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# The use of vibrational spectroscopy to study the pathogenesis multiple sclerosis and other neurological conditions

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**RUNNING HEAD:** Spectroscopy for multiple sclerosis

**KEYWORDS:** Multiple sclerosis, normal-appearing white matter, pathology, spectroscopy

1 **Abstract**

2 Spectroscopy techniques are valuable tools in biomedical research and have been used  
3 extensively in the study of disease. However, neurological conditions such as multiple  
4 sclerosis (MS) have received little attention and the available spectroscopy studies are  
5 limited, both in overall numbers of patients studied and the disease samples considered. MS  
6 is a complex immune-mediated disease, with variable clinical courses and limited  
7 therapeutic options. This review aims to summarize current literature in the area,  
8 demonstrating how spectroscopy techniques can provide valuable information to inform and  
9 advance research into the most common neurological condition affecting young adults.

## 10 Introduction

11 Biophotonic techniques are now widely used in biomedical research targeting better  
12 diagnosis, prognosis and surveillance of disease. Vibrational spectroscopy methods such as  
13 Fourier-transform infrared (FTIR) and Raman spectroscopy are so called because they  
14 probe the intramolecular vibrations and rotations of a sample when irradiated with a light  
15 source (1). The vibrational energy levels can be probed by both techniques, using different  
16 physical processes. Raman spectroscopy studies the Raman effect, the spontaneous  
17 inelastic light scattering process of photons, following the interaction of monochromatic  
18 radiation (e.g. a laser) with the sample. In contrast, FTIR spectroscopy studies the samples'  
19 absorption characteristics arising from the molecular motion due to atomic displacement  
20 upon interaction with an infrared source (2, 3). In both cases, the recording of vibrational  
21 energy level transactions results in a spectrum composed of peaks/bands that can be  
22 interpreted qualitatively (peak position) and quantitatively (peak intensity/area) (4). In FTIR  
23 spectroscopy, the spectral bands arise from a change in the electric dipole moment of the  
24 molecules, whereas in Raman spectroscopy, they arise from a change in molecular  
25 polarizability. FTIR and Raman spectroscopies are therefore complementary and provide a  
26 "fingerprint" or "signature" of the specific molecules contained within a biological sample  
27 (proteins, lipids, DNA), depending upon whether their bonds exhibit infrared or Raman  
28 activities. Both FTIR and Raman can be used for imaging tissue sections and are non-  
29 destructive, label-free techniques with sub-micron spatial resolution (5).

30 In biomedical research, scientists are continually investigating and exploring the application  
31 of new technologies that can detect early signs of disease and thereby reduce disease  
32 morbidity and mortality. The detection of biomarkers plays an important role in this  
33 exploration. In oncology, such biomarkers, have been used extensively to determine risk  
34 factors, aid diagnosis and prognosis, and in the assessment of treatment response as well  
35 as determining disease recurrence (4). However, from amongst the vast numbers of  
36 candidate biomarkers, only a limited few have been validated for clinical use. Vibrational  
37 spectroscopy is new investigatory tool in biomarker (re)search which is not restricted to the  
38 analysis of a specific protein, nucleic acid and/or lipid. As such, FTIR and Raman spectra  
39 are able to give spectral "signatures" or "biomarkers" which reflect the overall molecular  
40 composition of the studied samples (4).

41 Despite being extensively used in the field of cancer research (6, 7), FTIR and Raman  
42 spectroscopy are currently under explored in the study of diseases which affect the central  
43 nervous system (CNS) including multiple sclerosis (MS). To date, there are very few  
44 published papers in this field including a review article published in 2012 (8).

## 45 **Multiple Sclerosis (MS)**

46 MS is considered to be an autoimmune, neuro-inflammatory and degenerative condition,  
47 which affects both the brain and spinal cord. Its precise aetiology remains unknown,  
48 although both genetic and environmental factors influence an individual's susceptibility to  
49 develop MS (9). The clinical course of this disease is variable but is divided into several  
50 categories reflecting the degree of clinical disease activity and disability progression rate,  
51 including relapsing remitting (RRMS), primary progressive (PPMS) and secondary  
52 progressive MS (SPMS) (10). Whilst the inflammatory component of MS pathogenesis is  
53 relatively well understood, the progressive neurodegenerative component of the disease, in  
54 both its primary and secondary progressive clinical courses, is yet to be elucidated. In  
55 PPMS, patients have a gradual and progressive decline in function from the outset, with  
56 minimal disease activity detectable on magnetic resonance imaging (MRI) whereas in  
57 SPMS, the gradual progression follows an initial relapsing remitting phase, usually over  
58 many years (11). The consensus is that MS is a spectrum of conditions with RRMS being  
59 one end of that spectrum and PPMS being at the other.

60 The key pathological features observed in MS are the influx of inflammatory immune cells  
61 across the blood brain barrier into the CNS which results in the loss of axons and their  
62 insulating myelin sheaths and the formation of lesions (plaques) in the white matter (WM)  
63 and to a lesser extent in the grey matter (12). This process results in the impairment of  
64 conduction along the affected axon leading to variable symptoms experienced by affected  
65 patients including cognitive impairment, visual disturbances, sensory and motor symptoms,  
66 impaired balance, sphincter disturbance and fatigue (13). Histopathological comparisons of  
67 CNS tissue shows that the classical perivascular inflammation seen in SPMS is much less  
68 prominent in PPMS and that more diffuse inflammatory changes and greater extent of  
69 axonal damage in the normal appearing white matter (NAWM) are seen in PPMS (14, 15). In  
70 addition, there is evidence that patients with PPMS have a reduced capacity for re-  
71 myelination (11). Understanding the underlying pathogenesis which underpins the clinical  
72 progression in MS at the molecular and cellular levels is therefore vital for the development  
73 of therapies targeting the neurodegenerative process and enhancing remyelination  
74 strategies.

75 The diagnosis of MS is usually based on the clinical presentation and the results of brain and  
76 spinal MRI, which reveals evidence of active and chronic lesions as well as focal and  
77 generalised atrophy (16). Current treatments for MS target the initial relapsing phase of the  
78 disease, by preventing inflammatory responses leading to a reduction in the number and  
79 severity of relapses (17). However, there are currently no treatments for primary and

80 secondary progressive MS although two therapeutic agents are waiting to be licenced (18). .  
81 The underlying pathogenesis of the initial inflammatory phase of MS has been well  
82 characterised at both the cellular and molecular level. However the pathogenesis of the  
83 progressive phase is still not fully elucidated, although changes in the NAWM appear to be  
84 pivotal (12). The progressive loss of axons seem to continues despite the reduction of  
85 relapses with the use of effective anti-inflammatory therapies resulting in irreversible  
86 disability which support the presence of two separate pathological processes: inflammation  
87 and neurodegeneration (19).

