, and visualized by immunostaining.^[31] Enzyme immunostaining of gangliosides on TLC plates was performed by spotting aliquots of gangliosides on a plastic TLC plate

and developing with chloroform:methanol:aq. 2.5 M NH_4OH (55:45:10, by vol.). The plate was dried and soaked in blocking solution and monoclonal antibody was applied and the TLC was shaken at room temperature for 2 h. Then, TLC was next soaked in blocking solution for 15 min and re-incubated with horseradish peroxidase conjugated goat anti-mouse immunoglobulin for 2 h. After several successive washes with PBS, the chromatogram was exposed to the substrate solution (4-chloro-1-naphthol and H_2O_2) for visualization.

Several forced flow TLC methods have been introduced in recent years, and a compact and simple instrument for overpressure layer chromatography (OPLC) is now commercially available.^[32] By covering the TLC plate with an inert membrane sheet under external pressure, a mobile phase can be pumped through the sealed silica gel stationary phase. The plate represents a planar column resulting in shorter analysis times, higher efficiencies, and lower solvent consumption than conventional TLC. Analysis is carried out with UV detection or staining after solvent development. On-line OPLC-ESI-MS was accomplished using with a Q-TOF mass spectrometer.^[33] Aluminum-backed 5 cm \times 20 cm silica gel TLC plates with perimeter seals were used for OPLC and elution was with CHCl_3 : CH_3OH : H_2O (60:35:8, by vol.). A sensitivity of 5 pmol of glycosphingolipid was achieved for OPLC-ESI-MS and 20 pmol for OPLC-ESI-MS/MS to obtain saccharide sequence and identify the fatty acid composition of the ceramide. Initial preconditioning of TLC plates was required to achieve high sensitivity detection and the plates can be used at least 10 times for analysis. Following development, the TLC plates can be visualized in the conventional way by nondestructive staining or UV absorption or fluorescence.

NEOGLYCOLIPIDS

Neoglycolipids contain a single lipid moiety, in contrast to natural glycolipids that are comprised of a heterogeneous mixture of lipids. Neoglycolipids were conjugated by reductive amination of oligosaccharide with the aminolipid. The aminolipid, 1,2-dihexadecyl-sn-glycero-3-phosphoethanolamine (DHPE), which has been used to generate neoglycolipids was modified to incorporate a fluorescent label, anthracene. This new lipid reagent is *N*-aminoacetyl-*N*-(9-anthracenylmethyl)-1,2-dihexadecyl-sn-glycero-3-phosphoethanolamine (ADHP). Following conjugation of mixtures, each oligosaccharide remains a discrete entity and mixtures of neoglycolipids are amenable to resolution by TLC, and for binding experiments. The excellent ionization properties of neoglycolipids also enable MS based analyses by liquid secondary ion mass spectrometry (LSIMS).^[34] ADHP (10 μM) in chloroform:methanol:water

(10:10:1, by vol.) was added to dry oligosaccharide (typically 20 nmol). After sonication for 5 min, the mixture was heated at 60°C for 2 h. Tetrabutylammonium cyanoborohydride (100 nmol in 2 mL methanol) was added (per 200 nmole of ADHP), and after sonication for 5 min, the mixture was incubated at 60°C from 16 h to 3 days. The conjugation progress was monitored by HPTLC. Chromatographic separations of ADHP-neoglycolipids derived from reference oligosaccharides are shown in Figure 3. Quantification of nanomole or larger amounts of ADHP or the derived neoglycolipids was carried out by comparing the absorptions of solutions, in chloroform:methanol:water (60:35:8, by vol.), with a standard solution of 9-methylanthracene. For picomole amounts, quantification was by scanning densitometry on thin layer chromatograms using, as standard, an ADHP neoglycolipid previously quantified by the solution method. Binding of monoclonal antibodies, anti-sialyl-Le^A and anti-sialyl-Le^X, to neoglycolipids on HPTLC plates was assessed. The introduction of fluorescence to the neoglycolipid technology broadens

