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PHD THESIS

**THE USE OF MASS SPECTROMETRY IN ANALYZING AND
DESCRIBING GLYCANS AND GLYCOCONJUGATES OF
BIOLOGICAL SIGNIFICANCE**

A B S T R A C T

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A B S T R A C T

The PhD thesis aligns with contemporary research directions in biochemistry, harmoniously combining the investigation of molecular mechanisms involved in carcinogenesis with the advanced use of mass spectrometry for the analysis and characterization of glycans and glycoconjugates of biological significance. This approach leverages both the capabilities offered by cutting-edge analytical techniques and advanced bioinformatics resources, contributing to the elucidation of the role of these biomolecules in oncogenetic processes. The work is briefly described in the following sections:

The **general section** covers the following topics:

1. General aspects of glycans and glycoconjugates
2. Structural characteristics, classification, and nomenclature of glycosphingolipids
3. Analysis of glycosphingolipids by MALDI mass spectrometry (Matrix-Assisted Laser Desorption/Ionization)

The **special section**, which includes the original contributions of the experimental work, is composed of two major chapters:

4. Analysis of the glycolipid profile in secondary brain tumors (introduction, materials and methods, results, discussions, and conclusions)
5. Analysis of glycans modified at the reducing end with the purpose of obtaining glycoconjugates using modern mass spectrometry techniques (introduction, experimental section, results, and conclusions).

The general section begins with an overview of glycans, a diverse class of biomolecules composed of sugar chains that are essential components of glycoproteins and glycolipids. These molecules play a crucial role in numerous biological processes due to the specific sequences of monosaccharides and glycosidic linkages they contain. The discussion also includes glycosphingolipids (GSLs), a subtype of glycolipids integrated into the plasma membrane and involved in essential biological functions, such as cell adhesion and cell signaling. Each component of GSLs plays a specific role in complex biological processes, influencing molecular recognition, cell differentiation, and apoptosis. Alterations in the structure or expression of GSLs are associated with various pathological conditions, including neurodegenerative diseases, cancer, and autoimmune disorders.

Mass spectrometry (MS) has become an essential tool for elucidating the structure and profiles of GSLs, although analyzing these complex molecules remains challenging. Advanced methods, such as MALDI-MS, offer significant advantages in investigating GSLs, contributing to our understanding of their roles in health and disease.

The general section further elaborates on the classification system of GSLs, the specific terminology adopted, and their structural characteristics as detailed in the literature. It also reviews the studies conducted so far using non-imaging MALDI-MS of GSLs, including the fragmentation map of GSLs, the influence of different matrices used in MALDI spectrometry on the results obtained, and the outcomes of MALDI-MS studies conducted on both healthy and pathological human tissues.

The special section is comprised by three chapters, the first two ones addressed for:

1. **Analysis of the glycolipid profile in secondary brain tumors.**

Introduction: Metastatic brain cancer occurs when a neoplasm spreads to the brain from other parts of the body, and it is far more common than primary brain tumors. The risk of brain metastasis varies depending on the type and grade of the primary tumor, with the most common sources being lung, breast, colon, kidney, and skin cancers. Notably, lung cancer is recognized for its aggressiveness and its propensity to metastasize early to the brain, which is associated with a poor prognosis.

Glycosphingolipids (GSLs), a subtype of sphingolipids, are ubiquitous components of cell membranes and play a crucial role in cell adhesion and signal transduction. They are implicated in various types of cancer, including lung cancer and brain neoplasms. Alterations in the expression and structure of GSLs are associated with tumor progression and the development of chemotherapy resistance. These changes involve modifications in cell surface glycosylation, which affects cellular recognition and signal transduction.

In recent decades, mass spectrometry (MS) has become a crucial tool for analyzing the structure of glycoconjugates, including GSLs. Advanced methods, such as MALDI-MS, allow for the detailed characterization of GSLs, providing essential information on their molecular masses, sequences, and branching patterns. This study presents the GSL profile obtained by MALDI-TOF MS from a metastatic brain tumor of pulmonary origin, contributing to the understanding of cancer development mechanisms and the advancement of targeted therapies.