88 Approaches to studying MS pathogenesis have focussed on the analysis of post-mortem  
89 CNS tissue, as well as experimental work using both primary CNS cells such as astrocytes,  
90 microglia and oligodendrocytes isolated from CNS tissue, which allows manipulation of the  
91 individual cell's environment. In addition, a number of animal models of MS have been  
92 developed, primarily in rodents but also in primates, in order to investigate the disease  
93 course of MS. In these animals, experimental autoimmune encephalomyelitis (EAE) is  
94 induced through the injection of spinal cord homogenates, myelin proteins or peptides, such  
95 as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP) or proteolipid  
96 protein (PLP), in addition to adjuvant. This promotes the induction of an autoimmune  
97 response against myelin, leading to both an inflammatory response in the CNS as well as,  
98 dependent on the model used, demyelination (20). Nevertheless, the progressive aspects of  
99 the human condition seen in MS are more difficult to reproduce in animal models (21),  
100 although Peferoen has recently described an EAE model in Biozzi mice which, dependent on  
101 the age of the mouse at induction of disease, demonstrates progressive disease, with  
102 younger mice having an initial relapsing remitting phase followed by a secondary  
103 progressive phase and older mice showing progression at onset of disease induction (22). A  
104 further drawback of these animal models is that off the multitude of therapies found to be  
105 effective in preventing EAE, a very small number have been taken forward into clinical trials  
106 (23), suggesting that the models do not fully mimic the pathogenesis of MS in humans.

107 Current research approaches to investigating the aetiology of MS focus on the search for  
108 specific genes/proteins/lipids that are thought to be involved in the disease process using a  
109 variety of cell and molecular biology approaches. More recently DNA microarrays have been  
110 used to assess more global changes in gene expression in MS diseased human CNS tissue  
111 compared with normal age matched control (24, 25). The application of proteomic and  
112 metabolomic analyses in MS have focussed on biomarkers in biological fluids, including  
113 blood, cerebrospinal fluid and urine rather than in MS CNS tissue (26, 27).

114 The advantage of Raman and FTIR for analysis of human tissues is that the overall chemical  
115 composition of the tissue in terms of lipids, nucleic acids and proteins is obtained.  
116 Spectroscopic study of MS has been reported in the literature using both human post-  
117 mortem CNS tissue and animal models of the disease. A review of all available original  
118 research articles published to date is provided below and is summarized in Table 1.

119

## 120 **Human CNS tissue spectroscopy studies**

121 Initial studies applying spectroscopic techniques to the study of MS pathology were reported  
122 in the 1990s. Choo *et al.* were the first to use FTIR to study human white and grey matter  
123 tissue, obtained from healthy control subjects, and compared it with MS demyelinated  
124 lesions tissue from MS patients (28). This rapid communication reported that it was possible  
125 to discriminate between different types of MS tissue attributed to variations in intrinsic lipid  
126 and water content. Whilst FTIR spectra of white matter was dominated by lipids and protein  
127 absorptions and grey matter spectra showed reduced lipid content alongside an increase  
128 contribution of water to the spectra; MS lesion spectra were suggestive of both lipid and  
129 water depletion, as would be expected from histopathological tissue analysis (28).

130 Differences comparing white and grey matter as well as white matter with MS lesions were  
131 most notable in the 2800-3000  $\text{cm}^{-1}$  spectral region, where most infrared bands arise from  
132  $\text{CH}_2$  and  $\text{CH}_3$  stretching vibrations of lipid acyl chains. Four main assignments were made to  
133  $\text{CH}_3$  and  $\text{CH}_2$  asymmetric stretching vibrations at 2956 and 2922  $\text{cm}^{-1}$  and, to symmetric  
134 vibrations at 2871 and 2851  $\text{cm}^{-1}$  respectively. The overall intensity of these peaks were  
135 reduced in both grey matter and MS lesion tissue, compared to normal control white matter,  
136 which the authors explained was due to the expected lower lipid content of grey matter and  
137 MS lesions, due to demyelination. Similarly, the  $\text{CH}_2/\text{CH}_3$  ratio is also decreased as a  
138 decrease in lipid to protein ratio leads to methylene and methyl groups of amino-acid side  
139 chains dominating this spectral region; as the  $\text{CH}_2/\text{CH}_3$  ratio in proteins is much lower than in  
140 lipids, it was expected that the overall ratio would be decreased and band broadening would  
141 be observed. In order to distinguish between grey matter and MS lesion tissue, the authors  
142 reported the spectral region of 1200-1800  $\text{cm}^{-1}$  to be most useful (28).

143 The main feature in white matter' spectra was observed at 1467  $\text{cm}^{-1}$  and assigned to the  
144 scissoring vibration of  $\text{CH}_2$  groups of lipid acyl chains. In grey matter, the intensity absorption  
145 of this  $\text{CH}_2$  scissoring is reduced and is almost equal to the  $\text{CH}_3$  asymmetric bending  
146 vibrations at 1456  $\text{cm}^{-1}$ . This is explained by the reduction in lipid content, which is also

147 apparent by the decrease in intensity of the terminal methyl groups of lipid chains and of the  
148  $(\text{CH}_3)_3\text{N}^+$  symmetric bending of phosphatidylcholine headgroups, assigned to the bands at  
149  $1381$  and  $1415\text{ cm}^{-1}$ , respectively. In contrast the  $\text{COO}^-$  symmetric stretching band at  $1400$   
150  $\text{cm}^{-1}$  is increased in the spectra of grey matter in comparison to white matter. The same was  
151 also observed at  $1308\text{ cm}^{-1}$  which the authors assigned to amide III(28).

152 The spectral features of MS plaque tissue are similar to the ones described for grey matter.  
153 Nevertheless, the intensity of  $\text{CH}_2$  scissoring ( $1467\text{ cm}^{-1}$ ) to  $\text{CH}_3$  asymmetric bending  
154 vibrations ( $1456\text{ cm}^{-1}$ ) is now reversed with  $\text{CH}_3$  asymmetric bending vibrations being the  
155 main feature in this region. Similarly, the  $\text{PO}_2^-$  antisymmetric stretching band also displays  
156 greater intensity in the plaques' spectra comparatively to grey matter. Both these  
157 observations were suggested to indicate that lipid content of MS plaques is lower than that of  
158 the grey matter (28), which is known to be the case from histopathology studies (13).

