# Sphingosine and dihydrosphingosine as biomarkers for multiple sclerosis identified by metabolomic profiling using coupled UPLC-MS



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|---|---|
| Journal: | *Analytical Methods* |
| Manuscript ID | AY-ART-08-2017-001922.R1 |
| Article Type: | Paper |
| Date Submitted by the Author: | n/a |
| Complete List of Authors: | Ward, Sean; University of Huddersfield, Department of Chemical and Biological Sciences<br>Page, Michael; University of Huddersfield, Department of Chemical and Biological Sciences<br>McHugh, Patrick; University of Huddersfield, IPOS, Applied Sciences<br>Powles, Nicholas; University of Huddersfield, IPOS, Applied Sciences |
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ROYAL SOCIETY OF CHEMISTRY

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**Sean Ward, Michael I. Page and Nicholas T. Powles**

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## Supplementary Information

# Biomarkers for multiple sclerosis determined by metabolomic profiling using coupled UHPLC-MS

Sean Ward, Michael I. Page and Nicholas T. Powles

**Figure 1** PCA plot of raw data in MPP for plasma analysis of multiple sclerosis (blue), neuropathic pain (grey), multiple sclerosis and neuropathic pain (brown), control group (red) and QC injections (green). The principle component is plotted on the X-axis and represents 17% of data variation. Component 2 on the Y-axis represents 11% of the variation and component 3 on the Z-axis 6% of the variation.

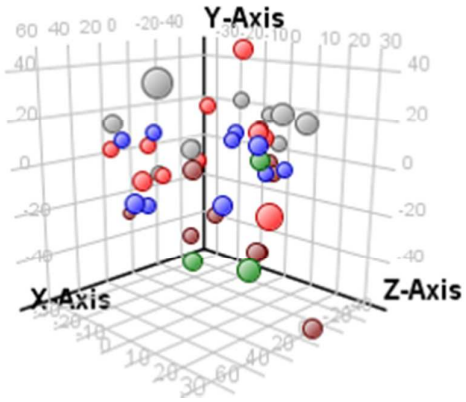


**Figure 2** PCA plot of re-processed data in MPP for plasma analysis of multiple sclerosis (blue), neuropathic pain (grey), multiple sclerosis and neuropathic pain (brown), control group (red) and QC injections (green). The principle component is plotted on the X-axis and represents 27% of data variation. Component 2 on the Y-axis represents 13% of the variation and component 3 on the Z-axis 7% of the variation.

