Chemical Profiling of Cerebrospinal Fluid by Multiple Reaction Monitoring Mass Spectrometry

Christina R. Ferreira, Karen E. Yannell , Brit Mollenhauer, Ryan D. Espy, Fernanda B. Cordeiro, Z. Ouyang, and R. G. Cooks

Method Development and Samples

Methanol was purchased from Avantor Performance Materials (Center Valley, PA, US), and acetic acid from Mallinckrodt Baker Inc. (Phillipsburg, NJ, US.). Cerebrospinal fluid (CSF) samples (N=10 clinical control and N=17 PD) supplied by Elena-Paracelsus-Klinik (Kassel, Germany) were used for method development.

For electrosonic spray ionization (ESSI) each CSF sample was diluted 16 times with 95% methanol, 5% water and 0.1% acetic acid. This solution was mixed well and injected via syringe into the spray source. ESSI is an ionization methodology that is comparable to electrospray ionization. It utilizes a high velocity nebulizing gas surrounding the sample emitter to rapidly evaporate the charged microdroplets and create gas phase ions.[1] ESSI is a good ionization technique to use in exploratory methodologies because it provides a direct injection of the sample removing any potential interferences from other ionization techniques. The ESSI source (**Figure 1S**) consists of a sample syringe connected to fused silica line that has a 100 µm inner diameter. This line is inserted through a Swagelock T fitting and allowed to protrude 0.1-0.2 mm to create a spray tip. Nitrogen at 100 psi is injected into the T fitting and serves as the nebulizing gas for the spray by covering the spray tip in a high velocity gas. The spray tip is placed 1 cm away from the mass spectrometer inlet. In this experiment, a high voltage of 3.5 kV was applied to the sample syringe and the sample solution was injected at a flow rate of 3 µL/min. The spray plume was observed to be stable before every sample was run.

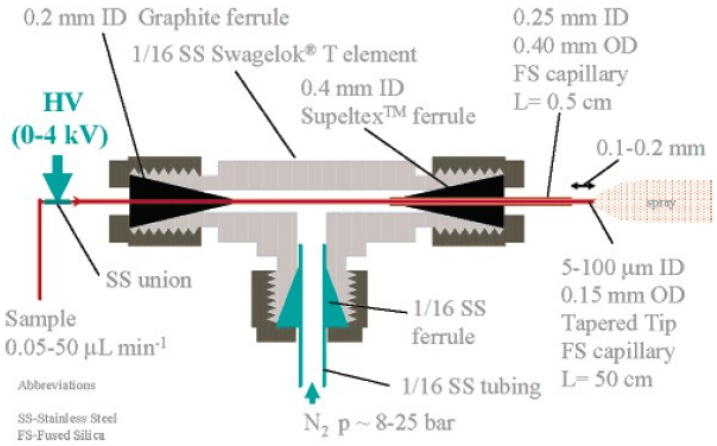

Figure S1: Illustration of the ESSI source with dimensional details of its parts (Takats *et al.*, 2004).

A triple quadrupole mass spectrometer (TSQ Quantum Access Max, Thermo Scientific, San Jose, CA) operated in the positive ion mode was used for all experiments. The MRM transitions were selected based on exploratory research by neutral loss and precursor ion scan experiments (**Table S1**) using pooled samples representative of the control and disease groups. Also screening was performed for MRMs reported in the literature[2]. Values of collision energy (CE) and tube lens (TL) were optimized experimentally for each MRM (**Table S2**).

Table S1. Examples of neutral losses and precursor ion scan that can be used to identify informative MRMs.

Mass units Lost	***Ion mode***	***Scan mode***	***Related common metabolites***
17	pos/neg	NL	*Amines*
18	pos/neg	NL	Carboxylic Acids, Aldehydes
28	pos/neg	NL	Aldehydes, Carboxylic Acids
44	pos/neg	NL	Carboxylic Acids
176	pos/neg	NL	Glucoronides
162	pos/neg	NL	Hexose Sugars
132	pos/neg	NL	Pentose Sugars
80	pos	NL	Phenolic Sulphates
79	neg	Prec	Phosphates
124	neg	Prec	Taurines
129	neg	NL	N-Acetylcysteines
85	pos	Prec	Acylcarnitines

NL = netural loss; Prec = precursor scan

Table S2. Ion pairs (precursosr and fragment), collision energy (CE) settings for the MRM profiling method, and compound possibly associated with the MRMs based on the HMBD database[*#]