159 In the  $1500$  to  $11800\text{ cm}^{-1}$  spectral range the main feature observed was the amide I band  
160 which arises from the  $\text{C}=\text{O}$  stretching vibration of amide groups of proteins and is centred at  
161  $1653\text{ cm}^{-1}$ . Other absorptions reported were assigned to  $\text{C}-\text{C}$  stretching of tyrosine at  $1517$   
162  $\text{cm}^{-1}$ ; the amide II band centred at  $1550\text{ cm}^{-1}$ ; and the acidic amino-acid and arginine side  
163 chains at  $1581$  and  $1580\text{-}1610\text{ cm}^{-1}$  respectively. In addition, the ratio of amide I to amide II  
164 was increased comparatively to that of isolated proteins. It was suggested this may result  
165 from non-protein contributions to the amide I region, and further proposing water to be the  
166 main source of this contribution (28).

167 Le Vine *et al.* (1998) assessed active lesions in MS tissue compared with healthy control  
168 white matter and reported an increased oxidation state of both lipids and proteins in MS  
169 lesions, indicative of a role for free radicals in MS pathogenesis (29). The spectra of WM  
170 tissue from control post-mortem cases was dominated by  $\text{CH}_2$  absorptions at  $2923$  and  $1468$   
171  $\text{cm}^{-1}$ ,  $\text{P}=\text{O}$  at  $1235\text{ cm}^{-1}$  and  $\text{HO}-\text{C}-\text{H}$  at  $1060\text{ cm}^{-1}$ , characteristic of lipids, phospholipids and  
172 glycolipids respectively. Areas of NAWM within MS cases were reported to display similar  
173 spectra to normal control white matter, whereas lesion areas display significant changes,  
174 such as a reduced ratio of  $\text{CH}_2$  to  $\text{NH}$  and  $\text{OH}$ , in comparison with control white matter.  
175 Further differences were revealed by the investigation of the oxidation products of lipids and  
176 proteins. Previous studies reported the amide I peak at  $\sim 1660\text{ cm}^{-1}$  to be broader when  
177 proteins are oxidised and the carbonyl absorption at  $1740\text{ cm}^{-1}$  to be increased when lipids  
178 are oxidised. This study reported the  $\text{C}=\text{O}$  ( $1740\text{ cm}^{-1}$ ) to  $\text{CH}_2$  ( $1468\text{ cm}^{-1}$ ) ratio to be  
179 increased and the peak at  $1657\text{ cm}^{-1}$  to be broader in MS lesions in comparison with white  
180 matter from control samples (29).



181 Furthermore, the authors followed the spatial spectroscopic profiles of these features by  
182 recording linear maps acquired partially or wholly within MS lesions sites and representative  
183 areas of control white matter. They reported the CH<sub>2</sub> (1468 cm<sup>-1</sup>) to amide II (1544 cm<sup>-1</sup>) ratio  
184 to be 0.644±0.053 for control samples (n=5), ranging from less than 0.1 to 0.7 in MS ones  
185 (n=5); 15.950±1.593 was the mean of the C=O (1740 cm<sup>-1</sup>) to amide II (1544 cm<sup>-1</sup>) ratio in  
186 control cases, which was in turn decreased for all MS cases; and four out of five MS cases  
187 presented one or more values above the mean of 24.047±3.22 for C=O (1740 cm<sup>-1</sup>) to CH<sub>2</sub>  
188 (1468 cm<sup>-1</sup>) ratio of control samples. Finally, whilst controls displayed an average of -  
189 0.033±0.010 at 1652 cm<sup>-1</sup>, MS cases displayed greater values all above -0.02 (29).

190 It was concluded that the higher carbonyl to CH<sub>2</sub> ratio detected in the spectra of MS cases is  
191 suggestive of lipids being oxidised, whilst oxidation of proteins cause the 1657 cm<sup>-1</sup> peak to  
192 broaden to 1652 cm<sup>-1</sup> in MS plaque tissue. This result may be caused by gliosis, which  
193 occurs in parallel with the demyelination process leading to higher expression of glial  
194 fibrillary acidic protein by astrocytes, which was also indicated as a potential factor  
195 contributing to the amide I broadening, as well as the relative greater expression of amide II  
196 (29).

197 More recently, Poon *et al.* used Coherent Anti-Stokes Raman Scattering (CARS) to study  
198 several regions of post-mortem MS brain, including areas of NAWM, remyelination and both  
199 active and chronic lesions (30). Investigating five chronic MS cases, they reported a novel  
200 instrument that allows acquisition of high resolution, label-free imaging whose pixels contain  
201 spectral information, together with a post-processing method, which allows isolation and  
202 quantification of these spectral images. The study showed the CH<sub>2</sub> symmetric stretch of  
203 2850 cm<sup>-1</sup> in NAWM, to shift to 2885 cm<sup>-1</sup> when myelin was contained within the phagocytic  
204 macrophages/microglia cells within the tissue (a CARS image is overlaid with  
205 immunostaining with the marker HLA-DR/LN3, confirming activated microglia). This was  
206 proposed to arise from the intermolecular chain disorder resulting from the breakdown of the  
207 myelin components during demyelination. Further CARS pseudo-colour images showed  
208 myelinated axons to have greatly reduced density within remyelinated areas in active lesion  
209 sites (30).

210 An additional study, also by Poon *et al.* reported lipid biochemical changes preceding myelin  
211 protein loss in peri-lesional areas and NAWM, when inspecting the CH spectral region from  
212 2750 to 3100 cm<sup>-1</sup> (31). CARS images were acquired from the NAWM region adjacent to the  
213 lesion and sequential images were acquired moving away from the lesion into the NAWM..  
214 Triplicate images were also acquired from an area furthest away from the lesion site,  
215 referred to as "true NAWM" and from matched brain regions in tissue sections from control

216 non-MS cases. The average "true NAWM" spectra did not overlap with region-matched  
217 control spectra, suggesting possible underlying pathology in MS tissue, which is not  
218 differentiated when using lipophilic histochemistry or immunostaining with conventional  
219 techniques (31).

