Rapid, sensitive analysis of sphingolipid variant profiles with simplified sample extraction

Using SelexION®+ Differential Mobility Separation Technology

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Plasma lysosphingolipids (LysoSLs) have recently emerged as potential biomarkers in a range of sphingolipidoses, including Gaucher disease, Krabbe disease, Fabry disease, Niemann-Pick types A/B disease (NPA/B), Sandhoff disease, Tay-Sachs disease and GM1 gangliosidosis.[1] LysoSL profiles are also of potential research importance as a biomarker of Niemann-Pick type C disease (NPC), where secondary storage of sphingolipids is believed to occur. Direct analysis of these groups can be complex due to extensive structural homogeneity between individual compounds. Thus, current methods of analysis of these lipids primarily involve either enzyme activity procedures or derivatization of compounds prior to analysis.

Differential mobility separation (DMS) using SelexION+ DMS Technology has the ability to separate stereoisomers through specific differential ion mobility characteristics in the presence of chemical modifiers and optimized compensation voltages (COV).[2] DMS also has the potential to eliminate the requirement for extensive sample extraction (e.g. SPE) through reduction of matrix interferences, thereby substantially improving the signal to noise ratio of the analytes in question. Figure 1 shows a research sample that was previously identified as positive for Krabbe disease, illustrating how DMS can distinguish between the structural isomers glucosylsphingosine (Glu-SPH) and galactosylsphingosine (Gal-SPH) without the need for specific, lengthy extractions or extended chromatography.

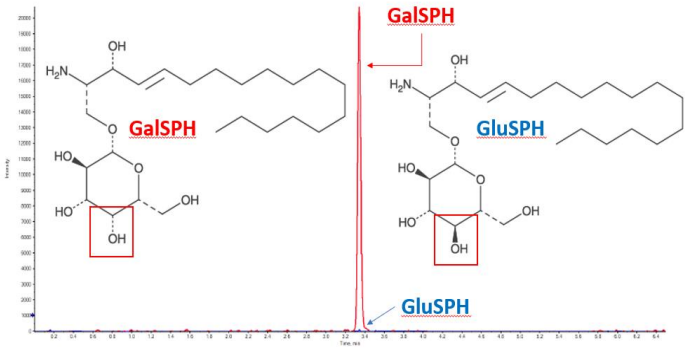

Figure 1. Resolution of Gal-SPH from Glu-SPH in a research sample previously identified as containing potential biomarkers for Krabbe disease.

A workflow has been developed employing simplified sample preparation combined with differential ion mobility and fast chromatography for the quantification of seven different lipids of interest in disease research. SelexION+ DMS Technology was key to providing definitive and specific separation and quantification, resolving the analytical challenges of previously developed workflows for these compounds.

Key features of differential mobility separation for the analysis of sphingolipid variants

- Separate isobaric compounds in the gas phase without the need for complex chromatography-based separations or extended runtimes
- Remove interferences from matrix allowing for use of streamlined, simplified extractions—avoiding lengthy incubations or derivatizations
- Exploit increases in signal to noise ratios to minimize sample volume needs and potentially allow the use of alternative matrices
- Employ simple optimization workflows as necessary when research dictates the need to expand compound coverage

Methods

Sample preparation: Calibrators and QCs were prepared by spiking known concentrations of analytes into 20 µL of human plasma. Samples were extracted by protein precipitation method using acetonitrile containing internal standards at working concentrations. After vortex mixing and centrifugation, the supernatant was separated and mixed with an equal volume of water, and 25 µL was injected on the LC-MS/MS system with DMS

UHPLC conditions: Chromatographic separation was achieved on a Phenomenex Kinetex C8 column (50 x 2.1 mm, 2.6µm). A gradient of water and acetonitrile (both containing formic acid) was used at a flow rate of 500 µL/min. The injection volume was set to 25 µL. The total run time for all compounds, including column equilibration time, was 6 minutes.

MS/MS conditions: A SCIEX QTRAP® 6500+ LC-MS/MS System, operated in low mass mode, was configured with the IonDrive™ Turbo V Ion Source and the SelexION+ DMS Device. Two MRM transitions per compound were analyzed. MRM transitions were optimized using individual standards and direct infusion. Source and gas conditions were optimized by flow injection of the lowest sensitivity compound (LysoGB3).

***Data acquisition and processing*:** Data was acquired using Analyst® Software 1.6.3 and processed using MultiQuant™ Software 3.0.3.