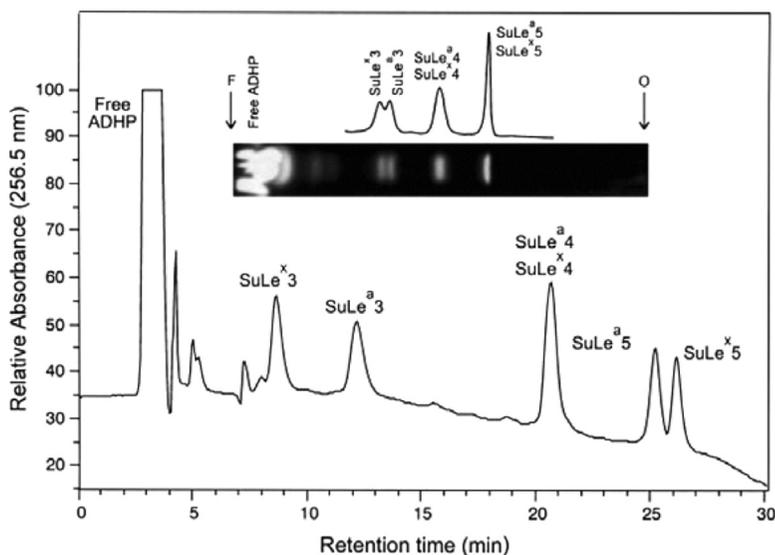


Figure 3. Chromatographic separations of ADHP-neoglycolipids derived from reference oligosaccharides. The UV profile of the reaction mixture of the neoglycolipids of oligosaccharides of the 3-sulfo Le^a- and 3-sulfo Le^x series (containing about 50 pmol of each neoglycolipid), resolved by HPLC. The insets are photographs taken under UV light (254 nm) and fluorescent densitometry scans of silica gel chromatograms of the respective neoglycolipids (100 pmol of each) developed with chloroform/ethanol/water, 45:60:15 (v/v). The arrows indicate origin, O, and the solvent front, F, of the chromatograms. (Reprinted from Ref. [34] with permission from Blackwell Publishing).

the range of possible applications, including the synchronization of resolution, bioassay, and sequencing.

The release of *O*-glycans ovarian cystadenoma glycoprotein using ethylamine was investigated in the search for epithelial derived oligosaccharide ligands of the endothelial adhesion protein E-selectin.^[35] Microscale assays of samples and standards were carried out on TLC plates. Orcinol staining was measured by densitometry, and hexose content in samples was determined from the calibration curve of density concentration. Direct TLC-LSIMS of neoglycolipids was carried out by cutting the sample bands out from the plate together with aluminum backing (typically 1.5 × 5.5 mm), and attached to the LSIMS probe tip by an electro-conducting adhesive. Extraction solvent CHCl₂:MeOH:H₂O (25:25:8, by vol.) and matrix were added to the silica gel surface prior to LSIMS analysis.

TLC-LSIMS of GAG-derived disaccharides was accomplished after conversion to neoglycolipid derivatives by reductive amination with an aminolipid (DHPE).^[36] DHPE conjugation mixtures (~1–2 nmol of oligosaccharide) was applied as a 5 mm band to an aluminum backed TLC plate and developed in CHCl₃:MeOH:H₂O (60:35:8, by vol.). The bands were located under long wavelength UV light after spraying with primulin reagent (0.001% of primulin in acetone:H₂O (4:1, by vol.)). The plate was primulin stained by immersion when quantitative measurements were required by comparison of fluorescence intensities.^[37] For LSIMS analysis, each band was carried out.^[35] Mobility on TLC, while largely determined by the number of sulfate groups present, was also influenced by the sulfate position, monosaccharide composition, and linkage. Mass spectra were acquired at high sensitivity directly from the TLC plate and provided quasi-molecular ions and fragment ions from which composition, including sulfate content, and sequence information was obtained. Lipid DHPE conjugation and TLC-LSIMS were performed to analyze the cleavage of unsaturated uronic acid (Δ UA) residues from disaccharide products of polysaccharide lyase by oxymercuration reaction. It was established that glycosidic linkages and *O*- and *N*-sulfo groups are preserved on removal of Δ UA. The combined use of HPTLC and *in situ* LSIMS analysis is a sensitive and reliable method for separation and structural identification of the GAG disaccharides as DHPE derivatives, possibly serving as an alternative approach to disaccharide compositional analysis of GAGs.