220 The three major features in the CH spectral region analysed, correspond to the symmetric  
221 and asymmetric and asymmetric stretching of acyl chain methylene at 2850 and 2886  $\text{cm}^{-1}$   
222 respectively, and the  $\text{CH}_3$  methyl chain end symmetric stretch at 2935  $\text{cm}^{-1}$ , which is thought  
223 to include protein contributions as well. Observing the intensity ratios of 2850/2880  $\text{cm}^{-1}$  and  
224 2935/2880  $\text{cm}^{-1}$  the authors noted a slowly decreasing trend across all measured intensity  
225 ratios, when moving away from the lesion site until reaching the "true NAWM" ratios,  
226 recorded from an area the furthest away from the lesion, and approaching the ratios of  
227 region-matched non-MS control samples. The 2850/2880  $\text{cm}^{-1}$  ratio is thought to relate to the  
228 intermolecular packing, interchain interactions and intrachain torsional motions, whereas the  
229 2935/2880  $\text{cm}^{-1}$  ratio allows monitoring intramolecular chain disorder and trans-gauche  
230 isomerisation. The authors conclude that biochemistry of myelin lipid content changes in the  
231 lesion periphery and in NAWM (31).

### 232 **Mouse models of demyelination and remyelination**

233 Animal models of MS have also been investigated by vibrational spectroscopy, where most  
234 studies aim to elucidate the mechanisms behind demyelination and remyelination. Heraud *et*  
235 *al.* used FTIR spectroscopy to investigate macromolecular components and protein  
236 conformational changes in the CNS of EAE versus control tissue sections(32). Using  
237 principal component analysis (PCA) and artificial neuronal networks (ANN) to analyse single  
238 data acquisition spectra, the authors demonstrated, without the need for chemical stains,  
239 subtle chemical and structural changes, particularly in the secondary structure of proteins in  
240 the white matter (33).

241 Fu *et al.* used resonant CARS imaging from the symmetric  $\text{CH}_2$  stretch vibration at 2840  $\text{cm}^{-1}$   
242 to characterize myelin changes induced by lysophosphatidyl choline (lyso-PtCho) (34).  
243 Although not directly relevant to demyelinating diseases including MS, the authors reported  
244 CARS was able to characterise the changes occurring in lyso-PtdCho-induced myelin  
245 breakdown and that together with electrophysiological data, it revealed involvement of a  
246  $\text{Ca}^{2+}$ , calpain, and cPLA<sub>2</sub>-dependent pathway (34).

247 In another study, CARS was used to study myelin loss in the mouse-model, Relapsing-EAE  
248 (R-EAE) (35). Two theories have been hypothesised for initiating demyelination, one where

249 the injury starts at internodal myelin, thinning layer by layer and the other, where it initiates  
250 with paranodal domain injury. The authors noted that the submicron spatial resolution of  
251 CARS images allowed not only the quantification of myelin thickness but also the ratio of  
252 myelin thickness to the axonal diameter at different stages of the disease process.  
253 Furthermore, two-photon immunofluorescence microscopy revealed that juxtaparanodal K<sup>+</sup>  
254 channels, paranodal myelin retraction and the displacement of K<sup>+</sup> channels was extensively  
255 observed at the onset of R-EAE and at lesion borders. Overall their results suggested loss of  
256 nodal integrity precedes the formation of myelin debris in the CD4<sup>+</sup> T-cell-mediated R-EAE  
257 model of MS and that remyelination is accompanied by reestablishment of the nodal makers,  
258 with myelin being only partially restored (35).

259 Furthermore, the Raman spectra of myelin were dominated by lipid assignments and the  
260 authors studied both C-C and C-H vibrational bands to determine the conformation of their  
261 hydrocarbon chains through: (1) lipid packing studied using prominent bands at 2850, 2885  
262 and 2930 cm<sup>-1</sup>, assigned respectively to stretching and asymmetric stretching of CH<sub>2</sub> and to  
263 CH<sub>3</sub> stretching: and, (2) lipid unsaturation using the <sup>1</sup>1650/<sup>1</sup>1445 ratio, which represents the  
264 C=C stretching bands to H-C-H deformation bands in lipid acyl chains. Myelin debris  
265 presented a higher intensity of the <sup>1</sup>2930/<sup>1</sup>2885 ratio, reflecting an increased intermolecular  
266 chain disorder; and regenerated myelin presented a higher lipid-packing disorder than  
267 normal myelin. Similarly, myelin debris presented the highest unsaturation degree, which  
268 was decreased in regenerated myelin but nevertheless was higher than normal myelin.  
269 Finally, the analysis of the <sup>1</sup>1122/<sup>1</sup>1076 ratio, revealed no significant change could be  
270 observed in the intramolecular chain ordering of myelin debris, normal and regenerated  
271 myelin (35).

272 A non-invasive multimodal CARS system, combining reflectance for visualizing axons,  
273 fluorescence to visualize green fluorescence protein (GFP) and Raman to visualize myelin  
274 and to monitor microglia induced neurodegeneration was reported by Imitola *et al.* (36).  
275 Using an EAE model, the authors reported fast *ex vivo* imaging of myelin, axons and  
276 microglia with great anatomical precision in live tissue. CARS images showed a global  
277 decrease in myelination, not seen before through other imaging techniques. This suggests  
278 that subtle alterations in the myelin lipid content may precede hallmark CNS demyelination,  
279 which is correlated with axonal loss and microglia activation (36).

280 Wang *et al.* reported DBT (3,3'-diethylthiatricarbocyanine iodide) to be a promising probe for  
281 Near Infrared Fluorescence (NIRF) imaging of myelination (37). Through *in vivo* NIRF  
282 studies on hyper and hypomyelination mouse models, the authors demonstrated DBT  
283 successfully enters the brain and selectively binds to myelin sheaths. Furthermore, aiming to

284 broaden NIRF-DBT imaging to MS disease, the authors studied a cuprizone-induced mouse  
285 model for demyelination and remyelination. NIRF imaging and quantitative analysis revealed  
286 DBT could successfully monitor the level of demyelination and subsequent remyelination in  
287 this mouse model, that could be correlated with histochemical staining (37).

## 288 **Future research directions**

289 The current literature, as reviewed above, considering human post-mortem CNS tissue  
290 specimens is limited, with most studies considering a nominal sample number as shown in  
291 Table 1, which also summarises the studies completed in models of MS.