***Optimization of the DMS device*:** Individual standards at 10 ng/mL were used to optimize additional DMS parameters by t-infusion into an LC flow at analytical conditions.

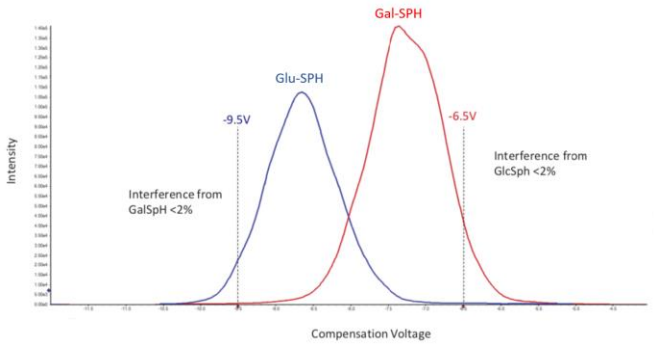

Figure 2. Optimization of compensation voltages (COV) for the two isobaric compounds. Through ramping the COV while infusing the two compounds, the full optimization profile can be observed. To achieve maximal separation, sometimes selecting COV settings not at the maximum but at the point of minimal signal of the competing compound is desirable.

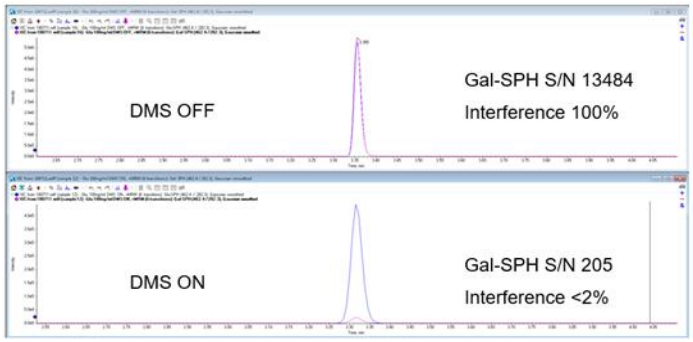

Figure 3. Comparison between "DMS off" and "DMS on" for a Gal-SPH extracted spiked sample. The Glu-SPH extracted spiked sample shows nearly complete isolation of Glu-SPH from the competing isobaric Gal-SPH lipid.

***Comparison of Glu-SPH and Gal-SPH performance with and without DMS*:** Pooled control plasma was spiked at high levels (100 nmol/L) individually with Glu-and Gal-SPH and extracted by simple protein precipitation as previously described. The separation and optimization of the two isobaric compounds, based on COV, is shown in Figure 2. Comparison between performances with and without the use of DMS is shown in Figures 3 and 4.

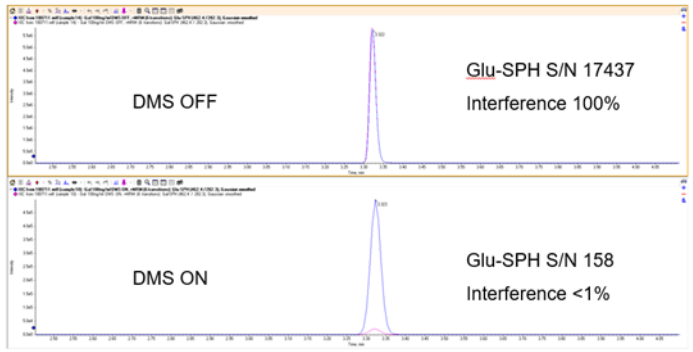

Figure 4. Comparison between "DMS off" and "DMS on" for a Gal-SPH extracted spiked sample. The Gal-SPH extracted spiked sample shows nearly complete isolation of Gal-SPH from the competing isobaric Glu-SPH

Simplified sample preparation

Existing sample preparation methodologies for the lipids analyzed are broadly categorized as either a solid phase extraction approach or as an approach that looks at the activity of the enzymes responsible for the creation or breakdown of the lipids in question. Both approaches have significant drawbacks. The SPE approach potentially requires significant laboratory preparation time and consumables, and the enzyme activity approach suffers from the need for lengthy incubations and longer sample turnaround time.