NEUTRAL POLYSACCHARIDES

The application of TLC for the analysis of neutral oligosaccharides has been undertaken by a variety of laboratories.^[38] One particularly

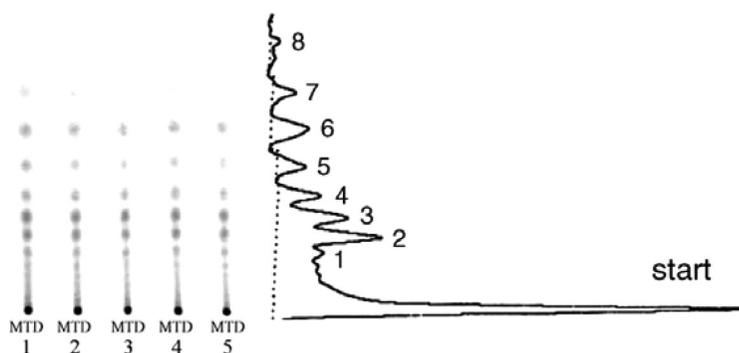


Figure 4. TLC chromatogram of five simultaneously analyzed. Raftifeed samples standard solutions (RFT) with a densitogram at wavelength 370 nm; peaks 1–7 on the densitogram represent individual fractions of Raftifeed IPX; chromatographic conditions: stationary phase: glass-backed precoated silica gel plates (Merck) impregnated by 0.02 M sodium acetate; mobile phase: butanol–ethanol–water (5:3:2, v/v); detection reagent: aniline–diphenylamine–phosphoric acid in acetone; volume of sample 0.2 μ g; (Reprinted from Ref. [38] with permission from Elsevier B.V.).

comprehensive study involved fructooligosaccharides as feed additives (prebiotics) and determined their presence in complicated biological samples with minimal pre-treatment. TLC was developed with butanol:ethanol:water (5:3:2, by vol.); visualization relied on a mixture of diphenylamine–aniline–phosphoric acid in acetone and was detected by reflectance densitometry at $\lambda = 370$ nm (Figure 4 & Table 2). TLC successfully monitored the fructooligosaccharides in samples from different parts of intestinal tracts of animals fed on a prebiotic diet. Statistical evaluation of qualitative chromatographic characteristics (R_F values) of each individual separated spot in analyzed dietetic products points to low RSD values ranging from 0.39 to 3.03%.

Table 2. R_F values of fructooligosaccharides analysis by TLC

MTD spots	R_F mean	Maximum	Minimum	R	SD	RSD (%)
1	0.523	0.520	0.525	0.005	0.0023	0.43
2	0.449	0.446	0.450	0.004	0.0018	0.41
3	0.375	0.372	0.378	0.006	0.0027	0.73
4	0.300	0.300	0.302	0.002	0.0009	0.30
5	0.238	0.228	0.240	0.013	0.0055	2.30
6	0.196	0.194	0.198	0.004	0.0018	0.93
7	0.157	0.156	0.162	0.006	0.0027	1.74
8	0.121	0.118	0.122	0.004	0.0018	1.51

ACIDIC PLANT POLYSACCHARIDES

Pectic acid is a plant polysaccharide consisting of a repeating structure of 1,4-linked galacturonic acid. Endo-polygalacturonase from *Saccharomyces fragilis* randomly hydrolyses pectic acid.^[39] The breakdown pattern of the methyl ester of pectate was investigated using TLC. Esterification was accomplished under dry conditions by adding 50 mL of 0.02 M methanolic hydrogen chloride for each gm of pectic acid oligomer and reacting for up to 15 days. The mixtures were neutralized with silver carbonate, filtered, concentrated, and analyzed by TLC on silica gel developed with 1-propanol:water (7:2, by vol.).

