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Review

Determination of sialic acid and gangliosides in biological samples and dairy products: A review

Ramón Lacomba[a], Jaime Salcedo[a], Amparo Alegría[a], M. Jesús Lagarda[a], Reyes Barberá[a,*], Esther Matencio[b]

[a] Department of Nutrition and Food Chemistry, Faculty of Pharmacy, University of Valencia, Avda. Vicente Andrés Estellés s/n, 46100, Burjassot (Valencia), Spain
[b] Hero Institute for Infant Nutrition, 30820, Alcantarilla, Spain

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ABSTRACT

Gangliosides are sphingolipids containing one or more moieties of sialic acid in their structure. Both gangliosides and sialic acid are bioactive compounds related to animal physiology. Due to their biological relevance, analytical methods adapted to each type of matrix have been developed over time. The present study reviews the main methods applied to the analysis of sialic acid and gangliosides in biological samples and dairy products.

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Contents

1. Introduction 347
2. Sialic acid 348
   2.1. Sample preparation 348
      2.1.1. Release of sialic acids 348
      2.1.2. Purification 348
   2.2. Sialic acid determination 348
      2.2.1. Spectrophotometric methods 348
      2.2.2. Liquid chromatography 352
      2.2.3. Gas chromatography 352
      2.2.4. MALDI-TOF-MS 352
      2.2.5. Amperometric methods 352
      2.2.6. Electromigration methods 352
3. Gangliosides 353
   3.1. Extraction, isolation and purification of gangliosides 353
   3.2. Determination of gangliosides 353
      3.2.1. TLC/HPTLC 353
      3.2.2. Liquid chromatography–mass spectrometry 355

Abbreviations: CHO, Chinese hamster ovary; CMP-NANA, cytidine 5′-monophospho-N-acetylneuraminic acid; DDB, 1,2-diamino-4,6-dimethoxybenzene; DMB, 1,2-diamino-4,5-methylenedioxybenzene; FTIC, Fourier transformation ion cyclotron; KDN, 2-keto-3-deoxy-D-glycero-D-galactonononic acid; LBSA, lipid bound sialic acid; Neu5,9Ac$_2$, N-acetyl-9-O-acetylneuraminic acid; Neu5Ac, N-acetylneuraminic acid; Neu5Ac2en, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; OPD, O-phenylenediamine; PAD, pulse amperometric detection; TBA, thiobarbituric acid; TEA, triethanolamine; TFA, trifluoroacetic acid; THF, tetrahydrofurane; TIP, triisopropanolamine; TOA-Br, tetraoctylammonium bromide; TOF, time-of-flight; Tos-Cl, p-toluenesulfonylchloride; VC, variation coefficient.

* Corresponding author. Tel.: +34 963544956; fax: +34 963544954.
E-mail address: reyes.barbera@uv.es (R. Barberá).

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4. Conclusions 355
Acknowledgments 356
References 356

1. Introduction

Gangliosides[1] are sialoglycosphingolipids—sphingolipids with one or more moieties of sialic acid in their structure. More in detail, gangliosides are composed of a sphingoid base linked to a fatty acid by an amide linkage (ceramide) bound to an oligosaccharide chain of variable size containing one or more sialic acid moieties (Fig. 1).

In relation to sialic acid, Blix et al. [1] proposed this term as a trivial name for the family of acylated derivatives of a 9-carbon carboxylated monosaccharide. Fig. 2 shows the basic backbone of the molecule. Under physiological conditions, the molecule has a negative charge (pK_a 2.2). Substitutions of the hydroxyl groups, modification of some carbons, and even unsaturation of the structure, result in the great variety of compounds that could be found in biological samples under this denomination. Since the 1940s, with the description of the first sialic acids found in brain gangliosides [2] and salivary mucins [3], more than 40 compounds have been associated to this family [4].

Sialic acids and gangliosides are natural compounds that could be found not only in all vertebrates and in higher invertebrate species, but also in lesser amounts in lower invertebrates, as well as in fungi (*Candida albicans*, *Aspergillus fumigatus*), bacteria (*Escherichia coli K1*, *Neisseria meningitidis*, *Campylobacter jejuni*), protists (*Entamoeba histolytica*, *Theileria sergenti*), the Archaea domain, viruses and prokaryotic cells [4–6]. Each species has characteristic sialic acids extensively distributed in all tissues and fluids. An example of note is their presence in milk, with amounts that decrease during lactation (−80% from colostrums to mature milk) [7].

The main representative form of sialic acid is the acylated (at the amino group of carbon 5) compound, known as N-acetylneuraminic acid (Neu5Ac). Another common form is N-glycolylneuraminic acid (Neu5Gc), where a glycolyl group is bound to the C5 amino group. The family of sialic acids was expanded with a new form where the amino group at carbon 5 is replaced by a hydroxyl group. This form is referred to as 2-keto-3-deoxy-D-glycero-D-galactonononic acid (KDN), and is not a neuraminic acid [8].

In dairy products and biological samples, sialic acids are usually present as glycoconjugates (glycoproteins, glycolipids and lipopolysaccharides), and as terminal moieties in glycan chains; they are rarely found in free form (3%). It is also common to find them forming polymers, where they are glycosidically linked via their hydroxyl group at C2 to position 3 or 6 of the penultimate sugar to C8 of another sialic acid residue [9].

Gangliosides are widely distributed in most tissues, forming part of certain organs and plasma cells. They are particularly abundant in neural tissue, although they also appear in high concentrations in extraneural organs as well as in biological fluids [10]. In the human body, the largest amounts are found in neural tissues (with a prevalence of G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}) and extra-neural organs such the lung, spleen and gut, where the majority representations are G_{D3} and G_{M3}.

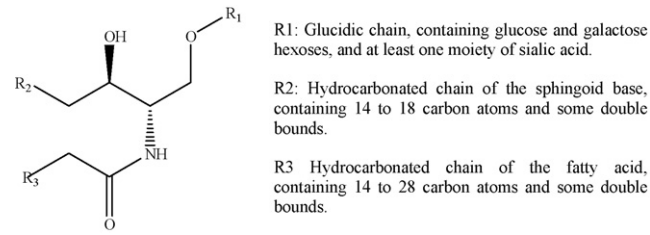

Fig. 1. General structure of gangliosides.

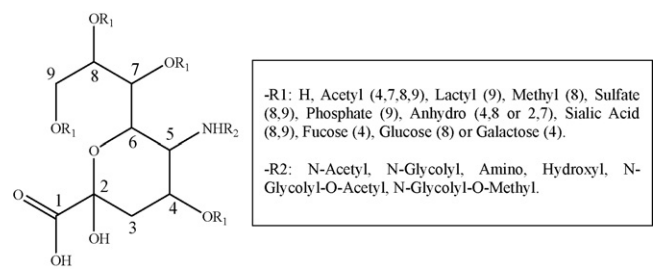

Fig. 2. General structure of sialic acid and main substitutions.

It has been reported that G_{D3} and G_{M3} are the main individual components of the gangliosides fraction in milk and dairy products. G_{D3} is the main ganglioside in human colostrum, cow's milk and infant formulas, representing 50%, 60% and 80%, respectively, of the total amount of gangliosides, while G_{M3} is present in trace amounts. From transitional to mature milk, the G_{M3} contents increase up to 50% in human milk, while the G_{D3} contents decrease [11].

Gangliosides are amphipathic molecules, this configuration being essential for the role they play. Forming part of cell membranes, gangliosides stabilize the latter, participate in cell-to-cell communication, in the regulation of axonal development [12] acting as modulators in synaptic transmissions [13], and in cell adhesion phenomena [14].

It has been demonstrated that gangliosides present protective action against enteric pathogens, acting as false receptors (*E. coli*, *V. cholera*, *Campylobacter*, *Helicobacter*) [15–19], and they have prebiotic functions—increasing bifidobacteria content in the gut of infants [16]. Likewise, in relation to the regulation of the immune system, gangliosides intervene in the differentiation of the Th1 and Th2 lymphocyte subpopulations [20], in the activation of T cells and/or lymphocytes [21,22], and increase the number of intestinal IgA-secreting cells as well as the percentages of Th1 and Th2 cytokine-secreting cells in the lamina propria and Peyer's patches [23–25]. The dietary intake of these bioactive compounds is important, especially during the first stages of life, when dietary gangliosides from breast feeding and infant formulas contribute to the aforementioned biological functions and prove essential for correct neuron development.