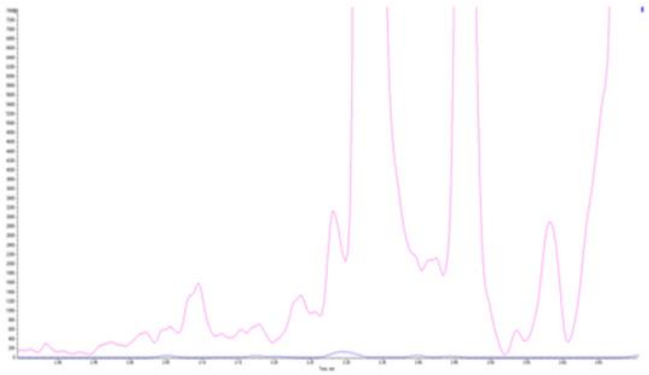

Figure 5. Significant selectivity improvement when using simplified sample preparation. Comparison of LC -MS/MS with DMS (blue) and LC-MS/MS (pink) for the same extract of a sample known to contain LysoSM (SPC). Sample preparation was a simple protein precipitation.

A simple protein precipitation using a small sample volume was proposed, to allow the assay to be run with higher throughput on more and varied sample matrix types, including those with significantly lower available sample volumes. Due to the added specificity of the SelexION DMS Device, matrix interferences inherent to a protein precipitation approach can be minimized. An example of a protein precipitation related matrix effect can be seen in Figure 5. Here the levels of the compound LysoSM (SPC) could have been overestimated by MRM and simple chromatography alone. The LC-MS/MS with DMS chromatogram, shown to a similar scale in blue, shows the peak without the matrix interference seen in the MRM trace in pink.

Analytical sensitivity

Due to the increase in signal-to-noise ratio, analytical sensitivity is maintained using SelexION DMS Technology. Figure 6 shows an extracted charcoal stripped serum spiked at 0.1 ng/mL (approximately 0.1-0.2 nmol/L for all compounds measured) and peaks are clearly seen with S/N values from 3 to 10.

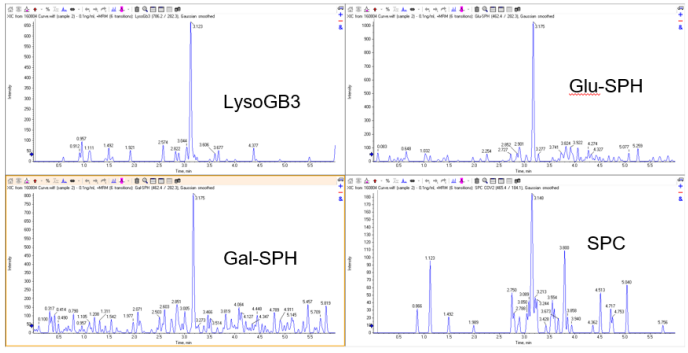

Figure 6. Good sensitivity achieved using DMS. Using charcoal stripped plasma, lipids were spiked in at 0.1 ng/mL. Four compounds are highlighted, showing clear detection with S/N > 3.

Analytical linearity

A series of extracts prepared in charcoal stripped serum were analyzed by the proposed methodology. Figure 7 shows the linearity for each compound analyzed over compound dependent ranges. Linearity (r^2) values ranged from 0.974 – 0.998.

Reproducibility

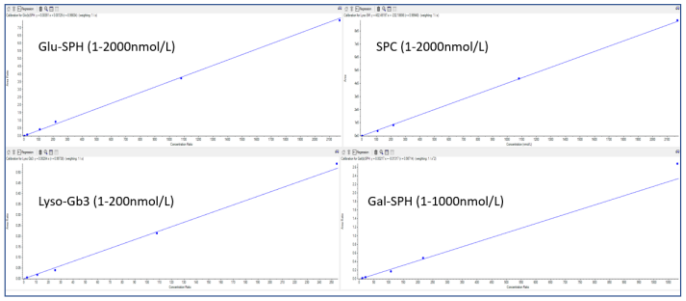

Figure 7. Analytical linearity in charcoal stripped serum. Example of linearity of compounds analyzed.

In order to determine the precision of this method, replicate QC samples were prepared from sample pools at known concentrations, extracted and quantified as described. The inter-day precision of all analytes across all concentrations was determined to be ≤18.2%. Results from accuracy and precision experiments are shown in Table 1.

Table 1. accuracy and precision values from pooled serum.

Compound	n	Mean (nmol/L)	% CV
LysoGB3	*5*	*105.9*	*18.2*
Glu-SPH	*5*	*5.5*	*8.0*
Gal-SPH	*5*	*37.2*	*9.6*
LysoSM (SPC)	*5*	*240.8*	*10.6*
LysoGM1	*7*	*49.6*	*7.7*

Grouped sample disease state stratification

Serum samples previously analyzed by established methods and identified as specific disease states were reanalyzed by the proposed method. Elevated levels of LysoSLs were detected in the following diseases: Lysosphingomyelin (LysoSM) in Neimann-Pick A/B; the carboxylated analogue of LysoSM (LysoSM509) in both NPA/B and NPC; LysoGM1 in GM1 gangliosidosis; LysoGM2 in both Tay-Sachs disease and Sandhoff disease, as shown in figure 8. LysoGb3 in Fabry disease; GluSPH in Gaucher disease; GalSPH in Krabbe, as shown in Figure 9.