292 Studies focusing on animal models have shown spectroscopy to be a valuable tool in  
293 probing the biochemical composition of samples otherwise deemed identical. The spectral  
294 imaging of myelinating and remyelinating processes, for example, further demonstrated the  
295 ability to differentiate between newly formed myelin and endogenous myelin, indicating the  
296 remyelinating process generates myelin of a different composition. Nonetheless, studies  
297 considering human samples are limited and concern only a small number of post-mortem  
298 tissues as human CNS material is difficult to obtain. Furthermore, most studies focused on  
299 the spectral distinction of MS lesions from control tissue of non-diseased subjects, which can  
300 readily be achieved by macro and microscopic evaluation using luxol-fast blue (LFB) stain or  
301 immunohistochemistry for myelin proteins to examine demyelination. As disease diagnosis  
302 through tissue sampling is not feasible, the advantages of spectroscopy techniques such as  
303 FTIR and Raman rely on their ability to reveal underlying biochemical changes not yet  
304 detectable either macro or microscopically, for example on NAWM of MS cases, when  
305 common techniques fail to recognize differences. Spectral data could potentially help to  
306 understand the underpinning mechanisms of disease and advance research in the field by  
307 probing deeper into the chemical composition of apparently normal areas of MS cases.

## 308 **FTIR and Raman spectroscopy analysis of post-mortem white matter MS tissue: 309 NAWM has a different signature**

310 Analysing four post-mortem brain samples obtained from UK MS Society Tissue Bank  
311 (Imperial College London) we show FTIR signatures allow the distinction of normal control  
312 WM from both active and chronic lesions, and more interestingly from the NAWM of MS  
313 cases despite no visible demyelination being observed when staining NAWM with LFB. The  
314 mean FTIR spectra of a brain tissue sample from control, NAWM, active lesion and a chronic  
315 lesion are represented in Figure 1, where it is possible to observe that the symmetric and  
316 anti-symmetric C-H stretches attributed to lipids  $\sim 2800-3000\text{ cm}^{-1}$  gradually decrease from

317 control to active lesion, as do the C-O and P-O stretches attributed to nucleic acids after  
318  $1000\text{ cm}^{-1}$ .

319 This decrease in lipid content seems to be in line with the previous findings acknowledged in  
320 this review of published work and is in agreement with the well characterised process of  
321 demyelination which occurs in MS, providing support to the validity of this approach to the  
322 study of the biochemical composition of brain tissue in MS.

323 Principal Component Analysis (PCA) was further employed to highlight the variability existing  
324 in the recorded spectral data set. PCA of FTIR signatures allowed the distinction of normal  
325 control WM from both active and chronic lesions, as expected, but also differentiated NAWM  
326 of the MS cases from control white matter cases. 2-D PCA scatterplot is shown in Figure 2.

327 A clear distinction between all sample groups can be observed. If the distinction between  
328 control and chronic and active lesions were expected due to MS pathology, the separation  
329 between NAWM and control WM provides novel insights into the alterations in white matter  
330 in MS which might contribute to disease progression.

331 PCA was also employed to compare FTIR data from NAWM and control white matter  
332 samples and results are shown in Figure 3. Figure 3A indicates that NAWM and control  
333 white matter FTIR spectra separate according to the 1<sup>st</sup> principal component (PC1) which  
334 accounts for 80.63% of the variation observed within the data set. The PC1 loading  
335 represented in Figure 3B shows that this separation is dominated by the negative loading of  
336 two main lipid assignments  $\sim 2800\text{-}3000\text{cm}^{-1}$  indicating these are more intense in the control  
337 WM samples (in black on the negative part of the PCA plot (Figure 3A)).

338 These preliminary results demonstrate that FTIR spectroscopy can be applied to analysis of  
339 post-mortem WM tissue and successfully discriminate not only between lesion and control  
340 WM but also between NAWM and control, without requiring any additional techniques.  
341 Furthermore, they are suggestive of a significant decrease in lipid content in NAWM tissue in  
342 MS cases, which is not detected by current staining techniques or documented in the  
343 literature, but ought to be further investigated to better understand MS pathogenesis and the  
344 biochemical changes that lead to lesion formation. Finally, the spectral signatures of the  
345 fingerprint region also pointed to additional differences at the protein and nucleic acid level;  
346 these pose further questions as to which specific species (i.e. proteins) are being 'lost' in  
347 NAWM samples, which could contribute to the disease process.

348 Similarly, the samples were also analysed using a Horiba XploRA PLUS confocal Raman  
349 microscope, operating with 532nm laser light and 1800nm lines grating. Raman signatures

350 of the fingerprint region were analysed using PCA and results are shown in Figure 4. PCA  
351 score plots, in Figure 4A, showed the separation of NAWM (black) and control WM (green)  
352 only to be achieved on the third PC which account for approximately 2% of the variance  
353 found within the dataset. PC 1 and 3 loadings are shown in Figure 4B. Our group is  
354 currently investigating the Raman signatures of NAWM samples further and a full research  
355 paper will be published in due course.

### 356 **FTIR and Raman spectroscopy analysis of biofluids**

357 Much like tissues, biofluids exhibit vibrational spectra that have characteristic bands  
358 reflecting their bimolecular composition (4). There are several reports of the application of  
359 Raman and FTIR spectroscopy to the study of body fluids. Although blood and serum are  
360 most commonly used due to their easy, less-invasive availability other biofluids including  
361 cerebrospinal fluid (CSF), bile, urine, saliva, pancreatic juice, synovial and pleural fluids,  
362 which are considered to more closely reflect ongoing pathology in the associated diseased  
363 tissue, have also been studied. In Alzheimer's disease, serum data from Raman  
364 spectroscopy allowed differentiation of Alzheimer's patients from other dementia cases (38),  
365 whereas in a different study, plasma spectral data was used to grade mild, moderate and  
366 severe Alzheimer's disease cases (39). FTIR spectroscopy showed Alzheimer's patients'  
367 plasma samples to be well delineated from normal ageing subjects (40) and the same was  
368 demonstrated for CSF (41). More recently, PCA-LDA allowed the distinction of the different  
369 types of mild, moderate and severe Alzheimer's disease cases and controls, with 85%  
370 accuracy, when using white blood cells from patients, using FTIR spectra and about 77%  
371 when using the plasma spectra. These 83% accuracy values increased to 83 and 89% when  
372 only moderate and severe patient groups were being considered (42).