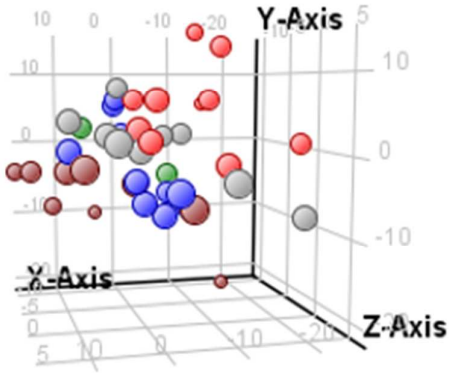

**Figure 3** An example of a missed integration in 7/10 samples in Profinder of compound m/z 805.0323.

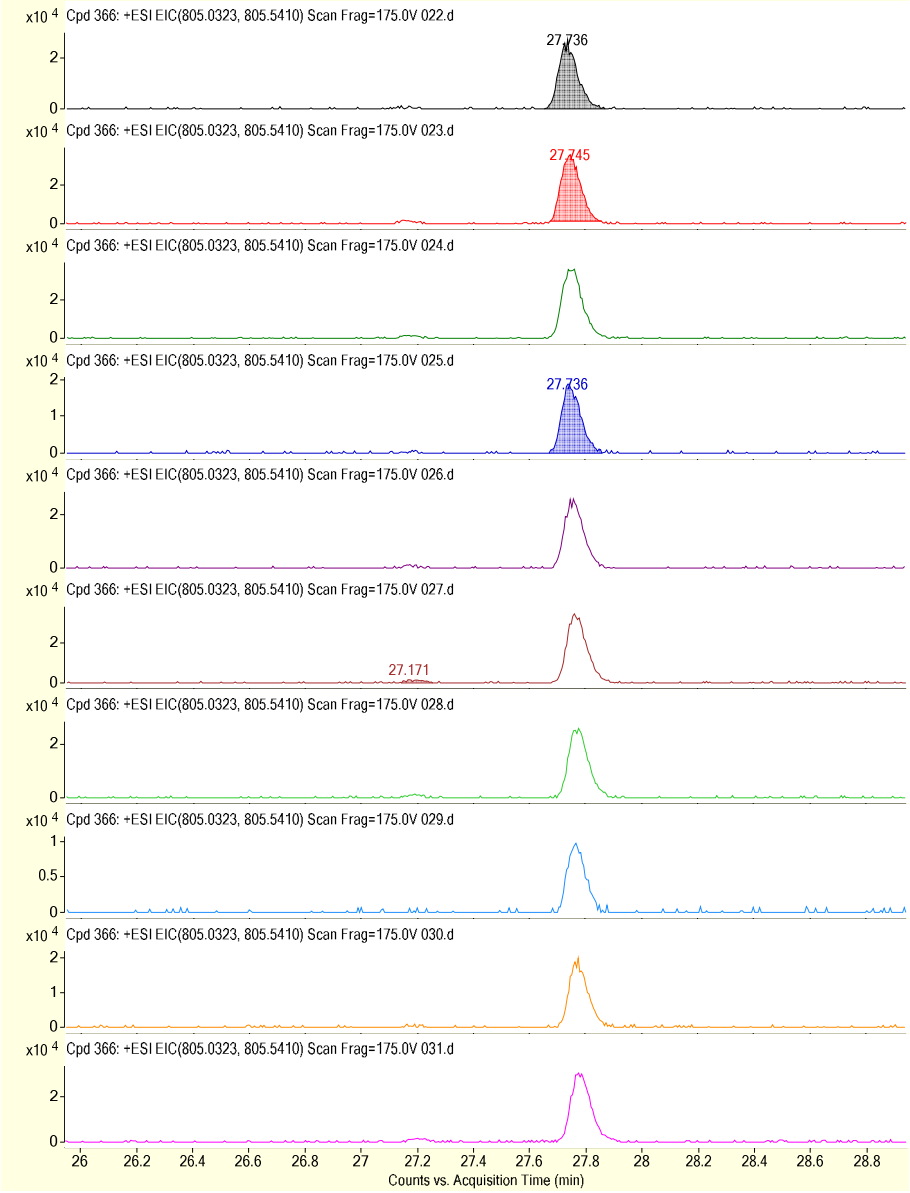

**Figure 4** EIC of m/z 300.2892 in control group sample showing the presence of two compounds with that m/z.

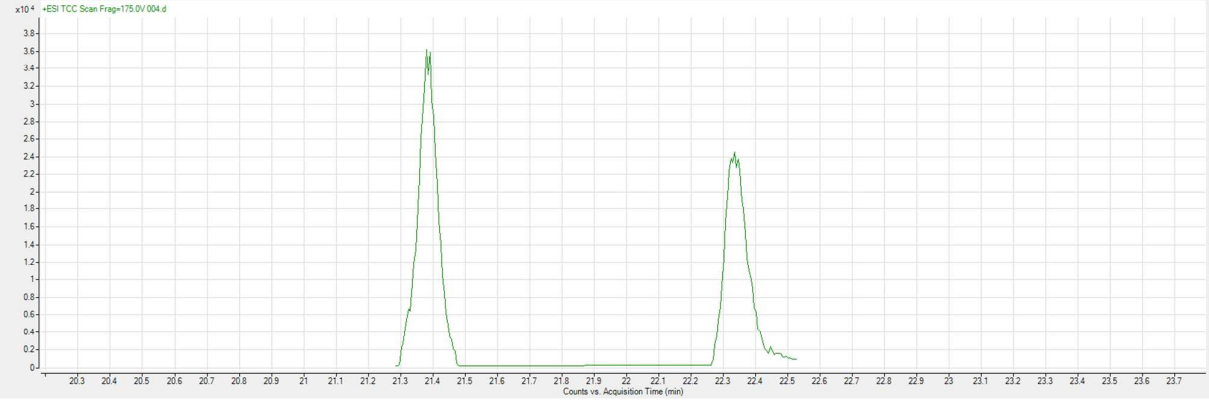


**Figure 5.** Isotope fit for formula $C_{18}H_{37}NO_2$ eluting at 21.3min in control group sample

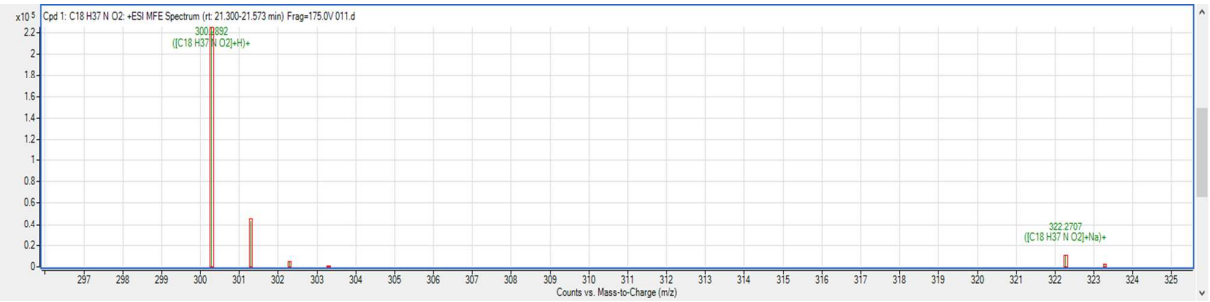

**Figure 6.** EIC's of m/z 300.2892 in control sample and sphingosine spiked control sample showing peak alignment for peak eluting at 21.4min.