Materials and Methods: In this study, we investigated a secondary brain tumor sample from a 66-year-old male patient diagnosed with poorly differentiated pulmonary adenocarcinoma (non-small cell lung cancer, NSCLC). The tumor sample, weighing 0.63 g, was obtained through surgical resection and subsequently underwent a complex extraction and purification process to isolate sphingoid glycosphingolipids (GSLs), the compounds of interest for this study.

In the first stage, the sample was precisely weighed and homogenized in a blender in the presence of ice to maintain a low temperature, resulting in a homogenate with approximately 10% dry substance content. The homogenate was then subjected to a two-step extraction using a solvent mixture (CHCl_3 : MeOH : H_2O = 1 : 2 : 0.75 by volume) at a

temperature below 5°C to extract lipid components. The upper phase, containing polar GSLs, was separated from the lower phase by the addition of methanol and water in a 1:1:1 volumetric ratio, followed by drying of the supernatant using a rotary evaporator (Speedvac SPD121P ThermoScientific).

To purify the crude GSL mixture, the dried sample was filtered through Sephadex G-25 gel and monitored by thin-layer chromatography (TLC) to identify the fraction of interest. The tubes containing the relevant fraction were combined, and the solvent was removed by nitrogen (N₂) purging, followed by final drying in a desiccator over P₂O₅, resulting in a crude GSL mixture with a final mass of 4.31 mg.

Preparation for mass spectrometry analysis (MALDI-TOF MS) involved dissolving this crude mixture in 2 ml of methanol with a purity of 99.8%, followed by vortexing and centrifugation at 2000 rpm for 20 minutes to sediment impurities. To optimize ionization and obtain a high-quality signal during analysis, multiple dilutions of the stock solution were performed, identifying that a 1:700 dilution provided the best quality spectrum. Thus, the final concentration of the methanolic GSL solution analyzed by MS was set at 2.45 pmol/μL.

Mass spectra were obtained using a Bruker Ultraflextreme MALDI-TOF spectrometer, operating in negative mode and equipped with a 355 nm Smartbeam II laser. Operating parameters included a laser frequency of 2000 Hz, an accelerating voltage of 20.07 kV, and a delayed extraction of 250 msec. Each spectrum was the result of accumulating 1000 laser pulses, and at least 10 individual spectra were combined to obtain a robust final spectrum. Spectra calibration was performed externally using a standard ganglioside mixture (ammonium salt, bovine brain), and data processing was carried out using FlexAnalysis software, version 3.3.

GSL samples were applied to polished metal supports (MTP 384, Bruker Daltonics) and coated with a 2,5-dihydroxybenzoic acid (2,5-DHB) matrix, prepared by dissolving it in a water/methanol/acetonitrile mixture (6:1:3 by volume). Optimal solubilization of the matrix was achieved with or without the addition of formic acid (HCOOH) or trifluoroacetic acid (TFA) at a concentration of 0.1%.

Through these rigorous methods, we successfully obtained and thoroughly characterized the GSL profile from the metastatic brain tumor sample, providing valuable insights into the molecular mechanisms involved in cancer and potential therapeutic targets.

Results: The study results highlighted the optimization of sphingoid glycosphingolipid (GSL) separation using thin-layer chromatography (TLC). Various stationary phases and solvent mixtures were tested to identify the optimal separation conditions, with the best results obtained using the elution system methanol: chloroform: water (40:50:10). The visualization of the fractions was achieved using a molybdate-ammonium and cerium sulfate-based reagent.

The mass spectrometry (MALDI-TOF MS) analysis of lipid mixtures from brain tumor tissue was optimized using a 2,5-DHB matrix without the addition of formic acid or trifluoroacetic acid, yielding the best spectra at a concentration of 20 mg/mL. The MS¹ spectra revealed the presence of multiple sphingolipid and complex glycolipid species, predominantly ceramides, hexosylceramides, and gangliosides.

In the study, the spectrometric analysis of sphingoid glycosphingolipid (GSL) mixtures isolated from metastatic brain tumor tissue revealed a range of complex ionic structures, each with important implications for understanding the tumor's molecular mechanisms. Among the ionic structures of interest discovered were simple ceramides, complex glycolipids, and gangliosides, all of which play significant biological roles.