Gangliosides and sialic acid can act as biomarkers for some pathologies as well. A high amount of Neu5Gc in plasma is associated with some cancers [26], since tumor cells induce alterations in the gene encoding for Neu5Ac, resulting in the production of

[1] The ganglioside nomenclature of Svennerholm [97] and the IUPAC-IUB JCBN (Joint Commission on Biochemical Nomenclature; Nomenclature of Glycolipids) recommendations (1999) was followed: G_{M1}, Galβ1-3-GalNAc β1-4-Neu5Ac α2-3)-Galβ1-4-Glc-Cer; G_{D1a}, Neu5Ac α2-3-Galβ1-3-GalNAc β1-4-(Neu5Ac α2-3)-Galβ1-4-Glc-Cer; G_{D1b}, Galβ1-3-GalNAc β1-4-(Neu5Ac α2-8-Neu5Ac α2-3)-Galβ1-4-Glc-Cer; G_{T1b}, Neu5Ac α2-3-Galβ1-3-GalNAc β1-4-(Neu5Ac α2-8-Neu5Ac α2-3)-Galβ1-4-Glc-Cer; LacCer, Galβ1-4-Glc-Cer; G_{D3}, (Neu5Ac/Neu5Gc) α2-8-(NeuAc/NeuGc) α2-3-Galβ1-4-Glc-Cer; *O*-acetyl G_{D3}, Neu5,9Ac2 α2-8-Neu5Ac α2-3-Galβ1-4-Glc-Cer; G_{M3}, Neu5Ac α2-3-Galβ1-4-Glc-Cer; G_{T3}, Neu5Ac α2-8-Neu5Ac α2-8-Neu5Ac α2-3-Galβ1-4-Glc-Cer.

Neu5Gc by the human body—this being a compound obtained only through the diet. Furthermore, a relationship has been demonstrated between anti-ganglioside antibodies (G_{M1}, G_{D1a}, Gal-NAcG_{D1} and G_{T1}) and Guillain–Barré syndrome [27,28] and Sandhoff–Jatzkewitz disease [29]. A correlation has also been seen to lead poisoning (G_{M1} and G_{M3} diminish, 9-O-Ac-G_{D3} increases, and G_{D3} remains stable) [30].

The present study reviews the analytical techniques, including the prior extraction and purification stages as critical steps to obtain valid results, for the identification and quantification of sialic acid and/or gangliosides in their different chemical structures, in dairy products and biological samples.

2. Sialic acid

Typically, the analysis of sialic acids starts with their release, followed by the purification of samples and determination by different analytical techniques.

Reviews of the methodology developed to isolate and release sialic acids from biological samples, as well as of the chromatographic, electromigration and hyphenated techniques available for their separation and analysis have been carried out by Lamari and Karamanos [9] and Karamanos et al. [31]. High-performance anion-exchange chromatography with pulsed amperometric detection has also been reviewed [32].

2.1. Sample preparation

2.1.1. Release of sialic acids

Two treatments for releasing the non-free form – enzymatic and acid hydrolysis – have been used.

Acid hydrolysis is the most widely used treatment. Mild acid conditions under heating (between 70 °C and 90 °C) with an oven, heater or block heater are used. Sulfuric acid diluted at concentrations of 25–100 mM is the most commonly used acid [33–38]. Others authors have used different solvents for hydrolysis: diluted HCl [32,39], microwave hydrolysis with acetic acid [9,40] that decreases the time of hydrolysis, sodium hydrosulfite [41], or trifluoroacetic acid [42,43].

A comparative study of different acid hydrolyses (25 mM HCl, 25 mM trifluoroacetic acid (TFA), 25 mM H_2SO_4 and 2 M acetic acid) was made for the determination of Neu5Ac and Neu5Gc in glycoconjugates [9]. Hydrolysis with HCl or TFA seems to be the best option, although 2 h of hydrolysis results in losses of about 20%.

Enzymatic hydrolysis secures the release of sialic acid moieties through the action of neuraminidases obtained from microorganisms (mainly *V. cholerae* and *Arthrobacter ureafaciens*) [44]. It is not easy to control the reaction cascade in biological samples, because release depends on the tissue structure, and on the origin of the enzyme—e.g., *V. cholerae* sialidase is preferentially used for mucins.

The enzymatic method is less aggressive than the hydrolytic approach, though release of all the non-free sialic acid content is not assured. In contrast, acidic hydrolysis releases all the non-free sialic acid, but may destroy part of it, and could take part in the rest of the process [9]. However, acid hydrolysis is the treatment most often used in both chromatographic and colorimetric techniques, due to the lesser cost involved and the greater reproducibility and ease of use.

2.1.2. Purification

Since biological samples and dairy products have a complex matrix, and sialic acids can form part of proteic, glucidic or lipidic fractions, determination cannot be carried out without a prior purification step.

The anionic exchange column is the most common option for the purification of samples, prior to the determination of sialic acid [9,32,37]. Other possibilities for purification are the use of solid phase extraction (SPE) such as SepPak C_{18} cartridges applied to biological samples [34] or ultracentrifugation in dairy products [45], generally after hydrolysis to eliminate protein residues. Another possibility is the combination of two systems using consecutive anion exchange column–ultracentrifugation applied to dairy products [37], or a CarboPac column followed by an anion exchange column applied to biological samples [32]. The anion exchange column is the most effective purification method, but it is also a tedious and costly method. As a result, it must be used when the sample is very complex, and the method moreover does not offer the best selectivity for the desired analyte. In contrast, the use of SPE cartridges is rapid and easy to carry out, but the effectiveness is high only in small quantities of interference—its use being recommended only for purified samples.

The conditions of release of sialic acids and of sample purification used by different authors are reported in Tables 1–3.

2.2. Sialic acid determination

2.2.1. Spectrophotometric methods

Spectrophotometric methods have been the techniques of choice for the determination of sialic acids, in view of their structural similarity to sugars. The inconveniences posed by such methods are their poor selectivity – it being impossible to perform determination without prior purification – and the impossibility of distinguishing between various forms of sialic acid. There are basically three colorimetric methods for the determination of sialic acids: the resorcinol method, thiobarbituric acid assaying, and enzymatic assay.

The most widely used spectrophotometric method is that developed by Svennerholm [46], and a later modification for its optimization, using anion exchange resins to improve purification [33]. In this method the reaction occurs between sugars and the resorcinol reagent. This method is developed for human tissues and biological fluids [33], although other studies apply it to dairy products such as bovine milk [47] and milk-based infant formulas [45].

The thiobarbituric acid (TBA) assay was developed and applied only for biological fluids by Warren [48]. In this method, sialic acid is measured by reaction with thiobarbituric acid, after being oxidized by periodate under acid conditions, via visible or fluorimetric detection. Using acid and basic conditions (borate in ethanolic medium), Neu5Ac has been determined in sialomucins—no differences having been observed between them [49].

The enzymatic assay is based on a reaction chain where sialic acid is first released by the action of neuraminidase, converted to pyruvate by N-acetylneuraminic acid aldolase, and finally obtained by pyruvate assay of hydrogen peroxide—which can be measured by spectrophotometry [44]. This method is distributed as a commercial kit. When the sample contains small amounts of sialic acid, use of the kit is recommended despite its higher cost, thanks to its greater rapidity and simplicity.

The three methods (resorcinol, TBA and enzymatic) were compared in biological fluids over a concentrations interval of 0–6.4 mM, no statistically significant differences being observed among them, with a limit of detection (LOD) of 0.12 mM, 0.20 mM and 0.06 mM sialic acid, a repeatability variation coefficient (CV) of 6.1%, 8.2% and 3.9%, respectively, and a reproducibility of less than 5% (4.5%, 3.1% and 4%, respectively) [50].

Table 1 shows the conditions of sialic acid determination by spectrophotometric methods.

Table 1
Sialic acids in biological samples or dairy products: determination by spectrophotometry.