Precursor	*Fragment*	*CE*	*Suggested compound from HMDB precursor mass*
76.2	58.3	5	Not attributed
76.2	59.4	16	Trimethylamine N-Oxide (HMDB00925)
78.2	61.5	5	Cysteamine (HMDB02991)
79.2	61.5	5	Dimethyl sulfoxide (HMDB02151)
88.1	57.5	15	Pyruvic acid/Putrescine (HMDB00243)
88.1	71.3	7	Pyruvic acid/Putrescine (HMDB00243)
113.1	59.2	20	Uracil (HMDB00300)
113.1	70	17	Uracil (HMDB00300)
113.1	77.3	14	Uracil (HMDB00300)
115.1	79.2	15	Fumarate/Maleate (HMDB00134)
115.1	97.1	5	Fumarate/Maleate (HMDB00134)
116.1	70	15	Proline (HMDB00162)
116.1	88.2	7	Proline (HMDB00162)
116.1	98.1	15	Proline (HMDB00162)
116.1	98.4	8	Proline (HMDB00162)
117.0	81.2	6	Not assigned
117.0	99.1	10	Not assigned
120.1	84	14	L-Threonine (HMDB00167)
120.1	102.4	5	L-Threonine (HMDB00167)
122.1	68.4	25	L-Cysteine/Nicotinate (HMDB00167)
122.1	77.3	10	L-Cysteine/Nicotinate (HMDB00167)
132.1	44.5	25	Creatine (HMDB00064)
132.1	90.5	13	Creatine/(iso)leucine/4-OH-proline (HMDB00064)
133.0	115.3	7	L-asparagine (HMDB00168)
134.1	72.4	18	Aspartate (HMDB00191)
134.1	115.8	5	Aspartate (HMDB00191)
136.1	100.2	12	Homocysteine (HMDB00742)
141.0	81.1	5	Methylimidazoleacetic acid (HMDB02820)
141.0	83.2	6	Not Assigned
142.1	124.2	8	Not Assigned
146.1	109.9	15	4-Guanidinobutanoic acid (HMDB03464)
150.1	114.3	5	Not Assigned
151.0	115.1	9	L-Threo-2-pentulose (HMDB00751)
151.0	83.23	18	L-Threo-2-pentulose/D-Xylulose/Xanthine (HMDB00751)
153.0	99.1	13	D-Arabitol/Cystamine (HMDB00568)
153.0	135	5	D-Arabitol/Cystamine (HMDB00568)
153.0	55.3	28	D-Arabitol/Cystamine (HMDB00568)
154.0	136.1	5	3-Sulfinoalanine/3-OH-anthranilate (HMDB00996)
155.0	*119.1*	*11*	*Orotate (HMDB00226)*

155.0	*137.1*	*5*	*Orotate (HMDB00226)*
166.0	*105.2*	*5*	*Quinolinic acid (HMDB00232)*
166.0	*103.2*	*28*	*Not Assigned*
166.1	*120.2*	*14*	*Not Assigned*
167.0	*131.1*	*7*	*Not Assigned*
168.0	*149.8*	*6*	*Quinolinic acid (HMDB00232)*
168.1	*150.2*	*5*	*Quinolinic acid (HMDB00232)*
169.0	*151.1*	*5*	*3,4-Dihydroxybenzeneacetic acid/DHAP (HMDB01336)*
169.0	*151.2*	*6*	*3,4-Dihydroxybenzeneacetic acid/DHAP (HMDB01336)*
169.0	*115.1*	*18*	*3,4-Dihydroxybenzeneacetic acid/DHAP (HMDB01336)*
169.2	*151.2*	*5*	*Not Assigned*
170.0	*152.2*	*6*	*Not Assigned*
171.0	*134.9*	*5*	*Not Assigned*
175.0	*139*	*5*	*N-Acetyl-L-aspartic acid (HMDB00812)*
175.0	*157.1*	*5*	*Suberic acid/N-Acetyl-L-aspartic acid/Ascorbate (HMDB00893)*
175.1	*70.3*	*25*	*Arginine (HMDB00517)*
177.0	*141.1*	*6*	*Serotonin (HMDB00259)*
177.0	*159.1*	*5*	*Serotonin (HMDB00259)*
177.0	*159*	*5*	*Serotonin (HMDB00259)*
177.1	*160*	*12*	*Serotonin (HMDB00259)*
181.2	*74.3*	*23*	*Arginine stable isotope*
182.0	*165.1*	*6*	*Hydroxyphenyllactic acid (HMDB00755)*
182.0	*91.2*	*30*	*Tyrosine (HMDB00158)*
182.8	*165.2*	*5*	*Homovanillic acid (HMDB00118)*
183.0	*164.9*	*6*	*Not Assigned*
183.0	*165.1*	*6*	*Homovanillic acid (HMDB00118)*
184.0	*125.2*	*7*	*Phosphorylcholine (HMDB01565)*
184.0	*166.1*	*5*	*Phosphorylcholine (HMDB01565)*
184.0	*166.2*	*5*	*Phosphorylcholine (HMDB01565)*
185.0	*166.8*	*5*	*Vanylglycol (HMDB01490)*
185.0	*167.1*	*5*	*Vanylglycol (HMDB01490)*
188.0	*171*	*10*	*Acetylspermidine (HMDB01276)*
192.5	*176.5*	*8*	*Isocitric acid (HMDB00193)*
192.8	*135*	*10*	*5-Hydroxyindoleacetic acid/5-Methoxytryptophol/Isocitric acid*
192.8	*175.1*	*5*	*5-Hydroxyindoleacetic acid/5-Methoxytryptophol/Isocitric acid*
192.8	*175*	*5*	*5-Hydroxyindoleacetic acid/5-Methoxytryptophol/Isocitric acid*
195.0	*99.2*	*18*	*Caffeine (HMDB01847)*
195.0	*158.9*	*7*	*Not Assigned*
196.0	*136.1*	*5*	*Not Assigned*
196.0	*159.8*	*5*	*Not Assigned*
196.0	*178.9*	*5*	*Not Assigned*