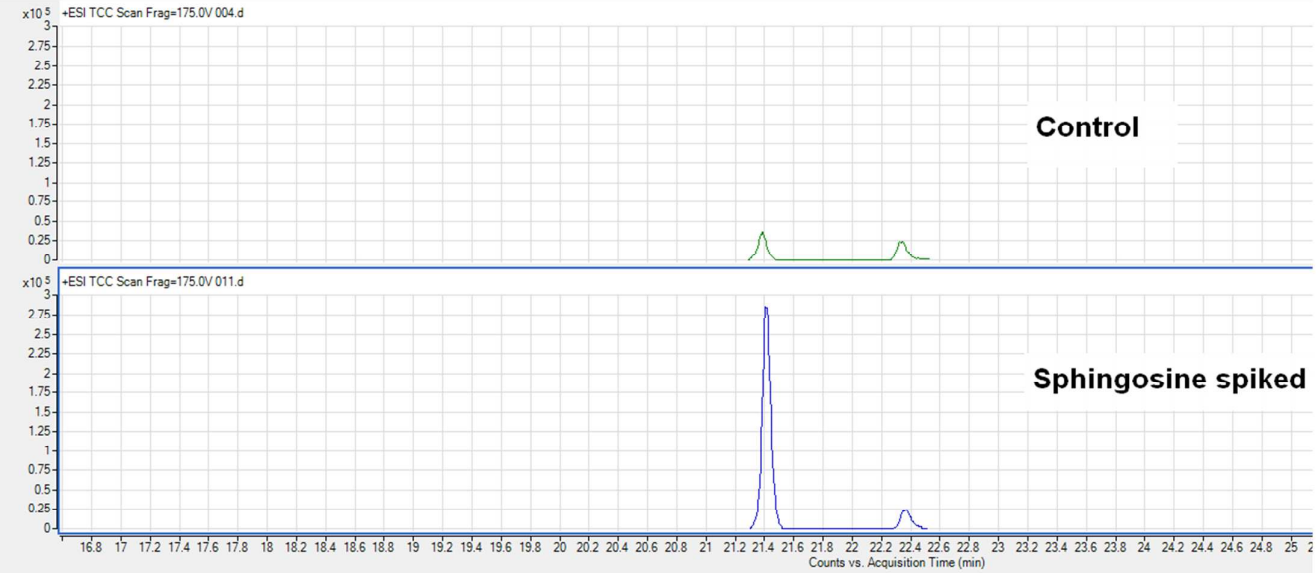

**Figure 7.** Fragmentation pattern for m/z 300.2892 in control group sample.

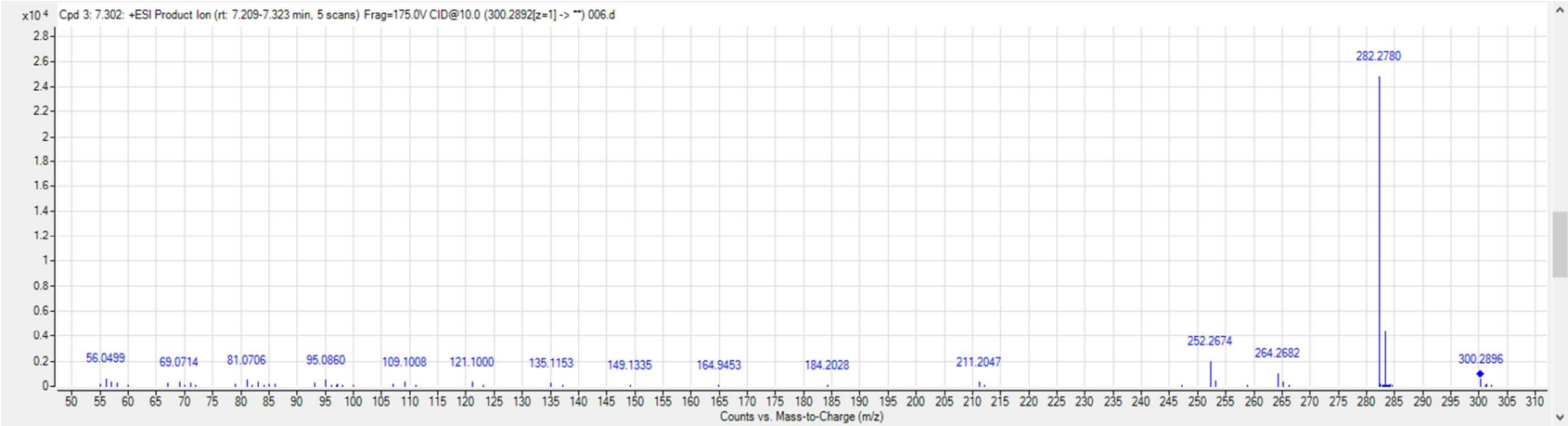


**Figure 8.** Fragmentation pattern for m/z 300.2892 in sphingosine standard.

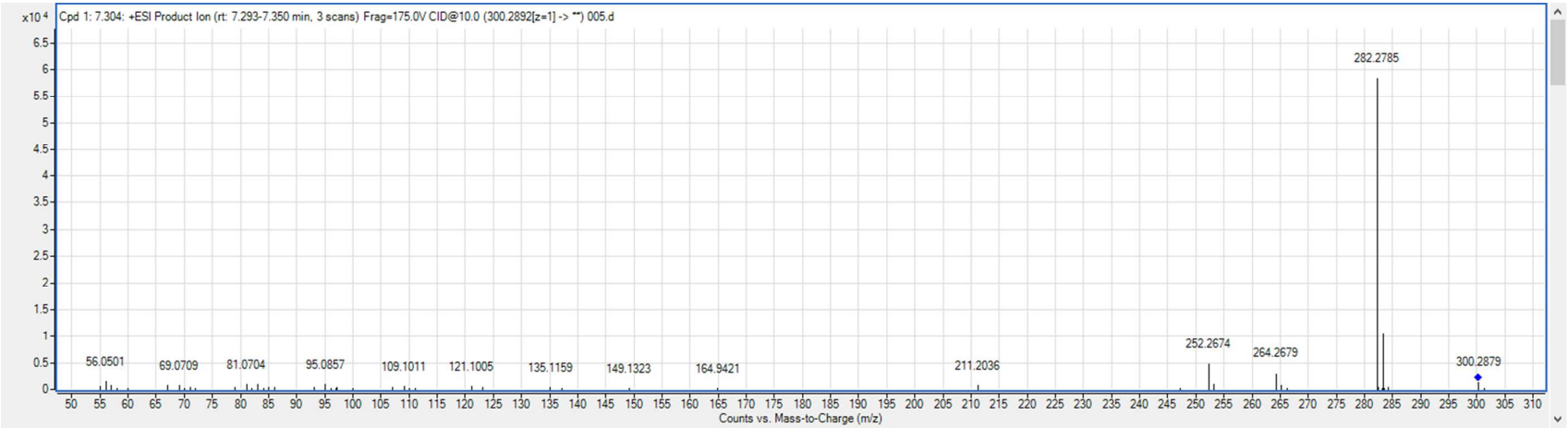

**Figure 9** Abundance of sphingosine in the multiple sclerosis, neuropathic pain, multiple sclerosis with neuropathic pain and control group.