Matrix	Analyte	Pre-treatment	Method conditions	Ref.
Biological tissues	Neu5Ac	(a) H_2SO_4 50 mM/80 °C/60 min Neutralization with NaOH 1M	(a) Periodate solution 10 mM/30 min/37 °C Arsenite solution (2%, v/v in 0.5N HCl)/3 min TBA solution/boiling water/7.5 min Cooled 0 °C/n-ButOH:12N HCl (95:5, v/v) 549 nm	[49]
		(b) H_2SO_4 50 mM/80 °C/60 min	(b) Boric acid 0.2M (pH 8.5)/100 °C/45 min/cooled EtOH/Ehrlich reagent (p-dimethylamino benzaldehyde in EtOH:HCl (1:1, v/v)/70 °C/20 min 558 nm	
		(a) H_2SO_4 0.1N or HCl 0.1N/80 °C/60 min (b) H_2SO_4 0.1N or HCl 0.1N/SDS 0.2% (w/v)/85 °C/60 min	Periodate solution 10 mM/30 min/37 °C Arsenite solution (2%, v/v in 0.5N HCl)/3 min TBA solution/boiling water/7.5 min Cooled 0 °C/n-ButOH:12N HCl (95:5, v/v) 549 nm	[93]
		H_2SO_4 50 mM/80 °C/60 min	Periodate solution 10 mM/0 °C/45 min Sodium thiosulfate 50 mM Ammonium acetate 4M (pH 7.5)/acetoanilide in EtOH 100 mM/10 min/λ_{exc}: 471 nm, λ_{em}: 388 nm	[92]
Biological fluids	TSA	Neuraminidase/N-Acetylneuraminic-aldolase/phosphate buffer 10 mM (pH 6.8)/45 °C/30 min	Pyruvate oxidase/phosphate buffer (pH 7.4)/FAD/peroxidase/4-aminoantipyrine (pH 7.0)/EDTA 50 mM 0.1M disodium phosphate/0.1M sodium citrate/Triton X-405 3 g/L (pH 9.0)/37 °C/15 min 550 nm	[44]
Biologial tissues and fluids	FSA TSA	TSA: H_2SO_4 0.05N/90 °C/60 min Dowex 2 × 8 (acetic form)	Resorcinol reagent (resorcinol dissolved in distilled water, HCl conc., copper sulphate 0.1M)/boiling water/15 min/cooled/amyl alcohol/shaking/0 °C/15 min/centrifugation/450 nm interferences/580 nm sialic acid	[33]
		TSA: H_2SO_4 0.1N/80 °C/60 min	Periodate solution (periodate 0.2M H_3PO_4 9M)/shaking/20 min/Arsenite solution (sodium arsenite 10%, w/v, sodium sulphate 0.05 M, H_2SO_4 0.1N)/shaking/TBA solution (TBA 0.6%, w/v, sodium sulphate 0.05M)/shaking/boiling water/15 min:	[48]
		Dowex 1 × 8 (acetic form)	Cyclohexanone (1:1, v/v)/centrifugation (3 min) 532 nm interferences/549 nm sialic acid	
		TSA: Neuraminidase/37 °C/180 min	Commercial kit: Tris reaction buffer (pH 7.5)/Neu5Ac aldolase/37 °C/10 min/NADH solution/340 nm blank Lactic dehydrogenase/37 °C/10 min/340 nm sialic acid	[50]
Milk and dairy products	FSA TSA	TSA: H_2SO_4 0.5N/80 °C/60 min	Periodic acid solution 0.04 M/0 °C/20 min/Resorcinol reagent/0 °C/5 min/100 °C/15 min/Tert-ButOH/37 °C/3 min/centrifugation 630 nm	[47]
Dairy products	FSA, TSA	TSA: H_2SO_4 0.12N/80 °C/60 min Dowex 2 × 8 (formic form) Lyophilization	Reconstitution in water/Resorcinol reagent/boiling water/15 min/amyl alcohol/0 °C/15 min/centrifugation 450 nm interferences/580 nm sialic acid	[45]

ButOH, butanol; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenin dinucleotide; FSA, free sialic acid; SDS, sodium dodecyl sulfate; TBA, thiobarbituric acid; TSA, total sialic acid.

Table 2
Sialic acid in biological samples or dairy products: determination by HPLC.

Matrix	Analyte	Pre-treatment	Derivatization Reagent/Conditions	HPLC Parameters	Ref.
Biological tissues	Neu5Gc	$NaHSO_4$ 0.5M/80 °C/20 min/cooled	OPD reagent (OPD 20 mg/mL in 0.25M $NaHSO_4$)/80 °C/40 min/cooled	Ultrasphere-ODS RP-C18 (250 × 4.6 mm, 5 μm)	[41]
	Neu5Gc			1-Butylamine:phosphoric acid:THF:water (0.15:0.5:1:98.8) (A): A:AcN (50:50) 87:13 1 mL/min. i.v. 20 μL, 230/425 nm	
		HCl or TFA 25 mM/80 °C/120 min	Benzoylation mixture (benzoic anhydride 10%. w/v, p-dimethylaminopyridine 5%, w/v)/80 °C/20 min/water/80 °C/10 min/Sep-Pack C_{18}	Supelcosil LC-18 (250 mm × 4.6 mm, 5 μm). Guard column Brownlee RP-C18 (5 μm). Water:AcN 33:67.1.5 mL/min i.v. 20 μL, 231 nm	[31]
		Lyophilization	Evaporation/AcN/centrifugation (10,000 × *g* 5 min)		
Biological fluids	Neu5Ac	H_2SO_4 0.05M/80 °C/60 min		LiChrosphere RP-C18 (250 mm × 4 mm, 10 μm). TIP_{aq} 60 mM (pH 3.5) 0.6 mL/min. i.v. 20 μL, 215 nm (Neu5Ac) 240 nm (Neu5Ac2en)	[34]
		Sep-Pack Plus C_{18}/HVLP 0.45 μm			
Biological fluids	Neu5Ac2en	HVLP 0.45 μm			
Biological fluids	Neu5AcNeu5Gc	TFA 2M/80 °C/120 min	Tos-Cl/60 °C/40 min/dryness/redissolution MetOH:water (1:8)/Sep-Pack C_{18}/evaporate/AcN	Supelcosil LC-18 (250 mm × 4.6 mm, 5 μm). Guard column	[43]
		Lyophilization/Dowex 50 × 8 (sulphite form)/lyophilization		Brownlee RP-C18 (5 μm). Water:AcN 85:15. 1 mL/min. i.v. 50 μL. 231 nm	
Biological tissues and biological fluids	Neu5AcNeu5Gc	H_2SO_4 0.025M/80 °C/60 min – 1 day	DMB solution 7 mM (DMB dihydrochloride in β-mercaptoethanol 1 M, sodium hydrosulphite 18 mM)/60 °C/150 min	Radial-Pak C18 (100 mm × 8 mm, 5 μm). MetOH:AcN:water 25:4:91. 1.2 mL/min, i.v. 10 μL, 373/448 nm	[52]
		CH_3COOH 2M/microwaves 200W/10 min	DMB solution 7 mM (DMB dihydrochloride in β-mercaptoethanol 0.75 M, sodium hydrosulphite 18 mM, acetic acid 1.4 M)/50 °C/150 min	Supelco RP-C18 (250 mm × 4.6 mm, 5 μm). Guard column Supelguard LC-18 (5 μm). Water (pH 3.0 with H_3PO_4):MetOH:AcN 86:6:8, 1 mL/min, i.v. 50 μL, 373/448 nm	[94]
Dairy products	Neu5Ac Neu5Gc	H_2SO_4 0.05M/80 °C/60 min Dowex 2 × 8 (formate form)/ultracentrifugation Microcon-10	DMB solution 8 mM (DMB dihydrochloride in β-mercaptoethanol 0.8M, sodium hydrosulphite 14 mM, acetic acid 1.5 M)/50 °C/150 min	LiChrosorb RP-C18 (250 mm × 4.6 mm, 5 μm). Guard column LiChrosorb RP-C18 (5 μm). MetOH:AcN:water 7:8:85 0.9 mL/min, i.v. 20 μL, 373/448 nm	[37]

AcN, acetonitrile; DMB, 1,2-diamino-4,5-methylenedioxybenzene; HVLP, high volume low pressure filters; i.v., injection volume; MetOH, methanol; Neu5Ac2en, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid; OPD, O-phenylenediamine; TFA, trifluoroacetic acid; THF, tetrahydrofurane; TIP, triisopropanolamine; Tos-Cl, p-toluenesulfonylchloride.

Table 3
Sialic acids in biological samples: determination by HPLC–MS.