373 FTIR spectroscopy analysis of synovial fluid has been shown to allow differentiation of joints  
374 affected by rheumatoid arthritis, osteoarthritis, spondyloarthropathies and meniscal  
375 injuries(43); whereas a Raman study showed the ability to discriminate patients with low and  
376 high osteoarthritis severity (44). More recently, FTIR analysis of blood plasma for diagnosis  
377 of schizophrenia and bipolar disorders against a healthy control group has also been  
378 reported (45). A separation of all sample groups was observed using PCA, with assignments  
379 to lipids from lipoproteins, polypeptides, and phosphates associated to the DNA backbone  
380 being responsible for the separation; whilst PLS-DA allowed for the correct classification of  
381 all sample groups. Sensitivity and specificity results were highest when the full spectral  
382 range was considered, being respectively 100 and 100% for schizophrenia and 100 and  
383 84.6% for bipolar disorder.

384 Overall, as demonstrated in this review and from our own FTIR preliminary data,  
385 spectroscopic techniques have the potential to advance our knowledge of MS pathogenesis.  
386 The analysis of post-mortem material, especially the comparison between NAWM and  
387 normal WM can provide insights into molecular changes unveiling novel disease  
388 mechanisms. And, although currently it cannot be applied for diagnostic purposes, due to the  
389 constraints of obtaining brain tissue specimens, other patient specimen samples such as  
390 CSF and blood might prove useful in the future to achieve a more rapid and accurate  
391 diagnosis and prognosis for people with MS, much like has been recently reported for other  
392 CNS diseases such as Alzheimer's.

### 393 **Conclusion**

394 The understanding of the underlying mechanism that lead to disease pathology and specially  
395 disease progression is of great importance in neurodegenerative conditions, such as MS.  
396 Spectroscopy techniques have the ability to unbiased characterisation of the biochemical  
397 composition of post-mortem and clinical samples alike, thus able of providing insights into  
398 the underlying changes occurring in tissue and biofluids (i.e. blood and CSF) which in turn  
399 could be helpful to guide future *in vitro* research aimed at novel therapeutics.

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403 for sectioning and staining the samples.

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**Table 1** Studies considered in this literature review. Sample specimen type and numbers included are indicated as well as the publication year and the spectroscopic technique used.

<b>Authors</b>	<b>Year</b>	<b>Tissue Sample species</b>	<b>No. samples</b>	<b>Spectroscopic technique</b>
<b>Choo <i>et al.</i></b>	<b>1993</b>	<b>Human</b>	<b>3</b>	<b>FTIR</b>
<b>LeVine <i>et al.</i></b>	<b>1998</b>	<b>Human</b>	<b>10</b>	<b>FTIR</b>
Fu <i>et al.</i>	2007	Mice	-	CARS
Heraud <i>et al.</i>	2010	Mice	12	FTIR
Belanger <i>et al.</i>	2011	Mice	14	CARS
Shi <i>et al.</i>	2011	Guinea pigs	-	CARS
Wang <i>et al.</i>	2011	Mice	7	NIRF
Imitola <i>et al.</i>	2011	Mice	-	CARS
Fu <i>et al.</i>	2011	Mice	-	CARS
Begin <i>et al.</i>	2013	Mice	4	CARS
<b>Poon <i>et al.</i></b>	<b>2013</b>	<b>Human</b>	<b>5</b>	<b>CARS</b>
Hu <i>et al.</i>	2014	<i>Xenopus laevis</i>	-	SRS
Marro <i>et al.</i>	2014	Murine retinal organotypic cultures	12	Raman Spectroscopy
<b>Poon <i>et al.</i></b>	<b>2015</b>	<b>Human</b>	<b>6</b>	<b>CARS</b>

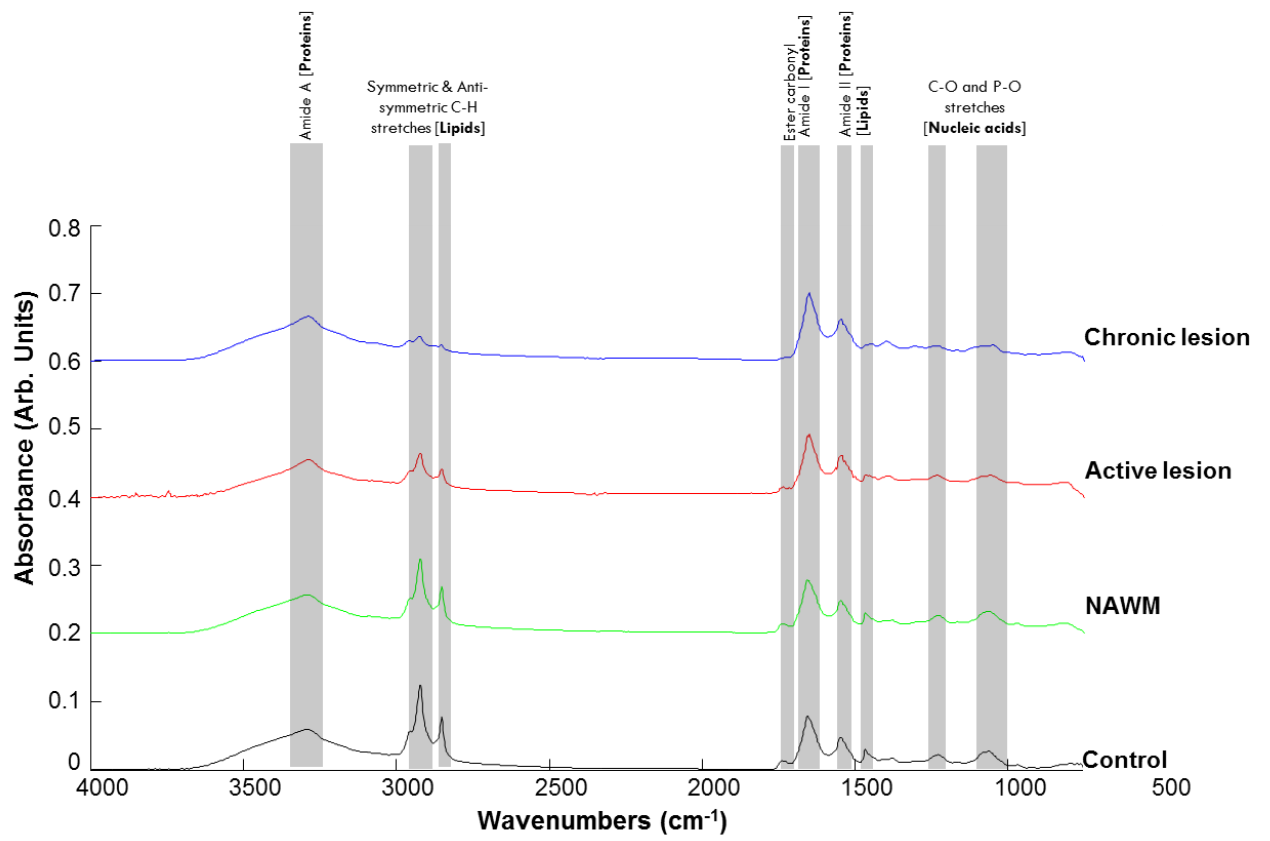
**Figure 1.** Mean FTIR spectra for four post-mortem samples analysed in the preliminary study: chronic lesion (blue), active lesion (red), NAWM (green) and control (black). All samples were subjected to FTIR analysis at the Focas Research Institute, DIT, using a Perkin Elmer Spotlight 400N FTIR imaging system, incorporating a liquid nitrogen cooled mercury cadmium telluride 16x1, 6.25 $\mu\text{m}$  pixel array detector, and acquired by the Spectral Image software. FTIR images from the tissue sections (10 $\mu\text{m}$  sections) mounted on  $\text{CaF}_2$  slides were recorded over the range 4000-800  $\text{cm}^{-1}$  in transmittance mode with a resolution of 4  $\text{cm}^{-1}$  and interferometer speed of 1.0  $\text{cm}^{-1}$ /second at continuously varying magnification. The scans per pixel for background were 120 and, for images, 16 per pixel respectively. Spectroscopic data analysis was carried out in Matlab, version R2013 (Mathworks, CA, USA) according to protocols developed and routinely used in-house at DIT.