Alginate is an acidic heteropolysaccharide from plants consisting of β -D-mannuronate (M) and α -L-guluronate (G) arranged as a poly(M)-block, a poly(G)-block, and an alternating or random poly(MG)-block. The purity of alginate oligosaccharides, derived from polyanionic alginic acid, polymannuronate, and polyguluronate, by partial depolymerization using either alginate lyase or mild acid hydrolysis were analyzed by TLC (Figure 5), and their structure then determined by ESI-MS-MS.^[40] The

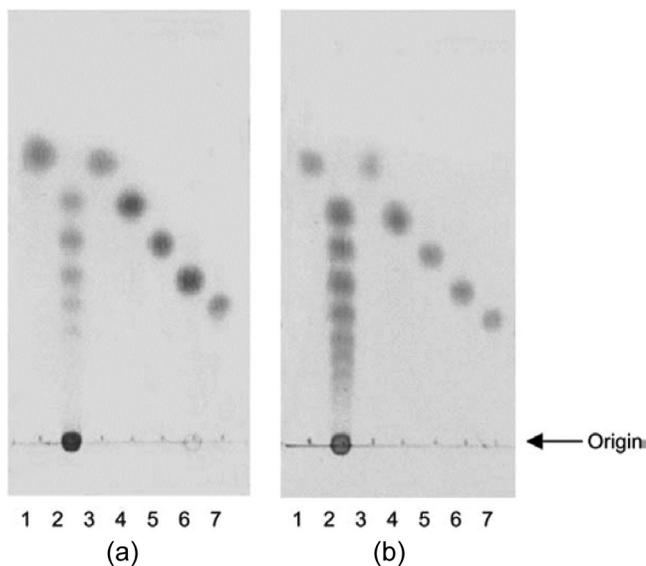


Figure 5. HPTLC of guluronate- and mannuronate-oligosaccharides. (a) Lane 1, D-glucuronic acid; lane 2, oligoguluronate mixture; lanes 3–7, mono- to pentasaccharides of guluronate, respectively. (b) Lane 1, D-glucuronic acid; lane 2, oligomannuronate mixture; lanes 3–7, mono- to pentasaccharides of mannuronate, respectively. (Reprinted from Ref. [40] with permission from American Society for Mass Spectrometry).

purity of alginate derived oligosaccharides was checked on a silica gel TLC developed with a solvent system of *n*-butanol:formic acid:water, 4:6:1, by vol.). The developed TLC was stained by dipping in cerium sulfate reagent (0.02% cerium sulfate crystal, 0.05% ammonium molybdate, and 5% H₂SO₄ in EtOH) for 3 s and heated at 100°C for 10 s.

A novel alginate degrading enzyme, HdAlex, was isolated from the digestive fluid of the abalone.^[41] HdAlex degrades alginate, mannuronate rich polymers and unsaturated trisaccharide (Figure 6). TLC of oligosaccharides was carried out on Silica Gel 60 TLC plates developed with 1-butanol:HOAc:H₂O (2:1:1, by vol.). The TLC plates were visualized by spraying with 10% (v/v) H₂SO₄ in EtOH, followed by heating at 120°C for 15 min, whereas unsaturated sugars were detected by the thiobarbituric acid method.^[42]