Matrix	Analyte	Pre-treatment	HPLC Parameters	MS Conditions	Ref.
Biological tissue	Neu5Ac Neu5Gc	Membrane extraction by centrifugation (100,000 × *g* 4 °C 60 min) Acetic acid 4M/80 °C/180 min Sep-Pack C_{18} DMB (commercial kit conditions)	Magic C18 (50 mm × 0.1 mm, 3 μm). Formic acid:AcN:water 0.1:2:97.9: formic acid:AcN:water 0.1:80:19.9 Gradient elution: 0–0.1 min 90:10, 0.1–30 min 10:90, 0.75 μL/min.	NanoLC/FTMS: 200 °C. spray voltage 1800 eV. AGC 5×10^4 SIM: Neu5Ac-DMB m/z 426.150 Neu5Gc-DMB m/z 442.145	[53]
Biological fluids	Neu5Ac Neu5Gc	HCl 0.01M/80 °C/60 min, addition of IS (Neu5Ac methyl ester)	Ultra IBD column (150 mm × 4.6 mm, 5 μm) formic acid 0.1%.0.5 mL/min, i.v.10 μL	LC/ESI/MS: Positive ESI mode Carrier gas N_2 440 L/h, 180 °C, 19 V capillary voltage 3.5 kV SIM: Neu5Ac m/z 310, Neu5Ac methyl ester m/z 324, Neu5Gc m/z 326	[95]
Biological fluids	Neu5Ac	Addition of IS (KDN)	Guard column LiChrosphere amino propyl (20 mm × 2 mm, 5 μm). Aqueous ammonia 0.114 g/L:AcN. Gradient elution: 0–2 min 0:100, 2–2.5 min 90:10, 2.5–6 min 90:10, 6–6.1 min 0:100, 6.1–10 min 90:10. 0.3 mL/min i.v. 10 μL	LC/ESI/MS/MS: Negative ESI mode. Nebulizer gas N_2. Collision gas Ar (0.25 Pa) 80 °C. 20 V. Capillary voltage 3 kV MRM: Neu5Ac m/z 308.3 → m/z 86.9 KDN m/z 267.2 → m/z 86.9 Acquisition: 4–8 min HPLC. i.v. 30 μL	[96]
Biological fluids	FSA	Addition of IS ($^{13}C_3$-SA)	Atlantis dC18 (20 mm × 3 mm, 3 μm). Guard column Atlantis dC18 (100 mm × 3 mm, 3 μm).	LC/ESI/MS/MS: Negative ESI mode. Nebulizer gas N_2 100 L/h collision gas Ar (0.003 mbar). 300 °C 35 V. Capillary voltage 3.2 kV	[24]
	TSA	FSA: Filtration (0.20 μm) TSA: H_2SO_4 0.063M/80 °C/60 min	Ammonium formate 0.05M (pH 3.0): AcN. Gradient elution: 0–2 min 0:100, 2–2.5 min 0:100, 2.5–2.8 min 100:0, 2.8–6 min 100:0, 6.1–10 min 90:10. 0.3 mL/min, i.v. 10 μL	MRM: SA m/z 308.2 → m/z 87.0 $^{13}C_3$-SA m/z 311.2 → m/z 90.0. Acquisition: 2-3.7 min HPLC	

AcN, acetonitrile, AGC, automatic gain control; DMB, 1,2-diamino-4,5-methylenedioxybenzene; ESI, electrospray ionization; FSA, free sialic acid; FTSM, Fourier transformation ion cyclotron resonance mass spectrometry; i.v., injection volume; IS, internal standard; KDN, 2-keto-3-deoxy-D-glycero-D-galactonononic acid; MALDI, matrix assisted laser desorption ionization; MetOH, methanol; MRM, multiple reaction monitoring; SA, sialic acid; SIM, selected ion monitoring; TOF, time-of-flight; TFA, trifluoroacetic acid; TSA, total sialic acid.

2.2.2. Liquid chromatography

Liquid chromatography (LC) is the most widely used method for the determination of sialic acid. Such determination can be made with or without derivatization.

When the sample is not derivatized, the most common technique is the ionic pair method with ultraviolet (UV) detection. The ionic pair method has been described by Spyridiaki and Siskos [35], who optimized the technique for the determination of Neu5Ac, Neu5Gc, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (Neu5Ac2en), N-acetyl-9-O-acetylneuraminic acid (Neu5,9Ac_2) and cytidine 5′-monophospho-N-acetylneuraminic acid (CMP-NANA) in model dissolution. It performs assay using various ionic pair reagents of a lipophilic nature and positive charged, such as tetraoctylammonium bromide (TOA-Br), triisopropanolamine (TIP), or triethanolamine (TEA). The reagent of choice is TIP, due to its superior performance in relation to intensity and separation. In this case caution with the pH is required (optimum value 3.5), since it is a determinant factor. When this method is applied to serum, urine and saliva [34], only two forms of sialic acid can be distinguished (Neu5Ac and Neu5Ac2en), and a purification step is required to eliminate proteins.

Studies have been published on the determination of sialic acid linked to glycoproteins, glycoconjugates and glycolipids of human serum transferrin and cultured cells (Chinese hamster ovary (CHO) cells and fibroblasts) using high-performance liquid chromatography–pulse amperometric detection (HPLC–PAD) [32]. In this case, the method is able to differentiate Neu5Ac, Neu5Gc and KDN. Caution is required with the solvent used during hydrolysis – since it can damage the electrode response – introducing a quadrupole potential pulsed amperometric device for preservation of the electrode.

When determination is made after derivatization, the most commonly used alternative is fluorimetric detection, in view of its specificity and selectivity, though derivative compounds can also be formed which absorb in the UV range (in this case sensitivity is 10-fold lower) [41].

The first derivatization reagent evaluated for HPLC determination with fluorimetric detection was 1,2-diamino-4,6-dimethoxybenzene (DDB), for the determination of Neu5Ac and Neu5Gc in human serum and urine, with a detection limit of 12 pmol for both analytes [51]. The same authors improved the method by changing the fluorimetric reagent from DDB to 1,2-diamino-4,5-methylenedioxybenzene (DMB)—a compound with superior selectivity and fewer interferences [52]. This method allows the determination of Neu5Ac and Neu5Gc in human and animal sera linked to glycoproteins and glycolipids. In this way, methods have been developed for the determination of Neu5Ac and Neu5Gc in human apolipoprotein E, using an internal standard (N-propinylneuraminic acid, which is very hard to prepare) [36], and in infant formula [37].

The total amounts of Neu5Gc and Neu5Ac in human cells by nano liquid chromatography-Fourier transformation ion cyclotron mass spectrometry (nanoLC-FTMS) or nanoLC-MS/MS prior to derivatization with DMB were used [53]. In the latter case, the determination was validated for the two analytes at levels of 7.8–50,000 fmol, yielding a LOD level of fmoles in the FTMS method (8.6 fmol for Neu5Gc and 5.6 fmol for Neu5Ac), versus pmoles in the MS/MS method. This method has the advantage of its low LOD and the identification of both sialic acid species with the use of small amounts of solvents and reagents, versus the fluorimetric method.

Finally, there have been other attempts of determination involving different derivatization reagents such as p-toluenesulfonylchloride (Tos-Cl), benzoyl derivatives and O-phenylenediamine (OPD). Derivatization with OPD is briefer than with DMB, and the determination can be carried out by UV or fluorescence—the latter being chosen due to its superior sensitivity. It also can be used for the determination of mono-, di- and tri-acetylated sialic acids by increasing the derivatization time to 2 h [41].

The use of derivative methods is currently more often used due to their high selectivity and sensitivity versus the non-derivative methods. Furthermore, if MS methods are used, the results obtained are better, because additional information can be obtained on the sialic acid moiety—though the treatment is more complex. In contrast, derivatization methods pose the inconvenience of prolonged periods of derivatization and higher costs. Mention must be made of the fact that while many methods have been described for biological samples, few have been published for dairy products.

Tables 2 and 3 show the conditions of sialic acid determination by HPLC methods with or without derivatization.

2.2.3. Gas chromatography

Gas chromatography (GC) coupled MS originally has been used for the characterization of glycosidic linkages and O-substituted neuraminic acids after derivatization. Zanetta et al. [40] developed a very useful determination of sialic acid in standard solutions and biological fluids (horse serum and bovine, ovine and equine submandibular gland mucin) via GC/MS electron impact mode by methyl esterification using diazomethane in the presence of anhydrous methanol and the formation of volatile derivatives using heptafluorobutyric anhydride. This method is capable of distinguishing a large number of sialic acids, including O-acylated forms of Neu5Ac, Neu5Gc and KDN, 8-O-methylated and 8-O-sulfated derivatives and 1,7-sialic acid lactones. In a later study [54], the same group developed minor modifications of the method for the determination of monosaccharides, fatty acids and amino acid composition in human mucin and human uromodulin. In this method the sample remains in the same reaction vessel, and following the analysis of sialic acids by GC/MS, some acid-catalyzed methanolysis steps are added, followed by the formation of heptafluorobutyrate derivatives, which are measured by GC/MS under the same chromatographic conditions. This can be done due to the great stability of the derivative compounds formed with the heptafluorobutyric anhydride.