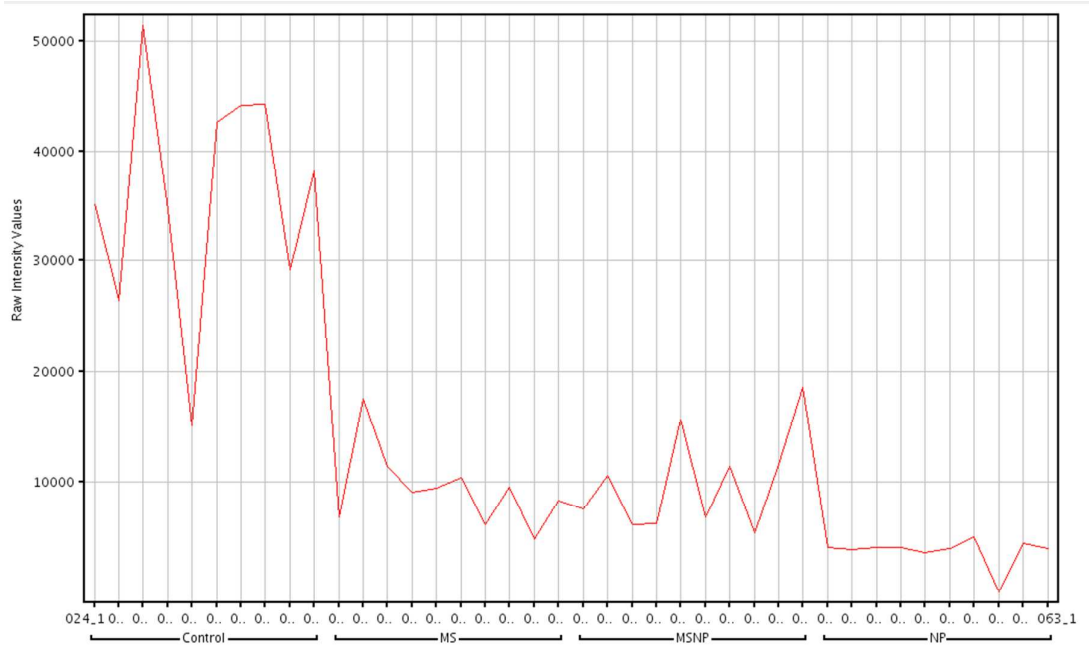

**Figure 10.** EIC's of m/z 302.30536 in dihydrosphingosine, control sample and spiked control sample.

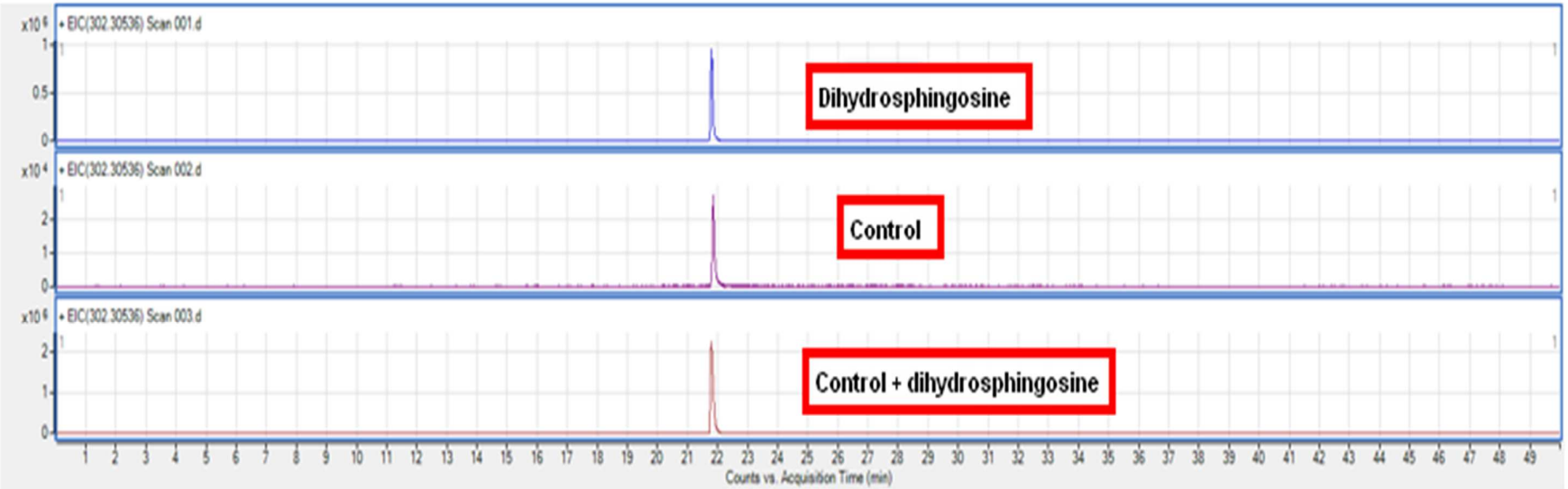

**Figure 11.** Fragmentation pattern for m/z 302.30536 in control and dihydrosphingosine standard sample.

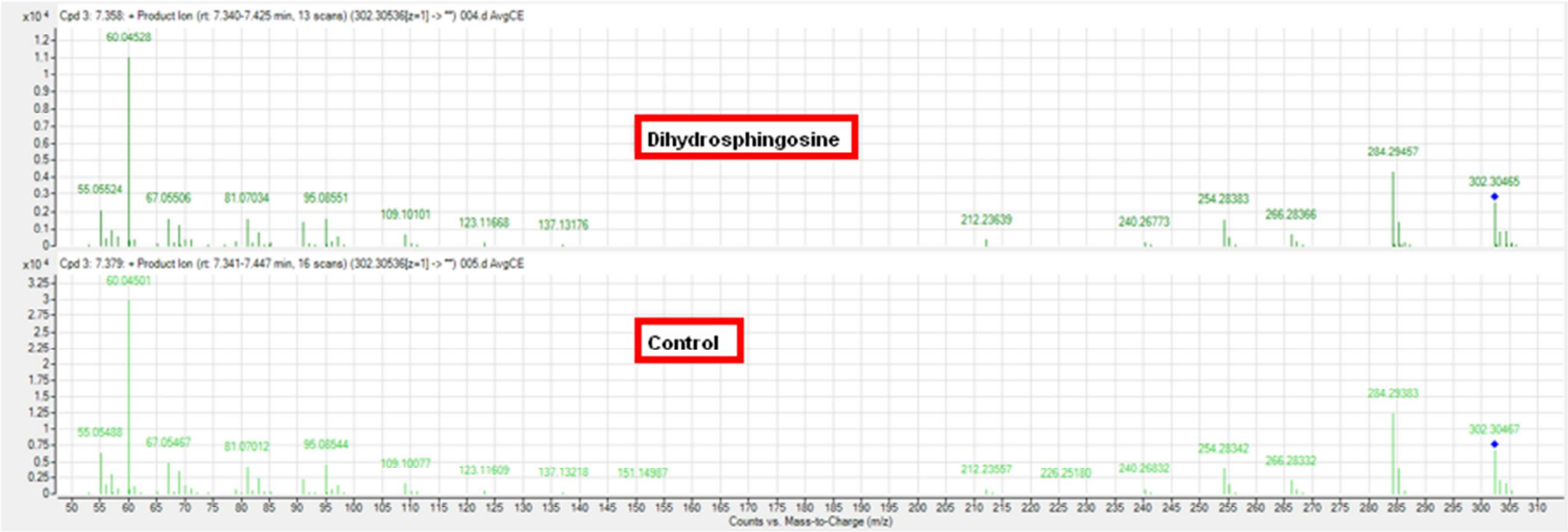

**Figure 12.** Sphingosine peak area for large sample set of control (blue), multiple sclerosis (red) and multiple sclerosis with neuropathic pain (green) groups.