196.9	*81.1*	*6*	*L-Dopa (HMDB00181)*
197.0	*179.1*	*5*	*L-Dopa (HMDB00181)*
198.0	*180.3*	*7*	*L-Dopa (HMDB00181)*
199.0	*81.2*	*10*	*Erythrose-4-P (HMDB01321)*
205.0	*145.2*	*10*	*L-Tryptophan (HMDB00929)*
205.0	*187.1*	*5*	*L-Tryptophan (HMDB00929)*
219.0	*201*	*5*	*Pantothenic acid/N-acetylserotonin (HMDB01238)*
219.1	*159.1*	*13*	*Pantothenic acid/N-acetylserotonin (HMDB01238)*
219.3	*202.3*	*8*	*Pantothenic acid/N-acetylserotonin (HMDB01238)*
220.0	*201.9*	*5*	*Pantothenic acid(HMDB00210)*
225.0	*164.7*	*5*	*Not Assigned*
225.0	*105.1*	*11*	*Not Assigned*
228.0	*210.1*	*7*	*Not Assigned*
285.0	*105*	*20*	*Not Assigned*
285.0	*225*	*5*	*Not Assigned*
289.0	*271*	*8*	*Not Assigned*
290.9	*273*	*5*	*Androsterone (HMDB00031)*
328.9	*246.8*	*5*	*Not Assigned*
338.8	*81.1*	*21*	*Not Assigned*
338.8	*256.8*	*10*	*Not Assigned*
344.0	*224.2*	*6*	*Not Assigned*
344.0	*283.5*	*5*	*cGMP (HMDB01314)*
346.0	*285.6*	*7*	*cGMP (HMDB01314)*
386.0	*303.9*	*8*	*Not Assigned*
386.3	*371.3*	*8*	*Not Assigned*
386.7	*244.7*	*10*	*Not Assigned*
386.9	*247*	*11*	*Not Assigned*
386.9	*326.5*	*7*	*Not assigned*
387.0	*245*	*12*	*Not assigned*
387.2	*371.2*	*8*	*Not assigned*
403.8	*288.2*	*17*	*Not Assigned*
407.1	*347*	*12*	*Not assigned*
442.2	*360.3*	*5*	*Not assigned*
448.0	*388.5*	*5*	*Not assigned*
471.9	*359*	*14*	*Not assigned*
514.6	*398.7*	*13*	*Not assigned*
522.9	*342.9*	*6*	*Not assigned*
678.1	*618.3*	*12*	*Not assigned*

Notes:

** Most of the attributed compounds have been already reported in the CSF metabolome database (http://www.csfmetabolome.ca/).*

[#]Parent ions and fragments matched MS/MS mass spectra from the human metabolome database (http://www.hmdb.ca).

Each MRM was scanned for 0.10 sec using a 0.7 Da isolation window and optimized values for collision energy (CE) and tube lens (TL) voltages. Ten scans per MRM were summed to obtain the final intensity value of each MRM. A minimum of three replicates was recorded for each sample.