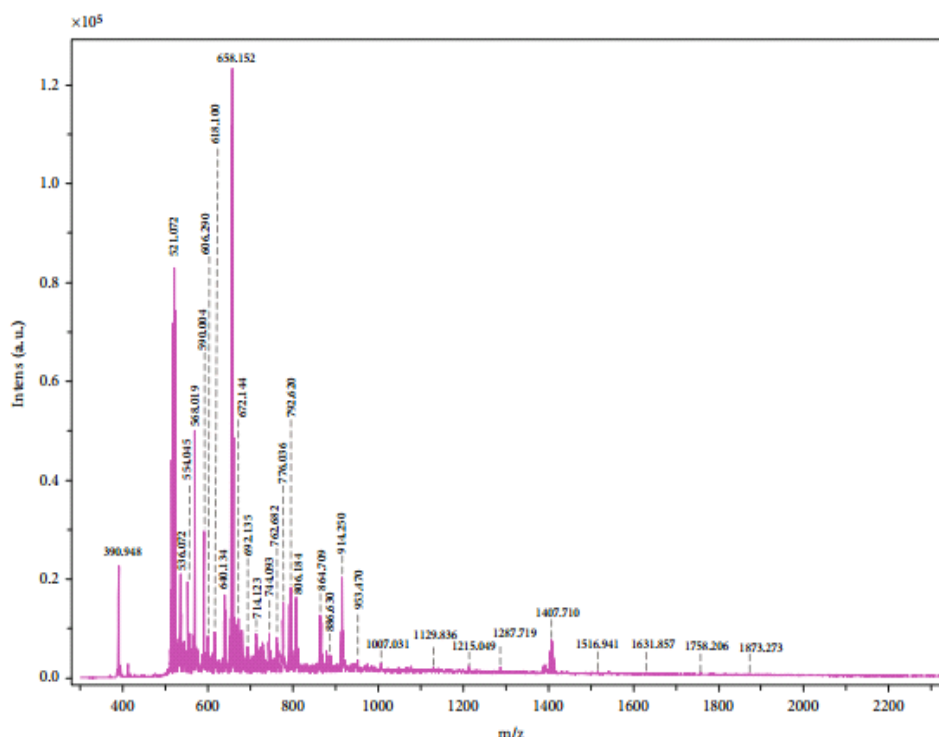


Figure 1: MALDI QTOF MS¹ of the native ganglioside mixture isolated from the secondary brain tumor. Ion source 1: 25.00 kV; ion source 2: 21.30 kV; lens: 10.50 kV; reflector: 26.30 kV; reflector 2: 13.85 kV; pulsed ion extraction: 50 ns. Matrix: 2,5-DHB. Solvent: MeOH.

Identified Structures are as follows:

a) **Simple Ceramides (Cer):** Simple ceramides are fundamental molecules in sphingolipid biosynthesis and were identified in mass spectra at various m/z values, including:

- **m/z 521.072:** This ion corresponds to a ceramide with sphingoid base (SPB) d18:1 and a fatty acid (FA) with 15 carbon atoms, suggesting a Cer 33:1; O₂ structure.
- **m/z 536.072:** Attributed to a ceramide with SPB d18:1 and FA C16:0 or d20:1/14:0, indicating a Cer 34:1; O₂ structure.
- **m/z 618.100:** Corresponding to a phosphorylated ceramide (CerP), with sphingoid base d18:0 and FA C16:0, suggesting the presence of an [M-H]⁻ ion indicative of a CerP 34:0; O₂ structure.

These ceramides are involved in various cellular processes such as apoptosis and cell proliferation. For example, phosphorylated ceramides (CerP) are known for their role in inhibiting apoptosis by blocking acid sphingomyelinase and activating the PI3K/AKT pathway, a crucial pathway for cell survival.

b) **Hexosylceramides (Hex-Cer):** Hexosylceramides are simple glycolipids with a sugar molecule attached to ceramide. In this study, several Hex-Cer were identified:

- **m/z 792.620:** This ion was attributed to a Hex-Cer with sphingoid base d18:1 and a fatty acid with 20 carbon atoms, indicating a Hex-Cer(d18:1/20:0) structure.
- **m/z 766.078:** Attributed to a sulfatide, a type of modified Hex-Cer with a sulfate group, suggesting a structure of HSO₃-3Gal β -Cer(d18:1/14:0).