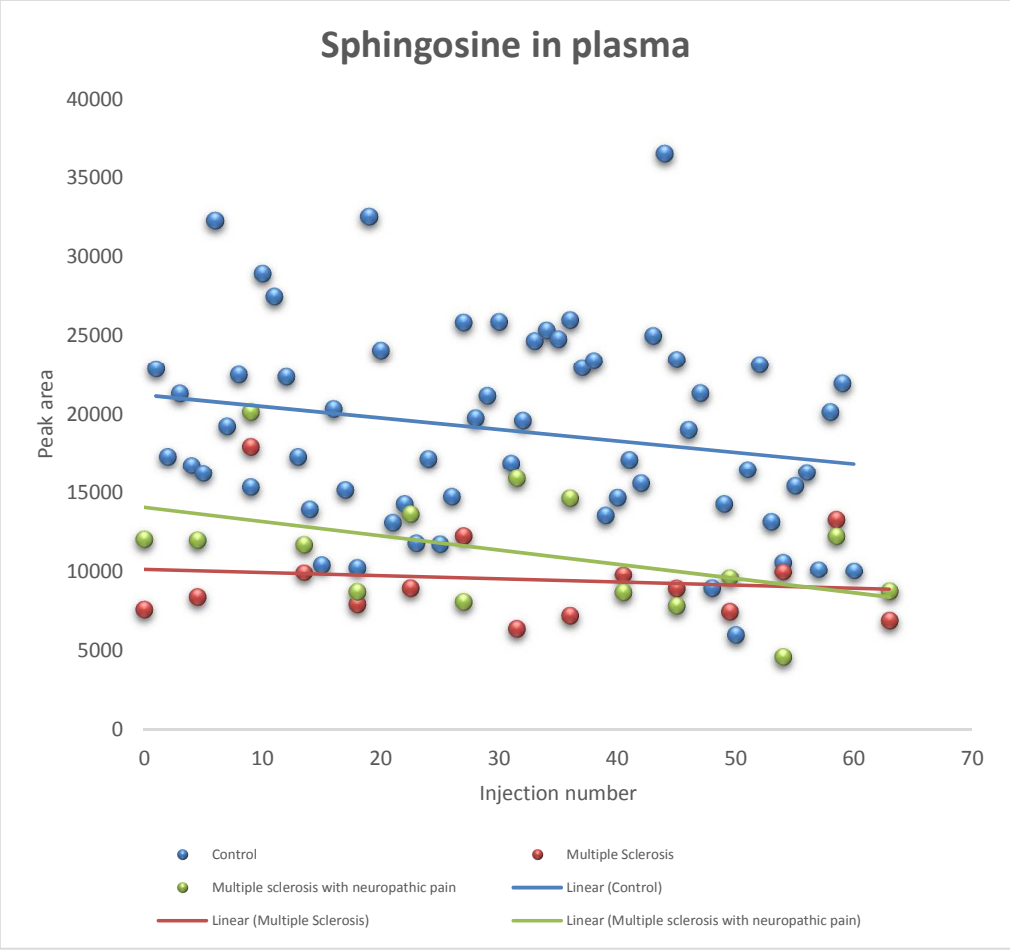

**Table:** Multiple sclerosis LC-MS conditions

| HPLC-MS | | | | | |
|---|---|---|---|---|---|
| Instrument | 6530 Q-TOF | | | | |
| Column | $C_{18}$ 1.8um 2.1X100mm | | | | |
| Oven (ºC) | 35 | | | | |
| Pump | Mobile Phase A | Water 0.1% formic acid | | | |
| | Mobile Phase B | Acetonitrile 0.1% formic acid | | | |
| | Flow (ml/min) | 0.5 | | | |
| | Isocratic/Gradient | Gradient | | | |
| | | Time/min | %A | %B | |
| | | 0.00 | 98 | 2 | |
| | | 1.00 | 98 | 2 | |
| | | 30.00 | 0 | 100 | |
| | | 35.00 | 0 | 100 | |
| | | 35.1.10 | 98 | 2 | |
| | | 40.00 | 98 | 2 | |
| | Runtime (min) | 40 | | | |
| Injector | Volume (ul) | 10 | | | |
| Detector | Wavelength Reference | N/A | | | |
| MS | QTOF/QQQ | QTOF/QQQ | QTOF | Mode | positive |
| | Source Duel jet stream Electrospray | Gas temp (°C) | 300 | Gas flow (l/min) | 8 |
| | | Sheath gas temp (°C) | 350 | Sheath gas flow (l/min) | 10 |
| | | Nebuliser pressure (psig) | 35 | | |
| | | VCap (V) | 3750 | Fragmentor (v) | 175 |
| | | Nozzle Voltage (V) | 1000 | | |
| | | Mass range | 100-1700 | Acquisition rate (Scans/s) | 3 |

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Journal Name

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x
www.rsc.org/

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The project aim was to identify differences in the metabolomic profiles in the serum of patients with multiple sclerosis (MS), those with neuropathic pain (NP) and those with both MS and NP compared with controls and to identify potential biomarkers of each disease state. Metabolomic profiling was performed using ultra-high-performance liquid chromatography coupled to mass spectrometry and the data analysis involved parametric methods, principal component analysis, and discriminating filter analysis to determine the differences between disease and control serum samples. Sphingosine and dihydrosphingosine were identified as significant biomarkers.

## Introduction

There is a long recorded history of people suffering from multiple sclerosis (MS) which spans back centuries before it was given its modern name. People who developed a progressive paralysis would be diagnosed as suffering from paraplegia, a general diagnosis which covered many different neurological disorders.[1] In the 19th century physicians performing careful pathology started to become aware of scattered grey patches, scar tissue in the nervous systems of young adults with a specific progressive disorder[2]. MS is now known to be a disease caused by the immune system attacking the myelin insulation surrounding the nerve cells in the central nervous system (CNS) causing the nerve impulses to slow down and eventually stop.[3] Because the effects of this nerve damage do not become evident until substantial damage has occurred people in the early stages of the disease often appear healthy. Myelin is made in the oligodendrocytes and its dry mass is composed about 70–85% lipids and about 15–30% proteins which allow it to stick only to a specific axon and insulate it. The CNS is isolated from the rest of the body by the blood brain barrier, reinforced by astrocytes which allow the transport of immune cells into the central nervous system.[3] Most of the cells in the body produce distinctive molecules that serve to identify them as being "self" and the immune system normally does not attack these cells. Auto immune diseases occur when the immune system wrongly identifies epitopes on self-cells as being foreign and launching an immune attack.[4] Naturally occurring auto reactive myelin T cells are normally under the control of the regulatory T cells, but in multiple sclerosis this control is lost and the T cells attack the myelin producing oligodendrocytes. This loss of T cell regulation leads to the T cells becoming activated, proliferating and circulating throughout the body. These T cells then produce adhesion molecules and changes in the endothelia which in turn allow access into the CNS across the blood brain barrier.[5] The epidemiology of multiple sclerosis reveals two consistent features, that the disease clusters in families and that its frequency depends on which part of the world you live in, implying that there might be a genetic and an environmental component to the disease.[6] The screening of the genome from tens of thousands of multiple sclerosis sufferers in comparison to a control group has revealed more than 100 common variables of genes in the MS

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Electronic Supplementary Information (ESI) available: [PCA plots, EIC's, MS data ]. See DOI: 10.1039/x0xx00000x

group, regularly found on genes which are involved in immunological processes and specifically in their regulatory regions.[6] More than thirty years ago it was proposed that vitamin D deficiency was a risk factor for MS and it has been shown that vitamin D has an immunomodulatory effect and sufficient levels can help protect against MS.[7] Vitamin D promotes the production of regulatory T cells which suppresses the presentation of antigens to the T helper cells also reducing the activation and recruitment of these cells.[8] Women are more likely than men, with a ratio 3:1, to develop MS and women with MS often show signs of improvement during pregnancy and worsen after childbirth. Testosterone and estrogen have been shown to have anti-inflammatory effects in animal models of the disease.[9]

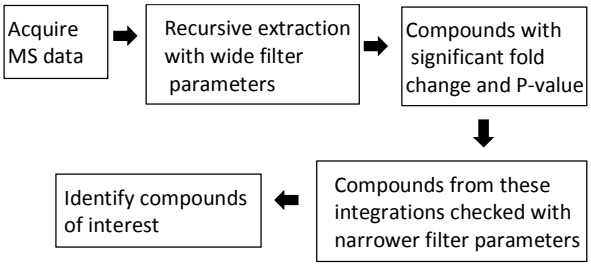
In recent years evidence that environmental factors which may affect MS to the greatest extent seems to be related to the gut microbiota as MS sufferers have an increased risk of gut permeability and inflammatory bowel disease which suggests that there may be a connection between the gut and the CNS.[10] It has also been shown that *Pseudomonas* peptides can activate myelin basic protein specific T cells which have been cloned from MS patients, but the difficulty in linking a specific microorganism with MS is that there are numerous microbial sequences that can activate the myelin basic protein specific T cells from MS patients.[11] *Chlamydia pneumonia* is commonly found in the cerebrospinal fluid of people suffering from MS and has been shown to induce the disease in an animal model.[12] Finally, there have been proposed links with mitochondrial dysfunction and multiple sclerosis along with other neurological diseases such as Alzheimer's and Parkinson's[13].