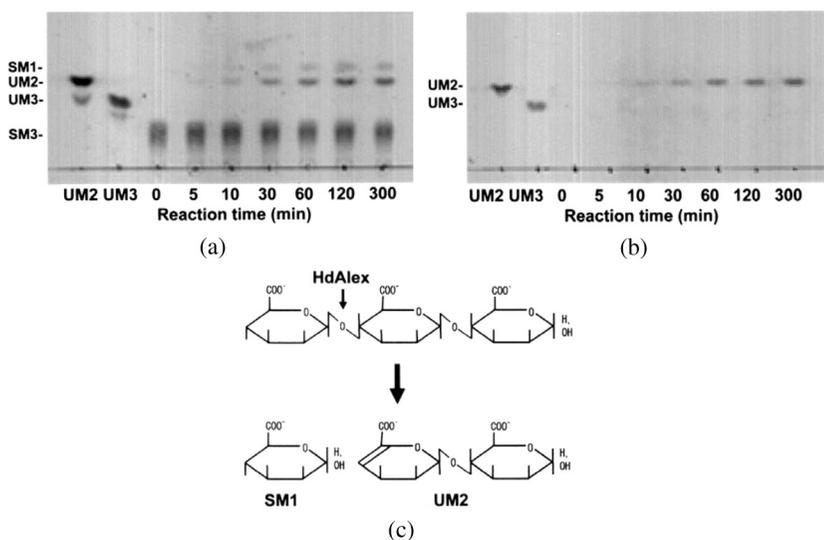


Figure 6. Degradation of saturated trisaccharide by HdAlex. Saturated trisaccharide (SM3) prepared by limited acid hydrolysis under the condition: 0.1% (w/v) poly(M)-rich substrate, 10 mM sodium phosphate (pH 7.0), and 4.4 U/mL HdAlex was incubated at 30°C for 5–300 min. Aliquots of the reaction mixture (2 μ L) were subjected to TLC. (a) Oligoalginates were detected with 10% sulfuric acid. (b) Unsaturated oligoalginates were detected with thiobarbituric acid. SM1, saturated monosaccharide; UM2, unsaturated disaccharide; UM3, unsaturated trisaccharide; SM3, saturated trisaccharide. (c) Schematic diagram for the action of HdAlex on saturated trisaccharide. (Reprinted from Ref. [41] with permission from Elsevier Ltd.).

GLYCOSAMINOGLYCANS

Glycosaminoglycans (GAGs) are linear acidic polysaccharides found on cell surfaces and in the surrounding extracellular matrix. GAGs participate in and regulates many cellular events in physiological and pathophysiological processes, such as cell proliferation and differentiation, cell-cell and cell-matrix interactions, and viral infections, through their interaction with different proteins.^[43] GAGs are divided into four main categories, hyaluronic acid (HA), chondroitin sulfate/dermatan sulfate (CS/DS), heparosan/heparan sulfate/heparin (HN/HS/HP), and keratan sulfate, based on monosaccharide composition and the configuration and position of the glycosidic bonds between their monosaccharides. The specificity of the interactions between GAGs and proteins results from the structural diversity of GAG type, size, saccharide composition, charge density, and sequence.

Hyaluronan (HA) is the simplest of all the GAGs, consisting of a repeating copolymer of (1→3) *N*-acetylglucosamine, (1→4) glucuronic acid, and (1→HA, extracted from rooster comb; it was digested by a mixture of β-*N*-acetylhexosaminidase and β-glucuronidase with concurrent dialysis for 96 h.^[44] A mixture of monosaccharides and oligosaccharides, was purified by gel chromatography and analyzed on silica gel developed with 1-butanol:acetic acid:diethyl ether:water (90:6:3:1, by vol.), ethyl acetate:acetic acid:water (6:3:2, by vol.) or 95% ethanol:pyridine:water (30:2:15, by vol.). Carbohydrate components were located by the H₂SO₄ charring. Arabinose, *N*-acetylgalactosamine, glucose, *N*-acetylglucosamine, glucuronic acid, mannose, ribose, rhamnose, HA disaccharide, HA trisaccharide, and HA tetrasaccharide could be resolved.

HA oligosaccharides can be prepared by treating HA with testicular hyaluronan hydrolase or *Streptomyces* hyaluronan lyase.^[45] Paper chromatography has been used in the past to analyze the products of these enzymatic reactions but this method is quite tedious.^[46] Oligosaccharides with odd numbers of sugar residues having uronic acids at both RE and NRE can be prepared by heating unsaturated oligosaccharides, obtained using *Streptomyces* hyaluronan lyase, in borate buffer at pH 9 and removal of residual boric acid as methyl borate by GPC.^[47] Odd number oligosaccharides having glucosamine residues at both RE and NRE were prepared by treating with β-glucuronidase. TLC separated even and odd number of residues on silica gel (Kieselgel 60, 5 × 10 cm plates) using 2-propanol:aq. 0.05 M NaCl (66:34, by vol.).^[45] A decrease in mobility was observed on the addition of NaCl, suggesting that sodium ions fixed to the gel and acted as an anion exchanger. The impact of replacing NaCl with CaCl₂ and LaCl₃ was consistent with the ion exchange hypothesis. A relationship between molecular size and R_f was established (Figure 7).