2.2.4. MALDI-TOF-MS

Sialic acid polymers using matrix assisted laser desorption ionisation-time-of-flight-MS (MALDI-TOF-MS), following acid hydrolysis with TFA, DMB as derivative reagent and on-target lactonization, were determined in purified capsular polysaccharide from *N. meningitidis NmC* and *E. coli K1,* in comparison with HPLC [42]. This technique determined the length of the polymeric chain and distinguished between α-2-8- and α-2-9-linked oligo- and polysialic acids (up to 40–50 sialic acid residues), due to their differing lactonization characteristics.

2.2.5. Amperometric methods

Recently, a method has been developed and discussed for the determination of sialic acid using an amperometric biosensor via the measurement of hydrogen peroxide [55]. This method has been optimized and applied to biological samples (human sera), with a LOD of 0.01 mM, reproducibility with RSD of 2.3%, and lineal response to 3.5 mM.

This method shows some advantages, such as simple preparation of the sample (direct if the objective is to measure free sialic acid), low cost and high selectivity and sensitivity—though with the disadvantage of only measuring small amounts of sialic acid (up to 3.5 mM).

2.2.6. Electromigration methods

This technique, and especially capillary zone electrophoresis (CZE), is one of the most widely used for the determination of the

structure and analysis of carbohydrates. Its application to sialic acid determination is therefore easy to understand.

In a recent study, Ortner and Buchberger [39] developed a simple, quick and reproducible method for the determination of Neu5Ac and Neu5Gc in human serum and standard glycoproteins by CZE-MS, using acetaminophen as internal standard. The combination of CZE and MS detection allows the quantitative analysis of these compounds without interferences from matrix compounds within a linear range (10–100 μg/ml), and with a LOD of 2 μg/ml.

3. Gangliosides

3.1. Extraction, isolation and purification of gangliosides

The first and most commonly used method for the extraction, isolation and purification of gangliosides is that described by Folch et al. [56] in animal tissues (brain). The first step is extraction of the total lipidic fraction using chloroform and methanol in different proportions. In a second step, gangliosides are isolated and purified from this fraction by a partition method with a salt in the aqueous phase. This basic procedure has received modifications over time, with the purpose of improving and adapting it to the target matrix.

Some authors [61] have developed methods for animal tissues and/or fluids, while others [45,62] have focused their research on dairy products and infant formulas. Table 4 presents the methods of extraction, isolation and purification of gangliosides in biological samples and dairy products.

The modification of Folch's method [56] developed by Suzuki [57] in brain and extraneural tissues—where gangliosides are isolated from the lipid extract through extractions with aqueous salt solution (NaCl or KCl), chloroform:methanol:aqueous salt (NaCl or KCl) and chloroform:methanol:water, and are purified by lyophilization followed by cold dialysis front distilled water—offers better ratios of isolation of total gangliosides, although the extraction of less polar gangliosides is not quantitative—this being an important fact if the matrix consists of extraneural tissue, where such gangliosides are abundant. A method for gangliosides extraction from brain, based on the use of tetrahydrofurane (THF) in a buffered medium (pH 6.8) has been developed [58]. This method offers greater gangliosides extraction, though with phospholipid contamination that is higher as well. On the other hand, the method described by Svennerholm and Fredman [59], based on Folch's technique [56], yields better brain ganglioside extraction than the use of THF, but is not superior to Suzuki's method [57]. Furthermore, the use of this method requires a large amount of sample, which in some cases may pose an inconvenience. Accordingly, this is the least useful method.

In biological fluids, where the total amount of gangliosides is very low (in the order of 10 times less than in biological tissue), or when only small amounts of sample are available, more effective elimination of interferences is required. In plasma, purification is proposed through the sequential use of partitioning in solvents, DEAE-Sephadex chromatography, base treatment and silicic acid chromatography—eliminating dipolar ions or uncharged molecules, sulfatides, free fatty acids and protein [60]. In addition, such purification is useful when evaluating the fatty acid composition of gangliosides. The method developed by Ladisch and Gillard [61] in biological tissue and fluids, incorporating the novel purification procedure involving partition of the total lipid extract in diisopropyl ether/1-butanol/aqueous NaCl, yields the same ganglioside recoveries as other aforementioned methods [58,59], with greater recoveries than those reported by Yu and Ledeen [60]. It is therefore the method of choice for samples with important interferences and a low ganglioside content.

As regards dairy products, due to their complex composition, high fat content and gangliosides located in the membrane surrounding fat globules, more complex lipid extraction is required (acetone/chloroform:methanol in different proportions) [45,62,63].

3.2. Determination of gangliosides

Total gangliosides could be quantified in isolated and purified fraction determining the sialic acids content (see Section 2.2)—the results being expressed as lipid bound sialic acid (LBSA) [45,63].

Regardless of whether the objective is the identification and/or quantification of individual gangliosides, a separation method is required. The methods described in the literature are separation by thin layer chromatography (TLC) or high-performance thin layer chromatography (HPTLC), and LC.

3.2.1. TLC/HPTLC

The TLC method is the classical technique, and is the most widely used option [64]. At present, HPTLC plates of silica 60 are used; they offer great resolution for the separation of gangliosides. Plastic and glass backed plates are used because staining is often done with resorcinol–HCl, and aluminum plates are unable to resist the acid conditions. For the application of samples, an amount of 5–10 μg of LBSA is recommended.

Regarding the developing solvents, there is a general mixture of $CHCl_3$:MetOH:0.2% $CaCl_2$ (55:45:5, v/v/v) that offers good reproducibility and is able to separate the most important gangliosides (G_{M3} and G_{D3}). It is even able to separate one same ganglioside based on its fatty acid and sphingoid base composition [65]. For example, in bovine milk, under these conditions, the bottom band of G_{D3} containing short chain fatty acids, and the upper band containing long chain fatty acids, are resolved in a double band. To differentiate between sialic acids (Neu5Ac and Neu5Gc) contained in the gangliosides, development with NH_4OH (1–5 M) in the aqueous phase could be done [62]. Once the TLC/HPTLC plate is developed, gangliosides are separated on it from the most polar to the least polar (beginning from the bottom).

3.2.1.1. Classical determination methods. Historically, the first reagent used for staining was orcinol, because it reacts with sugars bonded to lipids, yielding pink-violet stains; the inconvenience is that it is not a specific reaction for gangliosides [66]. Svennerholm [67] improved the detection by using resorcinol (see Section 2.2), which generates a violet-blue color after incubation for 15 min at 100 °C, and is the most specific and sensitive reagent used as a result of this feature. Neutral and sulfated gangliosides generate a yellow-brown color. Gangliosides could be identified by comparison running standard mixtures from bovine brain or other species or tissues [45]. Once the plate is stained with a color reagent, and with a view to quantification, optic densitometric measures could be made using a densitometer at 580 nm (Ando et al. [68] reported a linear relationship in the range of 0.09–10 nmol). This technique is reproducible, simple and rapid for ganglioside determination in animal tissues [69], and has even been used to determine gangliosides (G_{M1}, G_{M2}, G_{M3}, G_{D1a}, G_{D1b} and G_{T1b}) of cell lines [70] and G_{M3}, G_{D3} and G_{T3} contents in dairy products such as bovine milk [62] or infant formulas [45]. Another possibility is the quantification by computer-assisted image densitometry after two-dimensional HPTLC, where the image is acquired by an image analyzer over the resorcinol stained plate and processed by computer software, allowing the determination of G_{M3}, G_{M1a}, G_{D3} and G_{D1a} with a linear response ($r > 0.99$) between 0.01 nmol and 5 nmol. Optic densitometry yields more accurate and sensitive results than video image densitometry [71].

Table 4
Gangliosides in biological samples or dairy products: methods of extraction and isolation.