**Figure 2.** PCA of the four post-mortem samples FTIR data. (A) 2-D PCA scatterplot showing a separation between chronic (blue) and active lesion (red) and, NAWM (green) and control (black). (B) PC1 loading, responsible for the separation, is negatively dominated by peaks assigned to lipids around 2800-3000 $\text{cm}^{-1}$ .

**Figure 3.** PCA of the NAWM and control FTIR data. (A) The two dimensional PCA plot shows a separation between NAWM (black) and control (green) FTIR spectra in PC1 which explains 80.63% of the variation found in the data. (B) The PC1 loading is negatively dominated by peaks assigned to lipids around 2800-3000 $\text{cm}^{-1}$ .

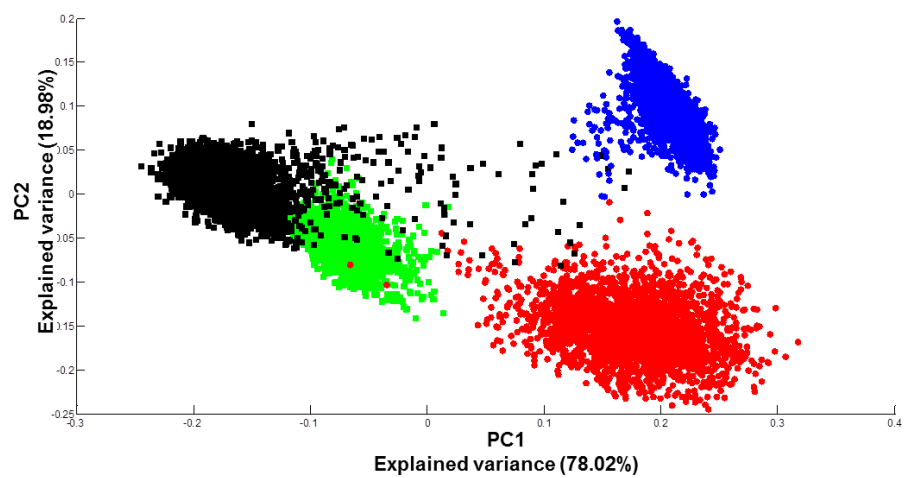
**Figure 4.** PCA of the four post-mortem samples Raman spectroscopy data. (A) 2-D PCA scatterplots showing a separation between chronic (blue) and active lesion (red) and, NAWM (green) and control (black). (B) PC1 and PC3 loadings, responsible for the separation.

Figure 1



**Figure 2**

**A**



**B**

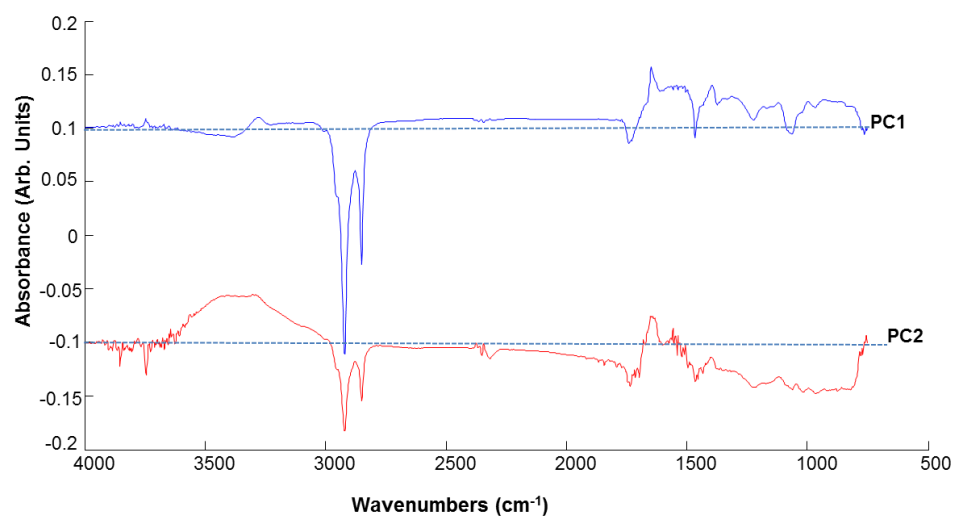
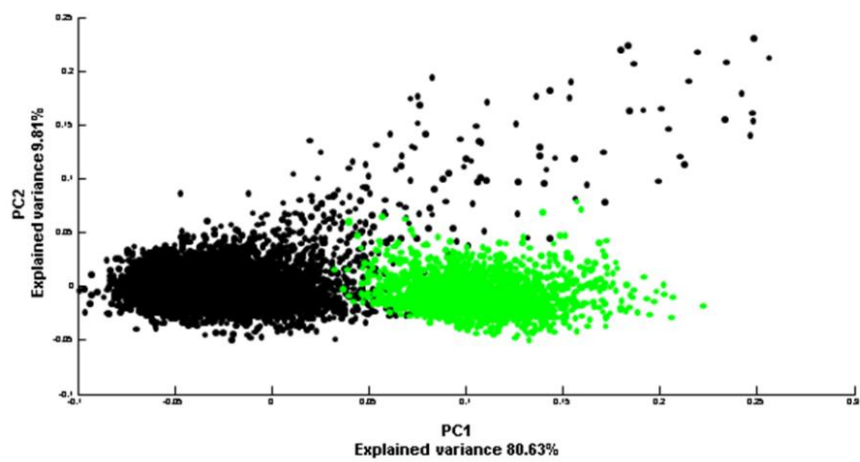