In summary, the full understanding of the causes of MS is far from complete. The metabolomics analysis of pathways combined with the identification and quantification of small molecules in disease patients may help to solve this problem. Liquid chromatography mass spec (LC-MS) is growing in popularity in the field of metabolomics[14] but is highly dependent on both the analytical method and data analysis steps.[15] The use of metabolomics techniques has found metabolic differences between control groups and people with MS using GC-MS and LC-MS.[16] Herein LC-MS of blood plasma samples from people suffering with multiple sclerosis (MS), neuropathic pain (NP) and multiple sclerosis with neuropathic pain (MSNP) along with a control group was used to identify specific biomarkers which could be a useful diagnostic tool.

## Results

For the comparison of LC-MS data sets the features first need to be extracted using their accurate mass and then be aligned by retention time.[17] Feature extraction and data alignment is a critical step for the reduction of false positives and negatives and also reduces data file size and complexity by the removal of non-specific information and removing features based on their accurate mass and elution time. It is very important to produce good quality raw data for feature extraction because poorly resolved data can lead to an increase in false peak detection, missing values and incorrect identifications.

Blood plasma samples from people suffering with (i) multiple sclerosis (MS), (ii) neuropathic pain (NP) and (iii) multiple sclerosis with neuropathic pain (MSNP) along with (iv) a control group were deproteinated by mixing 250µl of plasma with 1 ml of methanol, then centrifuged at 12000G for 5 min. before being filtered using syringe filters. Ten samples of each disease type and ten control samples were analysed along with quality control (QC) samples by taking equal portions of each sample and combining them. The samples were analysed by LC-MS on an Agilent 1290 HPLC combined with a 6530 Q-TOF. A dual jet stream ionisation source was used in positive mode using the LC and MS conditions given in the ESI. Because of the high number of samples and the complexity of the data it is difficult to check all of the compound integrations in the Profinder and Mass Profiler Professional (MPP) software. The workflow used was:




For the initial data extraction very lenient filtering parameters were used in order to catch as much data as possible. A peak height cut-off of 2000 total ion counts was used which was only six times the instrument noise level. The retention time drift in the QC samples

was 0.2 min but a retention time window of 0.5 min and a relatively large mass error window of 15 ppm. The use of these parameters yielded the detection of 3,494 compounds which were then further analysed, but with no further filtering at this stage. A volcano plot was used to find the differentiating compounds between the control group and the disease groups with a fold change above 2 and satisfying a P-value of 0.05. Each of the three entity lists generated from the volcano plots were then combined to give the Venn diagram (**Figure 1**).

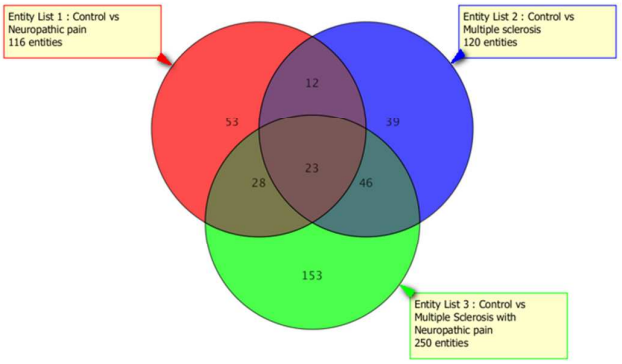



**Figure 1.** Venn diagram of entity lists created with volcano plots between multiple sclerosis, neuropathic pain, multiple sclerosis with neuropathic pain and the control group.

This entity list was then re-extracted, all of the integrations checked, the samples ordered by mass and duplicates removed to give 307 entities. Using principle component analysis of the re-processed data, creating new volcano plots, again comparing the controls to each disease group, but this time using the Bonferroni FWER multiple testing correction, a P-value of 0.05 and a fold change of 2 for each sample set to give the new Venn diagram (**Figure 2**).

There are 25 entities that differentiate the disease groups from the control group; 4 that only differ in the neuropathic pain group, 11 in the multiple sclerosis with neuropathic pain group and 1 entity that only differs in the multiple sclerosis group. There are 6 entities that differ in all of the disease groups and only 2 that differ in both the multiple sclerosis and neuropathic pain groups compared with the control group. There is just 1 entity that differs in both the multiple sclerosis and multiple sclerosis with neuropathic pain groups and this difference in the abundance of this entity is common to all of the samples collected from people with multiple sclerosis.

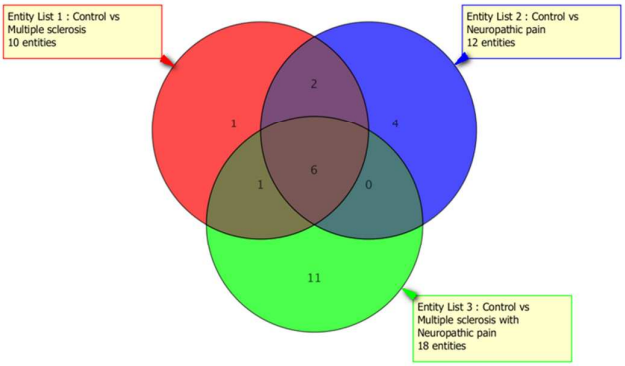
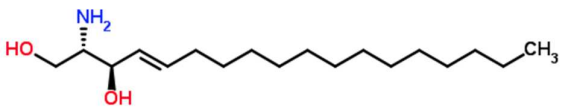



**Figure 2.** Venn diagram of entity lists created with volcano plots of re-processed data between multiple sclerosis, neuropathic pain, multiple sclerosis with neuropathic pain and the control group.