Hexosylceramides play crucial roles in membrane structure and cellular recognition processes. Particularly, sulfatides are involved in important cellular interactions and have been associated with pathological processes, including cancer.

c) **Gangliosides:** Gangliosides are complex glycolipids containing one or more sialic acid residues. In this study, various gangliosides were identified, including:

- **m/z 1407.710:** This ion corresponds to a complex ganglioside structure of KDN-GM1, containing a rare deaminoneuraminic acid (KDN) residue linked to sphingoid base t16:1 and a fatty acid with 12 carbon atoms. These structures are unusual and may indicate specific oncogenic processes or peculiarities in glycolipid metabolism within tumor cells.
- **m/z 1467.243:** Attributed to GM2 ganglioside, containing SPB d18:1 and FA C24:0, a common structure in gangliosides associated with tumors.
- **m/z 1759.163:** This ion suggests the presence of a disialyl Lea structure, a ganglioside associated with tumor antigens, indicating possible implications in cellular recognition processes and metastasis.

The identified gangliosides are involved in cellular signaling, intercellular adhesion, and are often overexpressed in tumor cells, contributing to cancer invasion and metastasis. Additionally, the presence of rare gangliosides such as those containing KDN indicates potential alterations in metabolic pathways in the tumor context, which could open new avenues for biomarker research and targeted therapies.

d) **Phosphorylated and Complex Glycosylated Species:** The mass spectra also identified other complex species, such as:

- **m/z 728.120:** Attributed to sphingomyelin (SM), an important phosphorylated sphingolipid involved in maintaining cellular membrane integrity and signaling.
- **m/z 2286.826:** Attributed to a Lewis antigen (sLea-x) linked to a ceramide, suggesting the involvement of this GSL in metastasis, given that Lewis antigens are well-known markers in cellular adhesion and migration processes.

The identification of these complex ionic structures and their detailed mass spectrometric analysis provided a comprehensive view of the lipid composition in metastatic brain tissue. The identified profile, dominated by complex glycolipids, suggests specific modifications associated with oncogenic processes, including adaptations in glycolipid synthesis and modification that may influence cellular behavior, tumor invasion, and cellular interactions.

These findings underscore the importance of continued research on glycolipids and sphingolipids in cancer, both for understanding the molecular mechanisms involved in tumorigenesis and for developing new targeted therapies aimed at these critical molecules.

Discussion

The discussions in this study highlight the complexity of lipid composition in tumor cells, emphasizing significant differences from normal cells and variability among different types of tumors. A central aspect is the metabolic reprogramming of cancer cells, which leads to alterations in the length and degree of unsaturation of fatty acids (FAs). These changes affect membrane properties, such as fluidity and the stability of lipid domains ("lipid rafts"). Such modifications are associated with critical processes for tumorigenesis and metastasis, including cellular signaling and resistance to chemotherapy.

An important point of discussion is the increased concentration of cerebroside in the plasma membrane of tumor cells, which may function as a metastatic and anti-apoptotic signal, contributing to resistance to chemotherapy treatments. Additionally, the upregulation of GlcCer synthesis and the accumulation of these glycolipids can neutralize pro-apoptotic signals, promoting a multidrug-resistant phenotype.

Aberrant glycosylation, particularly the altered sialylation of glycolipids, is discussed as a distinctive marker of malignancy. Changes in the content and exposure of sialic acid on the surface of tumor cells are associated with metastasis, and sialylated Lewis antigens, such as sLea and sLeX, play a crucial role in the adhesion of cancer cells to vascular endothelium, facilitating metastasis. The increased expression of these antigens is associated with poor prognosis in various types of cancer.

The discussion also addresses the abnormal expression of rare sialic acids, such as deaminoneuraminic acid (KDN) and N-glycolylneuraminic acid (Neu5Gc), in tumor cells. These changes are linked to reduced survival in patients with non-small cell lung cancer (NSCLC) and the potential of these compounds as negative prognostic markers. It has been observed that Neu5Gc is incorporated from dietary sources into the cellular glycocalyx, and its presence is correlated with an unfavorable disease progression.