Matrix	Lipid extraction	Isolation/Purification	Ref.
Brain tissue	$CHCl_3$:MetOH 2:1/homogenization/filtration in fat-free paper or sintered glass funnel	Mixing thoroughly with water, or $CaCl_2$ 0.04%, or $MgCl_2$ 0.034%, or NaCl 0.58%, or KCl 0.74%/centrifugation Removing upper phase $CHCl_3$:MetOH:water, or salt solution 3:48:47 (×3)/$CHCl_3$:MetOH 2:1	[56]
	K_2HPO_4–KH_2PO_4 0.01M (pH 6.8)/THF (1:8)/centrifugation (12,000× *g* 15 °C 10 min)	Ethyl ether/centrifugation/water/centrifugation	[58]
	K_2HPO_4–KH_2PO_4 0.01M (pH 6.8):THF (1:4) (×3)	Combined aqueous phase, cold dialyzation front distilled water (2–3 days)	
	$CHCl_3$:MetOH:water (4:8:3) (×2)	MetOH/KCl 0.01M/low speed centrifugation (×2)	[59]
	$CHCl_3$:MetOH:water (4:8:5.6)	Cold dialyzation front distilled water (2–3 days)	
Brain and extraneural tissue	(a) Fresh tissue: $CHCl_3$:MetOH (2:1) mechanical homogenization (5 min) (b) Dried samples: water addition/0 °C/filtration in fat-free paper/$CHCl_3$:MetOH (1:2) with 5% water filtration in fat-free paper Redissolution: $CHCl_3$ until $CHCl_3$:MetOH (2:1) reached or dryness/$CHCl_3$:MetOH (2:1)	KCl 0.1M, or NaCl 0.1M/low speed centrifugation/set aside upper phase $CHCl_3$:MetOH:NaCl 0.58%, or KCl 0.74% (3:48:47)/vigorously mixing/centrifugation/set aside upper phase $CHCl_3$:MetOH:water (3:48:47)/centrifugation/set aside upper phase Combined upper phases reduced to half volume by rotary evaporation/lyophilization/water reconstitution/dialyzation front distilled water (1–2 days, 4 °C)	[57]
Animal tissue and fluids	(a) $CHCl_3$:MetOH (1:1)/evaporation/cooling (−20 °C)/centrifugation/dryness (N_2)	DIPE:1-ButOH (6:4)/vortexing/sonication/NaCl 50 mM/vortexing/sonication/centrifugation (750 g 10 min) (×2) Lyophilization aqueous phase	[61]
	(b) $CHCl_3$:MetOH:water (4:8:3) or THF:K_2HPO_4–KH_2PO_4 0.01M (pH 6.8) (1:4)/dryness/redissolution in $CHCl_3$:MetOH (1:1)/centrifugation/dryness (N_2)	Redissolution in distilled water/sonication/Sephadex G-50 Elution with deionised double distilled water (aliquot 206 nm)/lyophilization Redissolution in $CHCl_3$:MetOH (1:1)/sonication/centrifugation	
Bovine milk	Lyophilized sample/cold acetone (−20 °C) washing Extraction solid residue $CHCl_3$:MetOH (2:1, 1:2, 1:1)/dryness	Folch partition Redissolution water/dialyzation front distilled water (5 days, 4 °C) Lyophilization/redissolution $CHCl_3$:MetOH:water (60:30:4.5)	[62]
Infant formula	Redissolution $CHCl_3$:MetOH (2:1)		[45]

1-ButOH, 1-butanol; DIPE, diisopropyl ether; MetOH, methanol; THF, tetrahydrofuran.

Another spectrophotometric approach was developed using fluorescence [72]. Spraying hydrochloric acid (18%) over the developed TLC stains gangliosides (G_{M1}, G_{D1a}, G_{T1b}) with fluorescence under 365 nm UV light. Image analysis is used for quantification; the intensity of the bands is proportional to the concentration of gangliosides ($r = 0.997$) ranging from 0.047 nmol to 4.5 nmol.

A review [64] on HPTLC as an analytical and preparative technique for the analysis of gangliosides includes aspects such as continuous and multiple development, the overlay technique and combination with other techniques such as MS.

Because the polarity of a ganglioside is determined by its fatty acid composition and sphingoid base, and samples and standards may differ in their composition, the same ganglioside does not always run in the same way. The development of the so-called overlay techniques is also useful in identifying gangliosides. Since these may be receptors for viruses, bacteria and cells, their identification using TLC plates involves the use of antibodies, various toxins, lectins and other proteins, as well as related compounds. These techniques begin with pretreatment of the developed plate, plasticizing the plate with polyisobutylmethacrylate, and preventing possible flaking of silica gel in the forwards steps of incubation and wash. Then, the plate is overlayed with the primary agent (antibody, toxin, etc.), incubated, washed, overlayed with a secondary agent if necessary (e.g., secondary labeled antibody), washed again and dried for autoradiography on X-ray film or revealed with the adequate reagent (e.g., enzymatic assays) [73–75].

Methods have been developed using specific antibodies against some gangliosides (G_{M1}, G_{M2}, G_{M4}, G_{D1a}, G_{D3}, O-Acetyl-G_{D3}, G_{T1a}, G_{T1b}, polysialogangliosides). A variety of monoclonal antibodies recognize specific types of sialic acid and linkages, but typically only in the context of specific underlying sugar chains; in some cases, polyclonal antibodies have been generated that can recognize a specific type of sialic acid [24,76]. In some cases enzymatic modifications are required (e.g., neuraminidase) to eliminate steric hindrance of sialic acid prior to antibody assay [28].

It has been demonstrated that ganglioside G_{M1} links specifically to the cholera toxin [77]. For different gangliosides (G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}) present in neuro-2A neuroblastoma and PC12 pheochromocytoma cells, the method is adapted with prior *V. cholerae* neuraminidase treatment before the reaction occurs [28].

Viruses such as Influenza C for 9-O-acetyl-G_{D3} [78] or BK virus for G_{D1b}, G_{T1b} [79] and bacteria, e.g., *E. coli* for G_{M3} and G_{D3} [63], can act in a way similar to toxins. As a result, they also can act as specific reagents in view of their specificity.

Due to the nature of lectins, which are carbohydrate-binding proteins, they also can act as agents for the identification of gangliosides [80].

3.2.1.2. Mass spectroscopy determination. MS allows the identification and quantification of gangliosides from TLC/HPTLC plates. Although the sensitivity of this method is less than that of TLC/HPTLC-immunostaining, both can be used on a complementary basis for the determination of gangliosides in TLC—offering the advantage of generating structural information on the ceramide moiety, polysaccharide chains and fatty acids [81]. The advantage of TLC-fast atom bombardment (FAB)-MS in relation to FAB-MS combined with GC, LC or supercritical-fluid chromatography is the potential for direct comparison with the results from the overlay of biological reactions. TLC-FAB-MS may be highly informative and time-saving, although conditions are still needed for the desorption of larger molecules, to increase sensitivity, and to minimize contamination of the ion source by the matrix. It is especially useful for the identification of mixtures of products formed by chemical modifications of known receptor-active glycolipids, and most of the predicted products may be identified and compared with overlay results [82].

In the classical FAB-MS methods, glycosphingolipids must be scraped from TLC before analysis. Such methods have been applied to the determination of G_{M1} in model systems [83]. Karlsson et al. [82] demonstrated that glycolipids with identical carbohydrate sequences were well resolved into molecular species with differences in long-chain base and fatty acids by TLC/HPTLC coupled FAB-MS, separating sulfatides, G_{M3} and $GgOse_4Cer$ of human tissues.

However, analysis by FAB-MS can be made directly over TLC/HPTLC development with aluminum- or plastic-backed silica gel plates and nondestructive localization [64,84], where the plate is cut, added with a small volume of methanol and matrix liquid, and introduced into the probe tip.

The main inconvenience of MS in relation to gangliosides is the ease with which the molecule can be destroyed during ionization. MALDI-TOF has been adapted to make measures over the plate without scraping the silica. In this sense, ganglioside G_{M3} has been determined from CHO cells by IR-MALDI-orthogonal-TOF-MS. The plates are pretreated with glycerol to create a homogenous medium where ionization is not affected by the irregularities of the silica surface and gangliosides do not suffer rupture during the ionization process. In negative mode, they yield a very low degree of ganglioside fragmentation, great precision, and the main ions detected are the deprotonated species of gangliosides and some adducts with glycerol and NaCl. In positive mode, more remnants of sialic acids dissociated from the gangliosides are generated, though precision is also good. With this method, G_{M3} could be quantified (above 0.05 μg) by a calibration curve (from 0.05 μg to 5 μg) obtained from the intensity of the signal for fatty acid C16:0 $[M-H]^-$ divided by the intensity of the signal of the main matrix ion [G3Na], versus the concentration of G_{M3} [85].