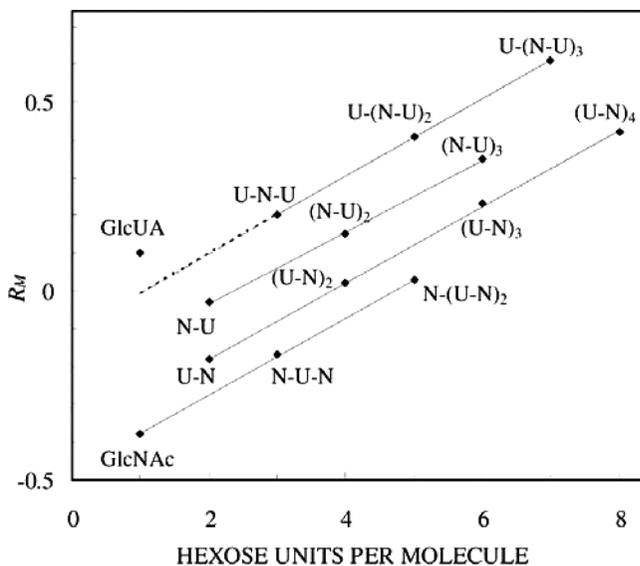


Figure 7. The relationship between R_M value and molecular size. U, glucuronic acid; N, *N*-acetylglucosamine. (Reprinted from Ref. [45] with permission from Japanese Biochemical Society).

Oligosaccharides were visualized by UV or by Morgan-Elson reagent^[48] reactive with hexosamine. The addition of electrolytes were necessary for resolution.

Chondroitin sulfate is a GAG consisting of a repeating copolymer of (\rightarrow 3) *N*-acetylgalactosamine, (1 \rightarrow 4) glucuronic acid, (1 \rightarrow 3) Chondroitin sulfate (CS), and HA oligosaccharides digested with chondroitin AC II lyase to prepare unsaturated disaccharides.^[49] These disaccharides were analyzed by TLC on cellulose desalted with 1-butanol:ethanol:water (52:32:16, by vol.) and then developed with 1-butanol:acetic acid: aq. 2 M ammonium hydroxide (2:3:1, by vol.), dried, and scanned by reflectance densitometry at 232 nm. Unsaturated disaccharides delDiHA, delDiOS, delDi4S, and delDi6S were resolved on an 18 cm cellulose TLC plate. Quantification was achieved as long as the loading of each disaccharide was $<10 \mu\text{g}$ with a coefficient of variation of $<22\%$.

TLC of HA and CS unsaturated disaccharides is possible on silica gel but these are difficult to visualize by carbazole reagents.^[45,50] GAGs were isolated from rabbit plasma and urine and treated with chondroitin B lyase to afford unsaturated disaccharides.^[51] These disaccharides were fluorescently derivatized by reaction with dansylhydrazine in ethanol and subjected to TLC. TLC on silica plates developed with 1-propanol: 2-propanol:1-butanol:aq. 0.04 M NaCl and 0.01 M ammonium hydroxide

Table 3. R_F values of dansylhydrazine derivatives of unsaturated disaccharides

Unsaturated disaccharide	R_F	Unsaturated disaccharide	R_F
Δ Di-0S	0.72	Δ Di-diS _D	0.52
Δ Di-4S	0.56	Δ Di-diS _E	0.41
Δ Di-6S	0.61	Δ Di-triS	0.30
Δ Di-UA2S	0.66	Δ Di-HA	0.75
Δ Di-diS _B	0.47		

(30:45:5:20, by vol.) could be separated and visualized with the naked eye (Table 3). This allowed the separation of the complex mixture of disaccharides afforded from a biologically significant sample.