In conclusion, the study underscores the importance of profiling glycolipids, particularly sialylated ones, in understanding cerebral metastasis processes and suggests that glycolipids could serve as diagnostic and therapeutic targets in cancer. However, further research on larger samples is needed to validate these findings and fully explore the role of glycosphingolipids in tumorigenesis and metastasis.

The **second chapter** of the special section is addressed for:

2. Analysis of End-Reducing Modified Glycans for Obtaining Glycoconjugates Using Modern Mass Spectrometry Techniques

Introduction. Polysaccharides, or glycans, can be chemically modified at their primary and secondary hydroxyl groups, with a particular emphasis on the reactivity of their reducing end. This reactivity is leveraged to produce biocompatible materials used in medicine. However, studies focusing on the preparative-scale modification of reducing ends of glycans are relatively limited, and the characterization of these products via mass spectrometry has been inadequately illustrated.

In this context, modern analytical techniques such as MALDI-TOF and ESI-MS mass spectrometry have been employed to analyze modified glycans and their derivatives. For instance, the derivatization of glycans with 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) via reductive amination, followed by capillary electrophoresis (CE) and ESI-MS analysis, has enabled the detection of oligosaccharides up to a polymerization degree of 13. Additionally, MALDI-TOF techniques have been used for the characterization of complex glycolipids and polysaccharides, demonstrating significant improvements in signal detection through chemical derivatization.

Further studies have reported various methods for sugar derivatization, such as using the ethyl ester of 2-(diethylamino)benzoic acid or the formation of benzimidazole derivatives, which enhance sensitivity and accuracy in mass spectrometric analyses. These methods have been successfully applied to analyze maltooligosaccharides and glycolipids from natural sources, such as glucan from *Ganoderma lucidum*.

In conclusion, derivatization of the reducing ends of polysaccharides and the use of mass spectrometry for their analysis and characterization represent advanced and effective techniques. They offer significant contributions to the development of biocompatible materials and the understanding of glycan and glycoconjugate structures.

Experimental Section

The experimental part of this study focuses on the functionalization of a maltodextrin with a medium molecular weight (Paselli 6, Mw=2800) through a reductive amination reaction using 4,4'-diaminodiphenylmethane (DADFM). The objective of this section was to synthesize a macromolecular derivative that retains the functional characteristics of the initial components and is suitable for subsequent applications.

Reagents and Materials. All reagents used were of analytical grade and were sourced from reliable suppliers. Maltodextrin Paselli 6 was used as the primary substrate, and DADFM served as the amination agent. Solvents such as dimethylformamide (DMF) and

dimethyl sulfoxide (DMSO) were tested to ensure adequate solubility of the reactants and their compatibility during the reaction.

Equipment. Various laboratory equipment was utilized, including a centrifuge XC 2145 for phase separation during the reaction and a UV-VIS spectrophotometer Biochrom Libra S12 for spectroscopic analysis. Mass spectrometry was conducted using an LTQ Orbitrap Velos Pro™ mass spectrometer, which provided precise data on molecular mass and fragmentation ion structure, essential for the characterization of the final product.

Synthesis Procedure. The functionalization of maltodextrin was achieved through a reductive amination reaction, which involved the following steps:

Preparation of the Reaction Mixture: In a 25 ml flask equipped with an upward condenser and magnetic stirrer, 1.25 g of maltodextrin Paselli 6 and 1.24 g of finely ground DADFM were dissolved in 4 ml of water. The mixture was stirred at room temperature, and the pH was adjusted to 4 by adding glacial acetic acid.

Reductive Amination Reaction: The temperature of the mixture was gradually increased to 55°C, at which point the solution became clear. Sodium cyanoborohydride (NaBH₃CN) was added gradually over five days while maintaining the temperature at 55°C. Overnight, the reaction was left without heating but with continuous stirring. Upon completion of the addition of the reducing agent, the reaction was deemed complete, with a final pH between 5.5 and 6.5.