The same technique has been used directly coupled to a immunostained/overlayed TLC [86] for tracing tumor-associated glycosphingolipids in hepatocellular and pancreatic cancer, detecting G_{M3}, G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}. The advantages are that the procedure works on a nanogram scale and with a LOD of less than 1 ng.

Another MALDI method avoiding the problem of fragmentation of gangliosides during the desorption phase has been developed. A first evaluation involved high pressure MALDI-FTIC-MS and a vibrational cooling system for thermal stabilization of labile molecules in general (small peptides and oxidized β-chain of insulin) [87]. This method was applied to the determination of G_{M1}, G_{D1a}, G_{T1b}, G_{Q1b} and $G_{P1b,1c}$ standard solutions [88]. Increasing the pressure in the MALDI source (1–10 mbar) during desorption, the authors were able to control fragmentation, avoiding even the sialic acid loss, and they added a cooling system for controlling adduct formation during ionization.

In posterior studies [89,90], the aforementioned methodology was applied to standards and mixtures of bovine brain gangliosides (G_{M1}, G_{M2}, G_{D2}, G_{D1a}, G_{D1b}, G_{T1b}, G_{D3}) directly coupling TLC with MALDI-FTIC-MS, resulting in great resolution and a detection limit of approximately 100 fmol.

3.2.2. Liquid chromatography–mass spectrometry

An LC/ESI-MS/MS technique was developed and validated for the quantitative determination of gangliosides G_{D3} and G_{M3} in bovine milk and infant formulas. The repeatability was less than 5% for G_{D3} and 14% for G_{M3}, and the recoveries ranged from 83% to 87% [91].

4. Conclusions

Spectrophotometric methods remain useful not only for quantifying sialic acids in biological samples, but also for total ganglioside determination—the results being expressed as lipid bound sialic

acid. Chromatographic techniques allow differentiation of the two principal sialic acid structures (Neu5Ac, Neu5Gc)—this being of interest not only for determining the origin of dairy products but also for detecting alterations in sialic acid metabolism in the clinical setting (early diagnosis of cancer).

Gangliosides determination in biological samples and dairy products requires long and tedious sample preparation (extraction, isolation and purification); this is the most critical step, since it is fundamentally responsible for the observed losses, and the elimination of all sources of interference (mainly phospholipids and ceramides) is difficult.

TLC/HPTLC remains the most important separating technique for the identification and/or quantification of individual gangliosides. Among the overlay techniques, immunostaining is the most useful for identifying gangliosides, while quantification is carried out by spectrophotometry and MALDI-TOF. The latter is the technique of choice, thanks to its specificity, greater sensitivity and capacity to generate structural information.

Future trends in ganglioside determination point to the use of mass spectrometry techniques for elucidating all the moieties that conform gangliosides, sugar chain, the fatty acids and sphingoid base, and which regulate their biological action. This is of interest for diagnostic purposes (diseases related to the accumulation or depletion of certain gangliosides—Guillain–Barré syndrome, Sandhoff–Jatzkewitz disease, lead poisoning, etc.).