The 25 differentiating compounds were searched against the Metlin data base and formulae only matched if the mass error was less than 5ppm (ESI). The one compound which differentiates people suffering from multiple sclerosis compared with the control group has a mass of 299.2818 and elutes at 21.3min with the method used in this analysis. The extracted ion chromatogram for m/z 300.2892 in a control group sample shows 2 peaks, one at 21.3min and one at 22.3min. The formula $C_{18}H_{37}NO_2$ was generated for mass 299.2818 eluting at 21.3min with good isotope fit. A possible structure for this is sphingosine (1), a component of sphingolipids. Sphingolipids are one of the well-defined lipid categories and their structural diversity and complexity involves N-acylation, O-phosphorylation, O-glycosylation and conversion to ceramides, and sphingomyelin.[18] As well as ensuring cellular membrane integrity[19] sphingolipids are play key roles in signaling and regulation of cell growth, proliferation, survival and apoptosis.[20] Metabolic disorders involving sphingolipids[21] include diabetes, cardiovascular diseases, various cancers[22] as well as central nervous system disorders.[23] None of the other compounds in the 25 differentiating set had masses corresponding to readily identifiable blood plasma components.




**(1)** sphingosine

A sample of plasma from one of the control group was de-proteinated with (i) LC-MS grade methanol and (ii) LC-MS grade methanol spiked with 0.2 ppm sphingosine. These two samples were then analysed using the previously described methodology and their extracted ion chromatograms compared (**Figure 3**). The peaks for m/z 300.2892 have shifted slightly to the right because the column had not been fully conditioned and are now at 21.4min and 22.4min. The peak at 21.4min is much bigger and there are no extra peaks. This means that the unknown compound with mass 299.2818 has the same mass isotope pattern and retention time as sphingosine.

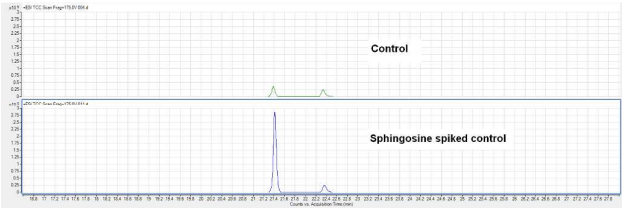



**Figure 3.** Extracted ion chromatograms of m/z 300.2892 in control sample and sphingosine spiked control sample showing peak alignment for peak eluting at 21.4 min.

The control sample and the sphingosine sample were then analysed by MS/MS at collision energies of 10, 20 and 40eV. A shorter 15min LC gradient was used to reduce analysis time. The fragmentation patterns for both have major fragments with m/z's of 282.278, 264.268 and 252.267. These fragments also have the same relative abundance in both samples. The MS/MS spectra for the control group sample were then exported into a database which gave sphingosine as one of the top hits. The fragments for the three main ions are those expected by mono- and di- dehydroxylation.

When the distribution of sphingosine was plotted across all disease groups (**Figure** 4) it appeared lowest in the neuropathic pain group compared with the control. In one of the replicates no sphingosine was detected.

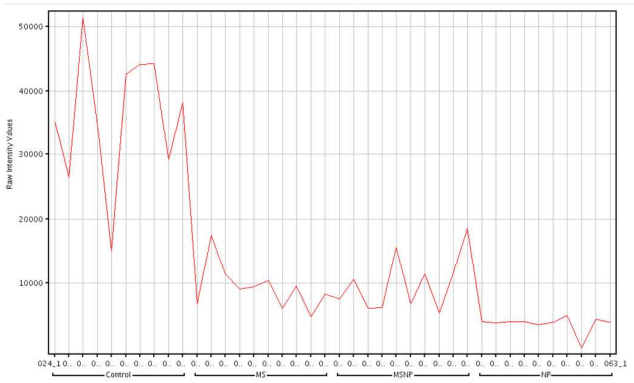



**Figure 4.** Abundance of sphingosine in the multiple sclerosis (MS), neuropathic pain (NP), multiple sclerosis with neuropathic pain (MSNP) and control group.

A second identifying compound that differentiated both MS and MSNP from the control group was that eluting at 22.0min with mass 301.2978 gives a good accurate mass and isotope pattern to the molecular formula $C_{18}H_{39}NO_2$. The isotope fit is shown in figure 5 of ESI for the singly charged protonated adduct. Dihydrosphingosine (1,3-dihydroxy-2-aminooctadecan) is a possible compound for this formula with mass 301.2981 and is a naturally occurring compound found in blood. A commercial sample of dihydrosphingosine was analysed separately at a concentration of 0.2ppm. When a blood sample was spiked with dihydrosphingosine, it showed a single peak at the same retention time with increased intensity. The samples were then analysed by MS/MS at collision energies of 10, 20 and 40eV with a shorter 15min LC gradient to reduce analysis time. The fragmentation patterns for dihydrosphingosine and the peak in the control at the same retention time have an identical pattern, with major fragments at 284.295, 254.284 and 60.045. The MS/MS spectra for the control group sample was then exported into the ChemSpider database which gave dihydrosphingosine as one of the top hits and the possible fragments for the three main ions corresponding to loss of OH, $CH_2O$ and $[H_2NCH_2CHOH]^+$ as expected from the structure.

In order to further investigate the difference in sphingosine concentrations between a multiple sclerosis group and a control group, a larger sample set was used. For this work 30 plasma samples from people with multiple sclerosis (15 of which also had neuropathic pain) were analysed along with 60 age and gender matched control samples. Sphingosine levels were significantly

lower in the multiple sclerosis groups compared with the control group giving $p=6.06x10^{-10}$. The control group had a mean peak area of 18,990 and standard deviation of 6,286. The multiple sclerosis groups had mean peak areas of 9,535 for multiple sclerosis and 11,254 for multiple sclerosis with neuropathic pain and standard deviations of 3,032 and 3,863, respectively. The p-value for the difference between the MS and MSNP groups was 0.186 showing no significant statistical difference between the two MS groups.

Similarly, the dihydrophingosine levels were significantly lower in the multiple sclerosis groups compared with the control group with $p=4.89x10^{-6}$. The control group had a mean peak area of 7,723 and standard deviation of 2,345, whereas the multiple sclerosis groups had mean peak areas of 4,936 for MS and 5,904 for MSNP with standard deviations of 1,389 and 1,581, respectively. The p-value for the difference between the MS and MSNP groups was 0.086 showing no significant statistical difference between the two MS groups.