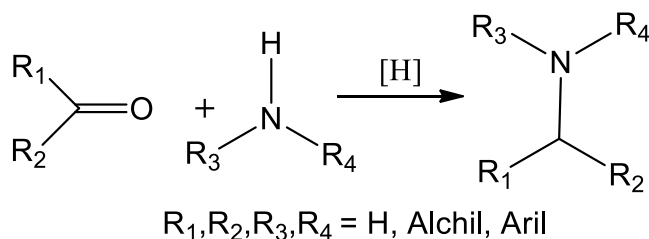


Figure 2: Reductive Amination Reaction

Purification of the Product: After completing the reaction, 15 ml of cold water was added to halt the reaction. The mixture was then transferred to a Berzelius beaker and treated with absolute ethanol to precipitate the product. The resulting precipitate was filtered under vacuum and suspended in acetone for 24 hours to remove impurities.

Quality Control: The obtained product was tested for unreacted DADFM using thin-layer chromatography (TLC) on silica gel plates, employing a solvent system composed of ethyl acetate (AcOEt) in a 1:2 ratio. If DADFM was detected, the product was resuspended in acetone, and the purification process was repeated.

Separation of the Final Product: The purified product, containing both functionalized polysaccharide and unreacted polysaccharide, underwent a separation process to isolate the functionalized dextran. It was dissolved in water and passed through a cation exchange column (Amberlite IR-120), using double deionized water and ammonia solution to elute the desired product.

Lyophilization and Storage: After dialysis, the solution was lyophilized to obtain a light beige powder, which was stored in a desiccator over P2O₅. This final product represents a polydisperse macromolecular compound exhibiting the specific characteristics of the reactants used in the reaction.

Analysis of the Final Product: To verify the success of the reductive amination reaction, the product was subjected to a comparative UV inspection with the initial reactants. UV-VIS spectra were recorded and compared to identify significant changes in characteristic absorbance, thereby confirming the functionalization of the polysaccharide. Mass spectrometry using an LTQ Orbitrap was employed to analyze the molecular structure of the product, providing detailed information about molecular masses and fragment sequences.

Experimental Conclusions: This detailed experimental procedure demonstrated the complexity of the functionalization process of medium- and high-molecular-weight polysaccharides. The success of the reaction largely depended on optimizing reaction conditions, including solvent choice, temperature control, and careful monitoring. The obtained products were characterized using a combination of advanced spectroscopy and mass spectrometry techniques, offering an in-depth understanding of the structure and properties of these compounds. These results are promising for the application of polysaccharide derivatives in various fields, including medicine and biotechnology.

Results and Discussions: The attachment of carbohydrate components to natural products is an essential biochemical process, manifested in proteins as a co- and post-translational event catalyzed by transferases. Other types of conjugations, such as those with lipids or terpenes, are also possible, resulting in bioconjugates with unique functions and cellular roles. Non-catalyzed attachment of sugar components is known as glycanation. Although this process is often described as glycosylation in the literature, the term is sometimes inaccurate, as it does not always imply the attachment of an actual glycosyl residue.

A common method for achieving this modification is reductive amination, a reaction where an aldehyde or ketone condenses with an amine in the presence of a reducing agent. The significance of this reaction is highlighted by its numerous applications in organic synthesis, biomolecule conjugation, and nanoparticle production. Reducing agents used in reductive amination include sodium or lithium cyanoborohydride, sodium borohydride, sodium triacetoxyborohydride, and other compounds from the borane class.

In our experiment, we successfully obtained DADFM-functionalized maltodextrin through reductive amination after testing various synthesis recipes and procedures to maximize yield. We demonstrated the binding of the aglycone to maltodextrin using thermogravimetric analysis (TG, DTG, DTA), and the results indicated significant differences between the reaction product and the physical mixture of reactants.