TLC can be used to determine the size and purity of GAG-derived oligosaccharides, analyze activity of polysaccharide lyases acting on GAGs, and monitor the preparation of GAG-derived oligosaccharides.^[52] Reaction product mixtures (0.3 μ L of each) were loaded onto a precoated Silica Gel 60 TLC aluminum plate (3 \times 5 cm, Merck, Germany), and developed with a solvent system consisting of 1-butanol:formic acid:water (4:8:1, by vol.). The developed plate was stained by dipping in diphenylamine–aniline–phosphoric acid reagent (1 mL of 37.5% HCl, 2 mL of aniline, 10 mL of 85% H₃PO₃, 100 mL of ethyl acetate, and 2 g of diphenylamine) for 3 s and heated at 150°C for 10 s.^[40] The TLC plate was scanned, digitized, and analyzed, and intensity was plotted as a function of distance from origin (Figure 8). TLC was used to separate GAG oligosaccharides on the basis of size and disaccharides on the basis of sulfation level. Furthermore, TLC represents a quick, easy, and reliable method for the parallel analysis of GAG oligosaccharide samples and can be used to examine the action pattern of polysaccharide lyases.

Isobaric oligosaccharides enzymatically prepared from HA and heparosan (HN), were distinguished using tandem ESI mass spectrometry.^[53] TLC was used to follow these enzymatic reactions. The progress of the polysaccharide based depolymerization was monitored by measuring the HA and HN oligosaccharides present in aliquots taken throughout the reaction by TLC on a precoated silica gel-60 aluminum plate, eluted with a solvent system consisting of 1-butanol:formic acid:water (4:8:1, by vol.). The eluted plate was stained by dipping in a reagent containing 1 mL of 37.5% HCl, 2 mL of aniline, 10 mL of 85% H₃PO₃, 100 mL of ethyl acetate, and 2 g diphenylamine) for 3 s and heating at 150°C for 10 s.

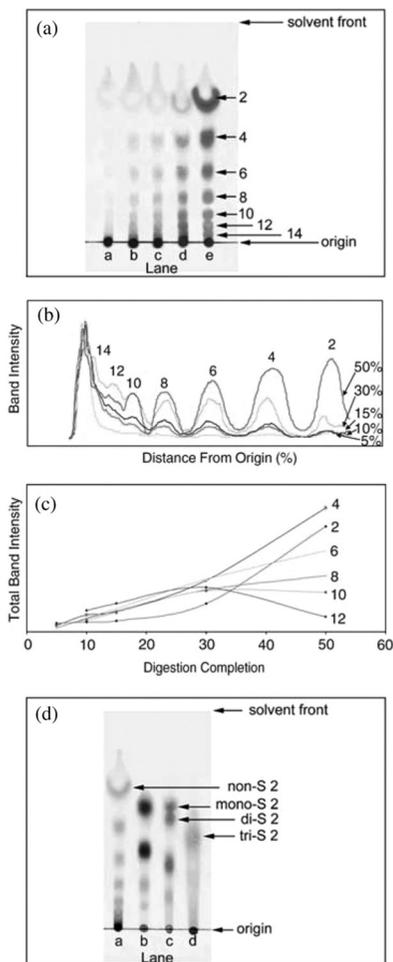


Figure 8. (a) TLC of partially digested HN. The reaction products obtained at 5% completion (lane a), 10% completion (lane b), 15% completion (lane c), 30% completion (lane d), and 50% completion (lane e) are shown. (b) Digitized TLC. (c) Plot of total band intensity as a function of percentage digestion completion for each size oligosaccharide. (d) TLC of other GAGs digested with polysaccharide lyases: HA (lane a), CS-A (lane b), DDS (lane c), and HP (lane d). The major disaccharides found in each GAG and their numbers of sulfo groups (non-sulfated [non-S 2], monosulfated [mono-S 2], disulfated [di-S 2], and trisulfated [tri-S 2]) are indicated. Their identities were confirmed based on the use of disaccharide standards. The numbers 2–14 in all four panels (a–d) correspond to the numbers of saccharide units present in the various oligosaccharide products. (Reprinted from Ref. [52] with permission from Elsevier Inc.).