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References

[1] G. Blix, A. Gottschalk, E. Klenk, Nature 179 (1957) 1088–11088.
[2] E. Klenk, Hoppe-Seyler's Z. Physiol. Chem. 268 (1941) 50–58.
[3] G. Blix, Hoppe-Seyler's Z. Physiol. Chem. 240 (1936) 43–54.
[4] A. Varky, in: R. Cummings, J. Esko, H. Freeze, G. Hart, J. Marth (Eds.), Essentials of Glycobiology, CSHL Press, New York, 1999, pp. 25–40.
[5] A. Reglero, I.G. Bravo, V. Fernandez-Martínez, Ann. R. Acad. Nac. Farm. 73 (2007) 833–871.
[6] F. Lehmann, E. Tiralongo, J. Tiralongo, Cell. Mol. Life Sci. 63 (2006) 1331–1354.
[7] B. Wang, J. Brand-Miller, P. McVeagh, P. Petocz, Am. J. Clin. Nutr. 74 (2001) 510–515.
[8] S. Inoue, A. Kanamori, K. Kitajima, Y. Inoue, Biochem. Biophys. Res. Commun. 153 (1988) 172–176.
[9] F.N. Lamari, N.K. Karamanos, J. Chromatogr. B 781 (2002) 3–19.
[10] L. Svennerholm, K. Bostrom, P. Fredman, J.E. Mansson, B. Rosengren, B.M. Rynmark, Biochim. Biophys. Acta 1005 (1989) 109–117.
[11] X.L. Pan, T. Izumi, Early Hum. Dev. 57 (2000) 25–31.
[12] J.A. Abad, A. Robotti, J. Neurochem. 103 (2007) 47–55.
[13] H. Rahmann, U. Balshüsemann, J. Neurochem. 66 (1996) 655–665.
[14] S. Sonnino, L. Mauri, V. Chigorno, A. Prinetti, Glycobiology 17 (2006) 1R–13R.
[15] A. Laegreid, A.B.K. Otnaess, J. Fuglesang, Ped. Res. 20 (1986) 416–421.
[16] R. Rueda, J.L. Sabatel, J. Maldonado, J.A. Molina-Font, A. Gil, J. Pediatr. 133 (1998) 90–94.
[17] S. Séverin, X. Wenshui, Crit. Rev. Food Sci. Nutr. 45 (2005) 645–656.
[18] Y. Hata, M. Murakami, S. Okabe, J. Physiol. Pharmacol. 55 (2004) 607–625.
[19] S. Usuki, K. Taguchi, A. Cawthraw, K. Shibata, T. Ariga, D.G. Newell, R.K. Yu, Exp. Neurol. 200 (2006) 50–55.
[20] F. Ebel, E. Schmitt, J. Peter-Katalinic, B. Kniep, P.F. Muehlradt, Biochemistry 31 (1992) 12190–12197.
[21] H. Yuasa, D.A. Scheinberg, A.N. Houghton, Tissue Antigens 36 (1990) 47–56.
[22] J.R. Ortaldo, A.T. Mason, D.L. Longo, M. Beckwith, S.P. Creekmore, D.W. McVicar, J. Leukoc. Biol. 60 (1996) 533–539.
[23] A. Gil, R. Rueda, Nutr. Res. Rev. 15 (2002) 263–292.
[24] N.M. Varki, A. Varki, Lab. Invest. 87 (2007) 851–857.
[25] J. Yin, A. Hashimoto, M. Izawa, K. Miyazaki, G.-Y. Chen, H. Takematsu, Y. Kozutsumi, A. Suzuki, K. Furuhata, F.-L. Cheng, C.-H. Lin, C. Sato, K. Kitajima, R. Kannagi, Cancer Res. 66 (2006) 2937–2945.
[26] G.N. Tzanakakis, A. Syrokou, I. Kanakis, N. Karamanos, Biomed. Chromatogr. 20 (2006) 434–439.
[27] H.J. Willison, J. Neurochem. 103 (2007) 143–149.
[28] G. Zhang, P.H.H. Lopez, C.Y. Li, N.R. Mehta, J.W. Griffin, R.L. Schnaar, K.A. Sheikh, Brain 127 (2004) 1085–1100.
[29] J.N. Thompson, A.C. Stoolmiller, R. Matalon, A. Dorfman, Science 181 (1973) 866–867.
[30] R. Pérez-Aguilar, S. Genta, S. Sánchez, J. Appl. Toxicol. 28 (2008) 122–131.
[31] N.K. Karamanos, B. Wikström, C.A. Antonopoulos, A. Hjerpe, J. Chromatogr. 503 (1990) 421–429.
[32] J.S. Rohrer, Anal. Biochem. 283 (2000) 3–9.
[33] L. Svennerholm, Acta Chem. Scand. 12 (1958) 547–554.
[34] P.A. Siskos, M.H.E. Spyridiaki, J. Chromatogr. B 724 (1999) 205–212.
[35] M.H.E. Spyridiaki, P.A. Siskos, J. Chromatogr. A 831 (1999) 179–189.
[36] M. Ito, K. Ikeda, Y. Suzuki, K. Tanaka, M. Saito, Anal. Biochem. 300 (2002) 260–266.
[37] M.J. Martín, E. Vázquez, R. Rueda, Anal. Bioanal. Chem. 387 (2007) 2943–2949.
[38] M. Van der Ham, B.H.C.M.T. Prinsen, J.G.M. Huijmans, N.G.G.M. Abeling, B. Dorland, R. Berger, T.J. deKoning, M.G.M.D.S.V. Velden, J. Chromatogr. B 848 (2007) 251–257.
[39] K. Ortner, W. Buchberger, Electrophoresis 29 (2008) 2233–2237.
[40] J.P. Zanetta, A. Pons, M.I. Wersen, C. Mariller, Y. Leroy, P. Timmerman, R. Schauer, Glycobiology 11 (2001) 663–676.
[41] K.R. Annumula, Anal. Biochem. 230 (1995) 24–30.
[42] S.P. Galuska, R. Geyer, M. Muhlenhoff, H. Geyer, Anal. Chem. 79 (2007) 7161–7169.
[43] E. Makatsori, N.K. Karamanos, E.D. Anastassiou, A. Hjerpe, T. Tsegenidis, J. Liq. Chromatogr. Relat. Technol. 21 (1998) 3031–3045.
[44] K. Sugahara, K. Sugimoto, O. Nomura, T. Usui, Clin. Chim. Acta 108 (1980) 493–498.
[45] A. Sánchez-Díaz, M.J. Ruano, F. Lorente, P. Hueso, J. Pediatr. Gastroenterol. Nutr. 24 (1997) 405–410.
[46] L. Svennerholm, Biochim. Biophys. Acta 24 (1957) 604–610.
[47] J.R. Nesser, M. Golliard, S. Del Vedovo, J. Dairy Sci. 74 (1991) 2860–2871.
[48] L. Warren, J. Biol. Chem. 234 (1959) 1971–1975.
[49] D. Aminoff, Biochem. J. 81 (1961) 384–392.
[50] M. Crook, M. Haq, P. Tutt, Clin. Biochem. 26 (1993) 449–454.
[51] S. Hara, M. Yamaguchi, M. Nakamura, Y. Ohkura, J. Chromatogr. 377 (1986) 111–119.
[52] S. Hara, Y. Takemori, M. Yamaguchi, M. Nakamura, Y. Ohkura, Anal. Biochem. 164 (1987) 138–145.
[53] N. Hashii, N. Kawasaki, Y. Nakajima, M. Toyoda, Y. Kagatiri, S. Itoh, J. Chromatogr. A 1160 (2007) 263–269.
[54] A. Pons, C. Richet, C. Robbe, A. Herrmann, P. Timmerman, G. Huet, Y. Leroy, I. Carlstedt, C. Capon, J.P. Zanetta, Biochemistry 42 (2003) 8342–8353.
[55] S.A.M. Marzouk, S.S. Ashraf, K.A. Al Tayyari, Anal. Chem. 79 (2007) 1668–1674.
[56] J. Folch, M.B. Lees, G.H. Sloane Stanley, J. Biol. Chem. 226 (1957) 497–509.
[57] K. Suzuki, J. Neurochem. 12 (1965) 629–638.
[58] G. Tettamanti, F. Bonali, S. Marchesini, V. Zambotti, Biochim. Biophys. Acta 296 (1973) 160–170.
[59] L. Svennerholm, P. Fredman, Biochim. Biophys. Acta 617 (1980) 97–109.
[60] R.K. Yu, R.W. Ledeen, J. Lipid Res. 13 (1972) 680–686.
[61] S. Ladisch, B. Gillard, Anal. Biochem. 146 (1985) 220–231.
[62] R. Puente, L.A. García-Pardo, P. Hueso, Biol. Chem. Hoppe-Seyler 373 (1992) 283–288.
[63] F. Sánchez-Juanes, J.M. Alonso, L. Zancada, P. Hueso, Biol. Chem. 390 (2009) 31–40.
[64] J. Müthing, J. Chromatogr. A 720 (1996) 3–25.
[65] A. Pörtner, J. Peter-Katalinic, H. Brade, F. Unland, H. Büntemeyer, J. Müthing, Biochemistry 32 (1993) 12685–12693.
[66] L. Svennerholm, J. Neurochem. 1 (1956) 42–53.
[67] L. Svennerholm, Biochim. Biophys. Acta 24 (1957) 604–611.
[68] S. Ando, N.C. Chang, R.K. Yu, Anal. Biochem. 89 (1978) 437–450.
[69] R.W. Ledeen, R.K. Yu, Methods Enzymol. 83 (1982) 139–191.
[70] L. Ferreira, E. Villar, I. Muñoz-Barroso, Int. J. Biochem. Cell Biol. 36 (2004) 2344–2356.
[71] M. Petrovic, M. K-Macan, D. Ivankovic, S. Matecic, J. AOAC Int. 83 (2000) 1457–1462.
[72] T. Hayakawa, M. Hirai, Anal. Chem 75 (2003) 6728–6731.
[73] J. Müthing, P.F. Mühlradt, Anal. Biochem. 173 (1988) 10–17.
[74] H. Yoshino, T. Ariga, N. Latov, T. Miyatake, Y. Kushi, T. Kasama, S. Handa, R.K. Yu, J. Neurochem. 61 (1993) 658–663.
[75] R.L. Schnaar, in: B. Ernst, G.W. Hart, P. Sinaÿ (Eds.), Carbohydrates in Chemistry and Biology, Part II: Biology of Saccharides, Wiley-VCH, Weinheim, Germany, 2000, pp. 1013–1027.
[76] I. Meisen, J. Peter-Katalinic, J. Müthing, Anal. Chem. 76 (2004) 2248–2255.
[77] K.A. Edwards, J.C. March, Anal. Biochem. 368 (2007) 39–48.
[78] C. Fahr, R. Schauer, J. Invest. Dermatol. 116 (2001) 254–260.
[79] J.A. Low, B. Magnuson, B. Tsai, M. Imperiale, J. Virol. 80 (2006) 1361–1366.
[80] H.J. Gabius, Biochem. Soc. Trans. 36 (2008) 1491–1496.
[81] J. Peter-Katalinic, Mass Spectrom. Rev. 13 (1994) 77–98.
[82] K.A. Karlsson, B. Lanne, W. Pimlott, S. Teneberg, Carbohydr. Res. 221 (1991) 49–61.
[83] P. Pahlsson, B. Nilsson, Anal. Biochem. 168 (1988) 115–120.
[84] Y. Kushi, S. Handa, H. Kambara, K. Shizukuishi, J. Biochem. 94 (1983) 1841–1850.
[85] K. Dreisewerd, J. Müthing, A. Rohlfing, I. Meisen, Z. Vukeli, J. Peter-Katalinic, F. Hillenkamp, S. Berkenkamp, Anal. Chem. 77 (2005) 4098–4107.

[86] U. Distler, M. Hülsewig, J. Souady, K. Dreisewerd, J. Haier, N. Senninger, A.W. Friedrich, H. Karch, F. Hillenkamp, S. Berkenkamp, J. Peter-Katalinic, J. Müthing, Anal. Chem. 80 (2008) 1835–1846.
[87] P.B. O'Connor, C.E. Costello, Rapid Commun. Mass Spectrom. 15 (2001) 1862–1868.
[88] P.B. O'Connor, E. Mirgorodskaya, C.E. Costello, J. Am. Soc. Mass Spectrom. 13 (2002) 402–407.
[89] V.B. Ivleva, Y.N. Elkin, B.A. Budnik, S.C. Moyer, P.B. O'Connor, C.E. Costello, Anal. Chem. 76 (2004) 6484–6491.
[90] V.B. Ivleva, L.M. Sapp, P.B. O'Connor, C.E. Costello, J. Am. Soc. Mass Spectrom. 16 (2005) 1552–1560.
[91] L.K. Sorensen, Rapid Commun. Mass Spectrom. 20 (2006) 3625–3633.
[92] K. Matsuno, S. Suzuki, Anal. Biochem. 375 (2008) 53–59.
[93] R.L. Matoo, S. Roseman, Anal. Biochem. 246 (1997) 30–33.
[94] A. Lagana, A. Marino, G. Fago, B.P. Martinez, Anal. Biochem. 215 (1993) 266–272.
[95] C.J. Shaw, H. Chao, B. Xiao, J. Chromatogr. A 913 (2001) 365–370.
[96] N.G.G. Valianpour, M. Abeling, M. Duran, J.G.M. Huijmans, W. Kulik, Clin. Chem. 50 (2004) 403–409.
[97] L. Svennerholm, J. Neurochem. 10 (1963) 613–623.