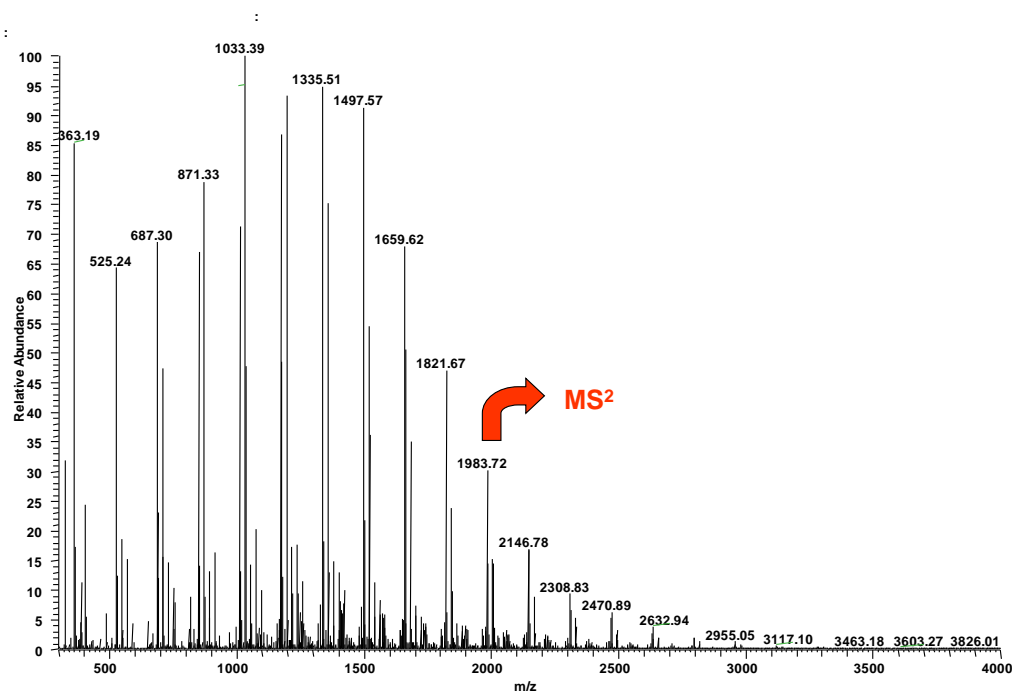


Figure 3. Analysis of Maltodextrin (Mw=2800 Da) Functionalized with DADFM (PARA) by (+) NanoESI Orbitrap MS; Solvent: MeOH. Sample Concentration: 1 ng/1 μ L; Source Voltage: 0.9 kV; Capillary Temperature: 250°C.

Subsequently, we analyzed the reaction product using mass spectrometry with an LTQ Orbitrap Velos ProTM spectrometer, obtaining detailed spectra (MS¹, MS², and MS³) that facilitated the sequencing and identification of the reaction-derived fragments. These analyses confirmed the successful attachment of the aglycone to maltodextrin, with no ring fragmentation observed, underscoring the success of the reductive amination process.

Conclusions: Based on the obtained results, the following conclusions can be drawn:

- The majority of the analyzed ions are doubly charged species (~60%). Predominant among these are disodiated, dipotasiated, monosodiated-monopotasiated, or monosodiated-monoprotonated or monopotasiated-monoprotonated species.
- The second significant group consists of singly charged ions (~18%). The most common species are monoprotonated and monosodiated. Less than 12% are represented by triply charged ions.
- In contrast to chip-based (+)-ESI-QTOF infusion using the nanoMATE robot or MALDI-TOF-MS, which favor multiply charged species, the nanoFLEX introduction method favors mono- and doubly charged ionic species.
- In addition to species containing the aglycone, approximately 40% of the ionic species correspond to the detachment of oligosaccharide fragments from the non-reducing end.
- About 60% of the detected ionic species correspond to mono- or multiply dehydrated fragments, which is explicable considering the desolvation transfer temperature (315°C).
- By ensuring a consistent signal and judiciously choosing operating parameters, we were able to isolate the singly charged ion at $m/z = 1983.71$, which was sequenced (leading to the MS² spectrum), gradually identifying the disaccharide corresponding to the fragment at $m/z = 525.24$. This fragment was isolated and fragmented via CID to produce the MS³ spectrum.