Figure 3

A



B

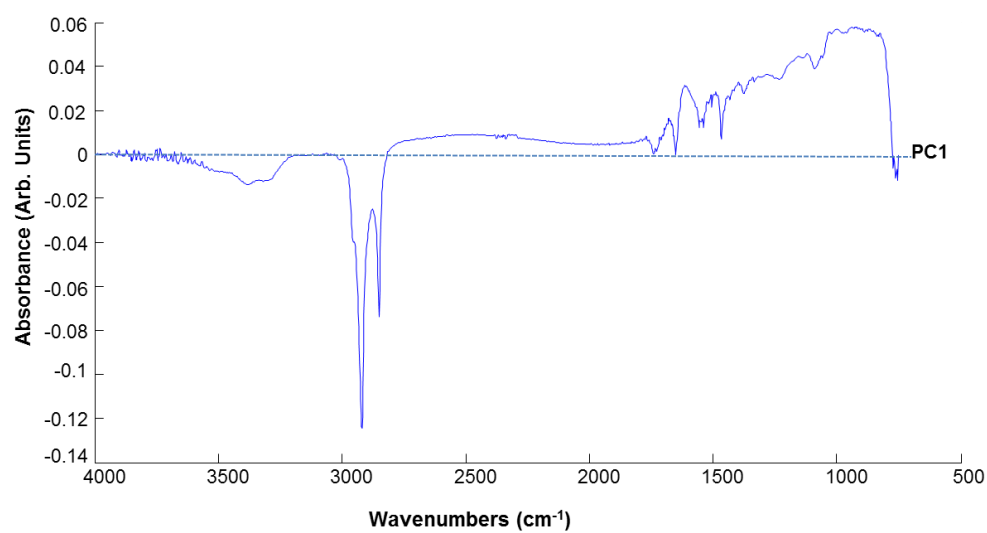
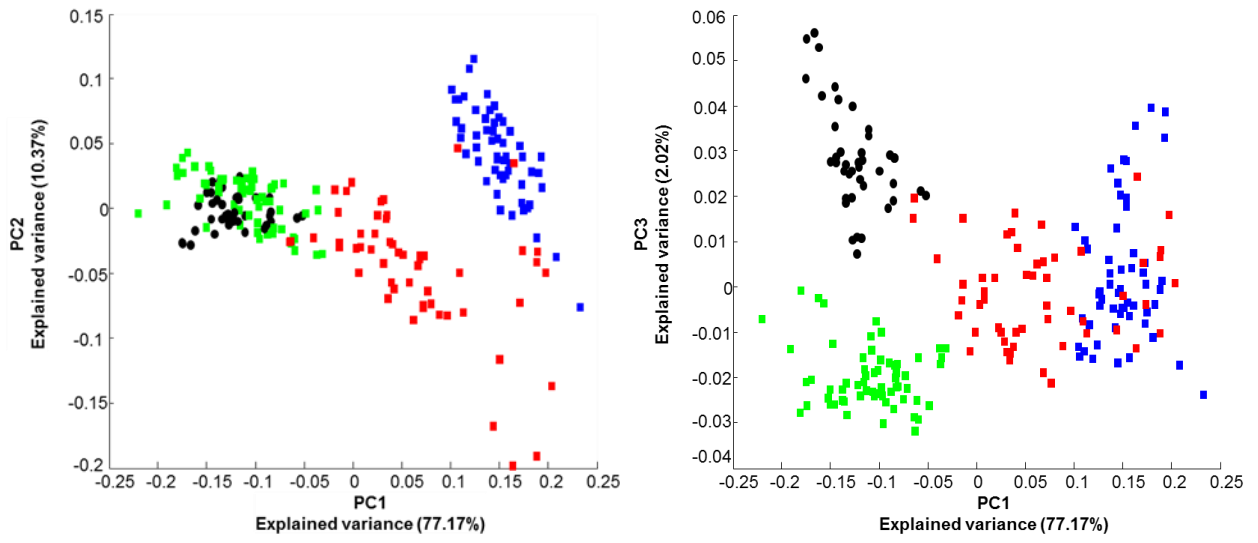
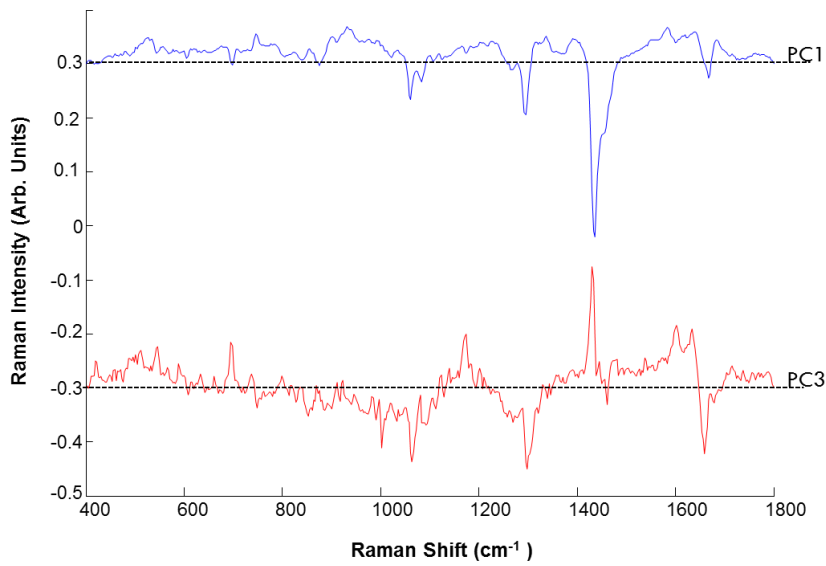


Figure 4

A



B



567