Table 4. TLC systems to separate acidic carbohydrates

Sample	Adsorbent	Solvent (proportions by volume)	Visualization reagent
Gangliosides ^[26]	Silica-gel	Methyl acetate:chloroform:methanol: 1-propanol:aq. 0.25% KCl (25:20:20:17)	Orcinol-sulfuric acid
Gangliosides ^[24]	Silica-gel with BSA	Chloroform:methanol:aq. 2 mM CaCl ₂ (20:85:20)	Resorcinol
Gangliosides ^[29]	Silica-gel	Chloroform:methanol:aq.0.2% CaCl ₂ (60:35:8);	Resorcinol-HCl;
Gangliosides ^[31]	Silica-gel	Chloroform:methanol:0.2% aq. CaCl ₂ (55:45:10)	Orcinol-H ₂ SO ₄
		Chloroform:methanol:aq. 2.5 M NH ₄ OH (55:45:10)	Immunostaining
Glycosphingolipid ^[33]	Silica-gel	Chloroform:methanol:water (60:35:8)	UV absorption
Neoglycolipids ^[34]	Silica-gel	Chloroform:methanol:water (60:35:8)	Fluorescence
Neoglycolipids ^[36]	Silica-gel	Chloroform:methanol:water (60:35:8)	Primulin
Fructooligosaccharides ^[38]	Silica-gel	1-Butanol:ethanol:water (5:3:2)	Diphenylamine-aniline- phosphoric acid
Pectic acid ^[39]	Silica-gel	1-Propanol:water (7:2)	H ₂ SO ₄
Alginate ^[40]	Silica-gel	1-Butanol:formic acid: water (4:6:1)	Diphenylamine-aniline-phosphoric acid
Alginate ^[42]	Silica-gel	1-Butanol:acetic acid:H ₂ O (2:1:1)	H ₂ SO ₄ -EtOH
Hyaluronan ^[44]	Silica-gel	1-Butanol:acetic acid:diethyl ether:water (90:6.3:1); Ethyl acetate:acetic acid: water (6:3:2); 95% Ethanol:pyridine: water (30:2:15)	H ₂ SO ₄
Hyaluronan ^[45]	Silica-gel	2-Propanol:aq. 0.05 M NaCl (66:34)	UV or Morgan-Elson reagent
Chondroitin sulfate disaccharides ^[49]	Cellulose	1-Butanol:acetic acid:aq. 2 M ammonium hydroxide (2:3:1)	UV

(Continued)

Table 4. Continued

Sample	Adsorbent	Solvent (proportions by volume)	Visualization reagent
Fluorescently derivative HA and CS disaccharides ^[3]	Silica-gel	1-Propanol:2-propanol:1-butanol: aq. 0.04 M NaCl; 0.01 M Ammonium hydroxide (30:45:5:20)	Naked eye visualized
GAG-derived oligosaccharides ^[52]	Silica-gel	1-Butanol:formic acid: water (4:8:1)	Diphenylamine-aniline-phosphoric acid
HA and heparosan derived oligosaccharides ^[53]	Silica-gel	1-Butanol:formic acid: water (4:8:1)	Diphenylamine-aniline-phosphoric acid

CONCLUSIONS

A number of TLC systems have been developed to separate acidic carbohydrates (Table 4). Separations, originally depending on paper chromatography, have moved to the use of silica gel stationary supports. Solvent systems have been designed that afford good separation of both derivatized and unmodified monosaccharides, disaccharides, and oligosaccharides. Since glycolipids are relatively easily resolved by TLC and are then amenable to LSIMS and protein probing by TLC overlay, neoglycolipids have been constructed as derivatives of a variety of oligosaccharides to improve their analysis. Substantial progress has also been made in the separation of unmodified acidic disaccharides and oligosaccharides by the careful design of developing solvent systems. Prospects are excellent for the more routine use of TLC for the rapid and inexpensive analysis of a wide variety of acidic carbohydrates.

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