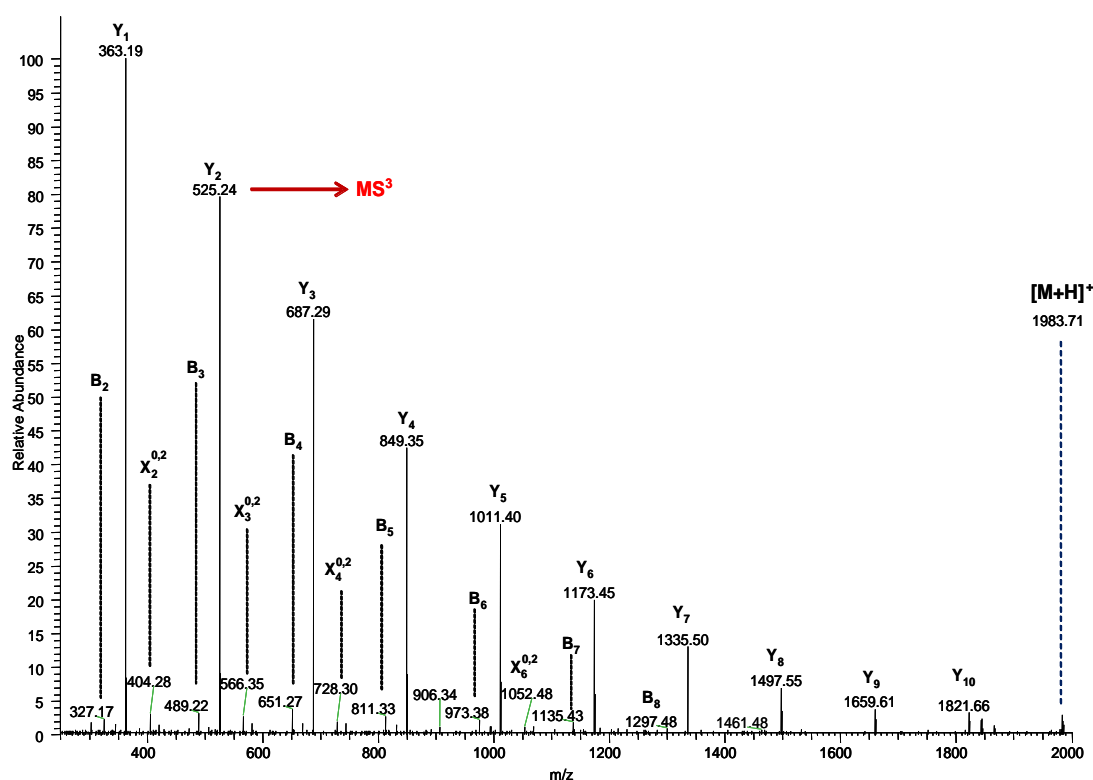


Figure 4: MS2 Spectrum of the $[M+H]^+$ ion at $m/z = 1983.71$ corresponding to $[PARA11+H]^+$ obtained through CID and multiple fragmentation (at a collision energy variable within the range of 30-70 eV); sample concentration: 1 ng/ μ L; source voltage: 0.9 kV; capillary temperature: 250°C; solvent: methanol (9:1); precursor ion isolation window: 2 m/z units; (fragment ion nomenclature follows Domon and Costello [28]).

Bibliography (Selected):

1. **Novaconi C.R**, Onulov R, Serb A.F, Sisu E, Dinca N, Pascariu M.C, Georgescu M, Assessing Glycosphingolipid Profiles in Human Health and Disease Using Non-Imaging MALDI Mass Spectrometry. *Appl.Sci.*, 2023; 13(7): 9922. <https://doi.org/10.3390/app13179922>
2. Serb A.F, Georgescu M, Onulov R, **Novaconi C.R**, Bolocan A, Sandu R.E, Mass-Spectrometry-Based Research of Cosmetic Ingredients. *Molecules*, 2024; 29(6): 1336. <https://doi.org/10.3390/molecules29061336>
3. Serb A.F, **Novaconi C.R**, Georgescu M, Puiu M, Dema A, Onulov R, Sisu E, Preliminary Analysis of the Glycolipid Profile in Secondary Brain Tumors, *Biomed Res. Int.* 2022, 1: 4293172. <https://doi.org/10.1155/2022/4293172>
4. **Novaconi CR**, Dema A, Georgescu M, Sisu E, Serb AF. Manuscript submitted for publication.