## Discussion

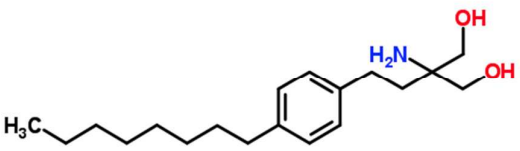
Two compounds have been identified in this study as potential biomarkers for multiple sclerosis, sphingosine and dihydrosphingosine, which are found at significantly lower concentrations in both the multiple sclerosis and the multiple sclerosis neuropathic pain groups. These sphingolipids have an eighteen carbon amino alcohol backbone and variations in this basic structure create a wide variety of sphingolipids that are utilised in constructing cell membranes and for acting as signalling molecules[24]. Phosphorylation of sphingosine and dihydrosphingosene at the C1 hydroxyl group creates the important cellular signalling molecules sphingosine-1-phosphate and dihydrosphingosine-1-phosphate, respectively. Sphingosine is synthesised from the hydrolysis of sphingomyelin or from the precursors serine and palmitoyl-CoA.[25]

Although a difference was anticipated between the neuropathic pain group and the multiple sclerosis/multiple sclerosis with neuropathic pain groups, with regards to levels of sphingosine and dihydrosphingosine the neuropathic pain group showed a similar trend as the multiple sclerosis/ MSNP groups indicating the possibility of underlying similar biochemical mechanisms in these three groups. Neuropathic pain can accompany multiple sclerosis as a result of de-myelination but not all cases of multiple sclerosis include neuropathic pain.[26]

Neuropathic pain without multiple sclerosis is a debilitating condition with limited treatment potential due to its unknown biochemical basis. Recently an LC-MS metabolomics study was carried out on rats which had been subjected to tibial-nerve transection (TNT). The results of this study showed alterations in sphingomyelin-ceramide metabolism. This was due to an increase in the levels of the enzyme sphingomyelinase which is responsible for catalysing the breakdown of sphingomyelin to ceramide and phosphoryl choline. In this study both sphingosine and dihydrosphingosine levels were *elevated*[27], in sharp contrast to the findings reported here conducted on human samples with real disease conditions.

Another study[28] found differences in lipid composition in the white and grey matter of multiple sclerosis patients. Patients with active multiple sclerosis showed higher levels of phosphorylated sphingolipid but *lower* sphingolipid levels in both white and grey matter. This is in agreement with the results found herein from blood plasma analysis. In patients with inactive multiple sclerosis only white matter had increased phosphorylation of sphingolipids. One of these was phosphatidylcholine which on hydrolysis yields lysophosphatidylcholine[29] which can be used for the *in vitro* demyelination of nerve fibres[30] which could be another contributing factor to the progression of the disease.

The drug fingolimod (**2**) has shown promise in clinical trials for the treatment of multiple sclerosis. Fingolimod is a sphingosine analogue which is phosphorylated in the body to form fingolimod-phosphate, this resembles sphingosine-1-phosphate (S1P). Currently five S1P receptors have been discovered ($S1P_{1-5}$) which are found on a variety range of cell types including lymphocytes and neural cells. The immune and central nervous system has a large number of $S1P_{1-3}$ receptors, $S1P_4$ receptors are usually found on lymphoid and heamatopetic tissue and $S1P_5$ receptors are found on the white matter of the central nervous system. The S1P receptors in the CNS could contribute to the neuropathology of multiple sclerosis effecting neurogenesis as well as neural function and migration[31].




**(2)**

Sphingolipids also play an important role in microbial pathogenesis regulating the balance between the microbe and the host. Most bacteria and viruses do not produce their own sphingolipids but instead use the sphingolipids of the host cell. The utilization of the host cells sphingolipids for the production of a microbial cellular membrane may be used by a microbe to hide from the immune system allowing colonisation. Alternatively the microbe may enzymatically functionalise the sphingolipid which may interfere with intracellular signalling thus avoiding removal and destruction from the host cell.[32]

## Conclusion

The project aim was to identify differences in the metabolomic profiles in the serum of patients with multiple sclerosis (MS), those with neuropathic pain (NP) and those with both MS and NP (MSNP) compared with controls and to identify potential biomarkers of each disease state. We found that the concentration of both dihydrosphingosine and sphingosine are lower in all groups compared with those in the controls and so the detection of the differences in the concentrations of these compounds in blood plasma may be a useful diagnostic tool to aid in the investigation of their role in the disease. This was achieved using a fast analytical methodology. Sphingosine and dihydrosphingosine have been previously found to be at lower concentrations in the brain tissue of patients with multiple sclerosis. The detection of these sphingolipids in blood plasma is advantageous because it allows the non-invasive monitoring of these and related compounds.

For this project the ability of MPP software to determine differences between disease groups and control groups quickly and easily was tested. Only reverse phase chromatography was used as this gave good separation of the detectable compounds present in the samples. For detection only Jet Stream ESI positive was used because this gave the largest number of compounds. The data processing is the most time consuming part of the MPP workflow so the data was first processed with Profinder then put into MPP unchecked. The data was filtered in MPP on fold change (2) and p-value (0.05) and the generated compound list re-processed with Profinder. This drastically reduced the number of compounds that had to be manually checked for proper integration. This process reduced the data processing time from weeks to days.

## Experimental

Blood plasma samples from people suffering with (i) multiple sclerosis (MS), (ii) neuropathic pain (NP) and (iii) multiple sclerosis with neuropathic pain (MSNP) along with (iv) a control group were deproteinated by mixing 250µl of plasma with 1 ml of methanol, then centrifuged at 12000G for 5 min. before being filtered using syringe filters. Ten samples of each disease type and ten control samples were analysed along with quality control (QC) samples by taking equal portions of each sample and combining them. The samples were analysed by LC-MS on an Agilent 1290 HPLC combined with a 6530 Q-TOF. A dual jet stream ionisation source was used in positive mode using the LC and MS conditions given in the ESI. Because of the high number of samples and the complexity of the data it was difficult to check all of the compound integrations in the Profinder and Mass Profiler Professional (MPP) software.

To further investigate the difference in sphingosine concentration between a control group and a multiple sclerosis group a larger sample set was used consisting of 30 plasma samples from people with multiple sclerosis (15 of which also had neuropathic pain) were analysed along with 60 age and gender matched control samples and the peak area for sphingosine integrated using Profinder.

## Acknowledgements

We are grateful to Simona Palusci for preparing the serum samples. SW is grateful to IPOS, Agilent Technologies and the University of Huddersfield for support.

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Manuscript ID: AY-ART-08-2017-001922
TITLE: Biomarkers for multiple sclerosis determined by metabolomic profiling using coupled UHPLC-MS

**Response to reviewers comments**

1. The title of this article did not highlight this study's result and focus on the concentration of sphingosine and dihydrosphingosine in MS blood.
   *Title amended*

2. The reason why the author choose the mass of 299.2818 from the 25 differentiating compounds as the biomarker could not be found in the article.
   *Text modified to explain this.*

3. The author did not clearly explained in this article why the entity list needed to be re-processed.
   *Text modified to explain this.*

4, Page 6, line 6, right panel, "figure 147" seems like a typo that needed to be modified.
   *Text modified*

*5.* How many the patients with multiple sclerosis (MS), those with neuropathic pain (NP) and those with both MS and NP, and controls?
*This was in the original text but is now also included in the experimental section*