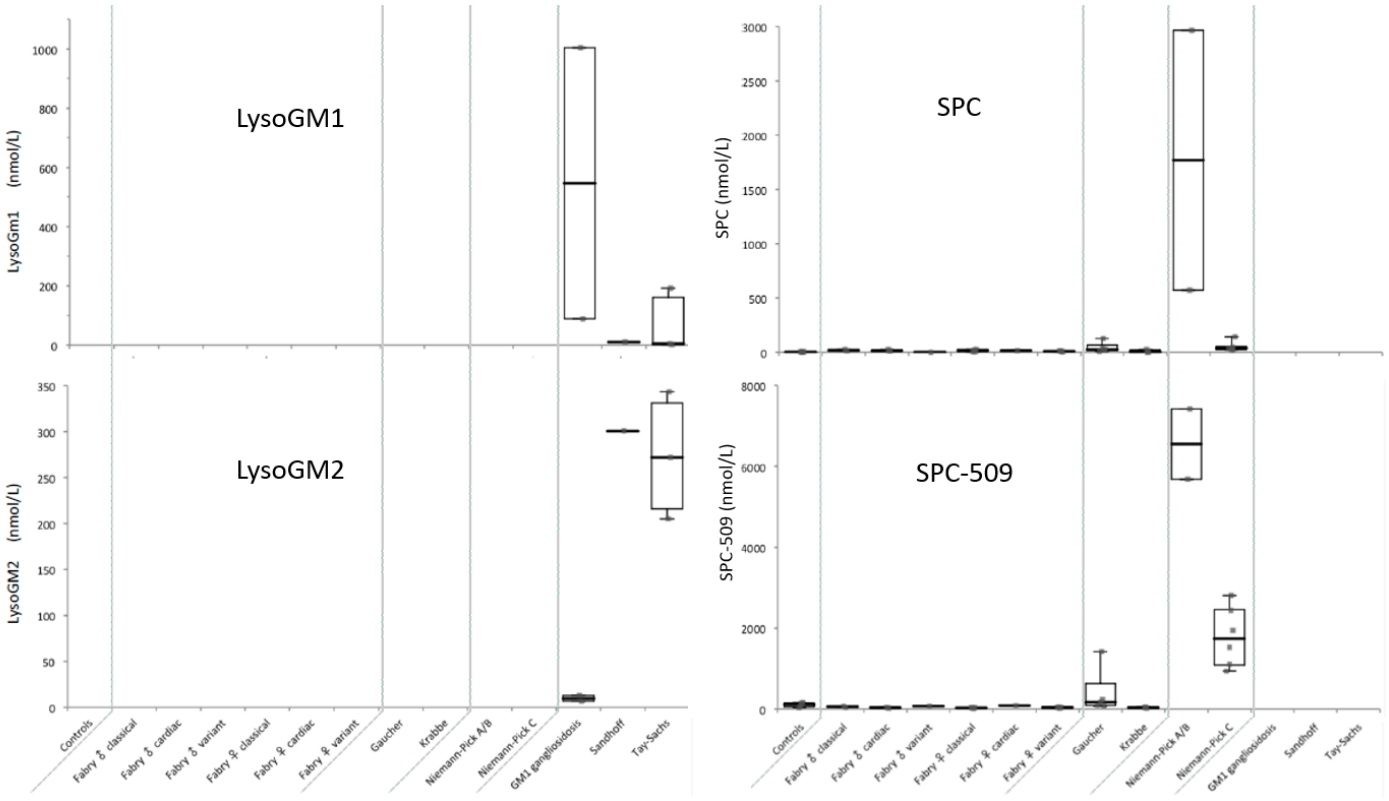

Figure 8. Lipid profiles observed across different disease states. Elevated levels of LysoGM1, LysoGM 2, LysoSM (SPC) and LysoSM509 could clearly be seen in specific disease samples.

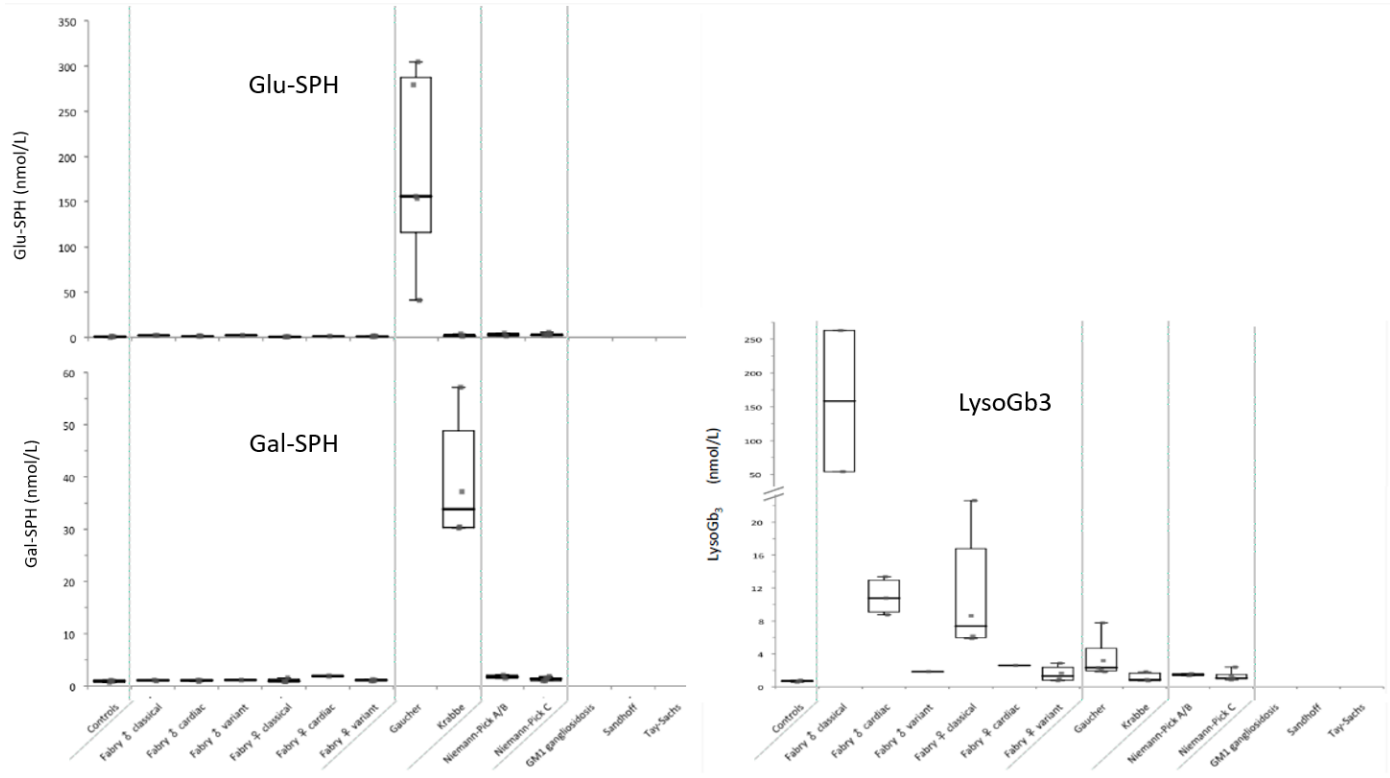

Figure 9. Lipid profiles observed across different disease states. Elevated levels of Gal-SPH, Glu-SPH and LysoGb3 could clearly be seen in specific disease samples.

Where comparisons were possible, the results indicating these lipids as potential biomarkers agreed with previously published results.[1]

Conclusions

This improved methodology for plasma sphingolipid analysis using SelexION DMS Technology has a number of advantages over previous methods, including:

- Simplified sample preparation procedure
- Removal of lengthy incubation steps
- Separation of structural isomers
- Reduced chromatographic runtimes
- Reduced sample volume

The proposed methodology also shows initial promise in areas where research into alternative methodologies is currently active, such as where sample volume is limited or more invasive methodologies (e.g. skin biopsy) are considered.

References

1. Pettazzoni M. *et al.* (2017) LC-MS/MS multiplex analysis of lysosphingolipids in plasma and amniotic fluid: A novel tool for the screening of sphingolipidoses and Niemann-Pick type C disease. *PLoS ONE* **12(7)**, 1-29.
2. SelexION® Technology: The solution to selectivity challenges in quantitative analysis. SCIEX technical note RUO-MKT-02-3251-C.

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