The final method was applied to the BioFIND samples. Details on the gender, age, years of diagnosis and staging of the patients for the BioFIND samples are shown **in Figure 2S.**

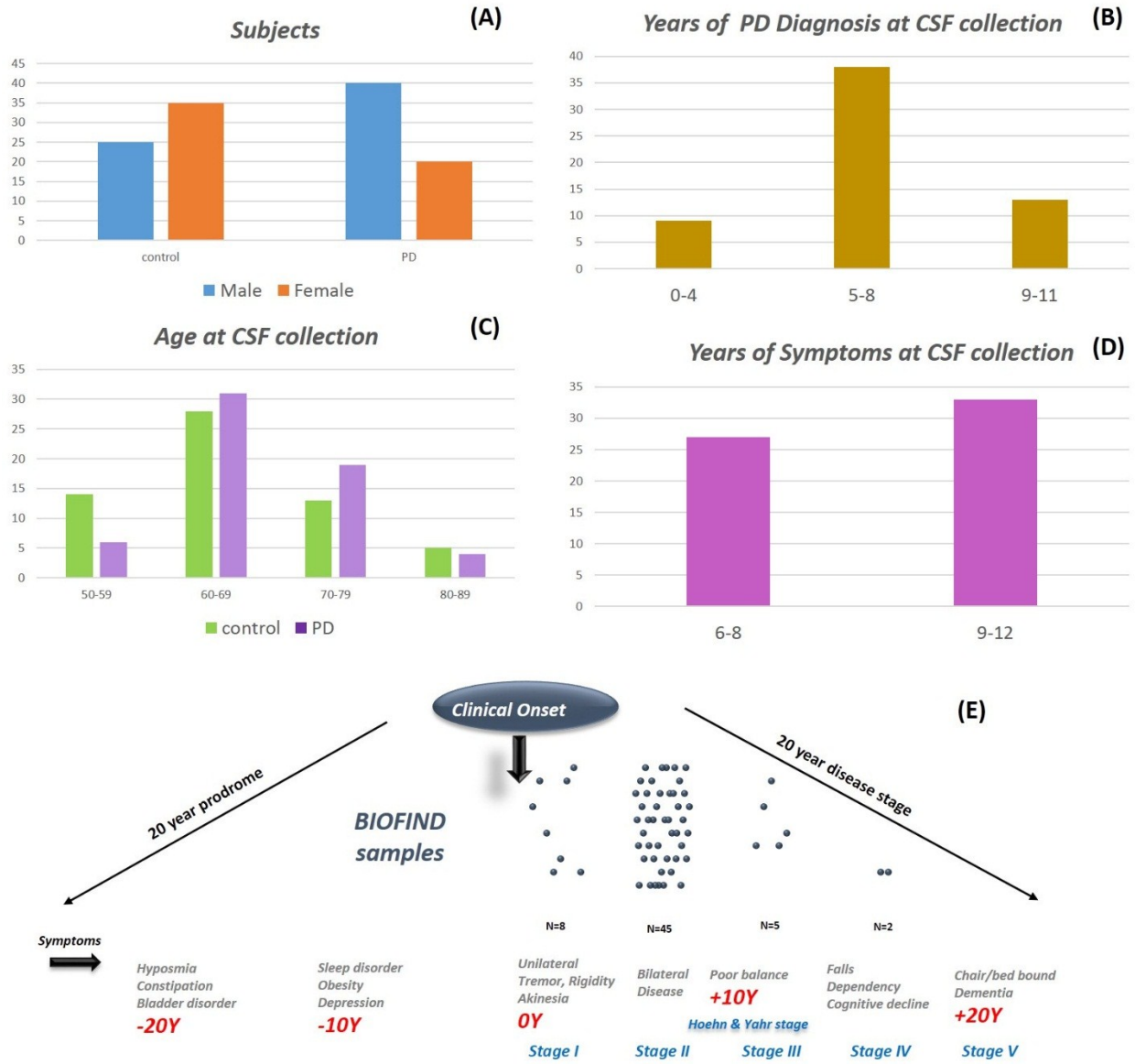

Figure ~~2S~~S2. Distribution of the 120 BioFIND samples according to **(A)** gender, **(B)** years of PD diagnosis at CSF collection, **(C)** Age at CSF collection; and **(D)** years of symptoms at CSF collection. **(E)** Distribution of BioFIND PD samples (dark dots, N=60) according to the Hoehn & Yahr stage scale. Most of the samples (75%) have been classified as Stage II. This schematics is adapted from Hawkes et al., 2010[3] to illustrate the complexity of PD. Prior to the motor phase of classical PD there is a prodromal period

spanning several years. Typical motor features appear in the initial clinical phase and the disease progresses up to 20 years with clinical sign heterogeneity.

Data processing

Ion intensities of each MRM were normalized to the ion intensity of the MRM of endogenous arginine because this compound has been reported to present stable levels in human CSF even in the presence of neurodegenerative conditions.[4] Experiments indicated that the use of endogenous arginine as a calibrator was appropriate since comparable discrimination and homogeneity was observed after multivariate analysis for three replicates performed on three different days. Also, arginine spiked into artificial CSF at different physiological concentration levels showed linearity in the concentration response (**Fig. 3S**).

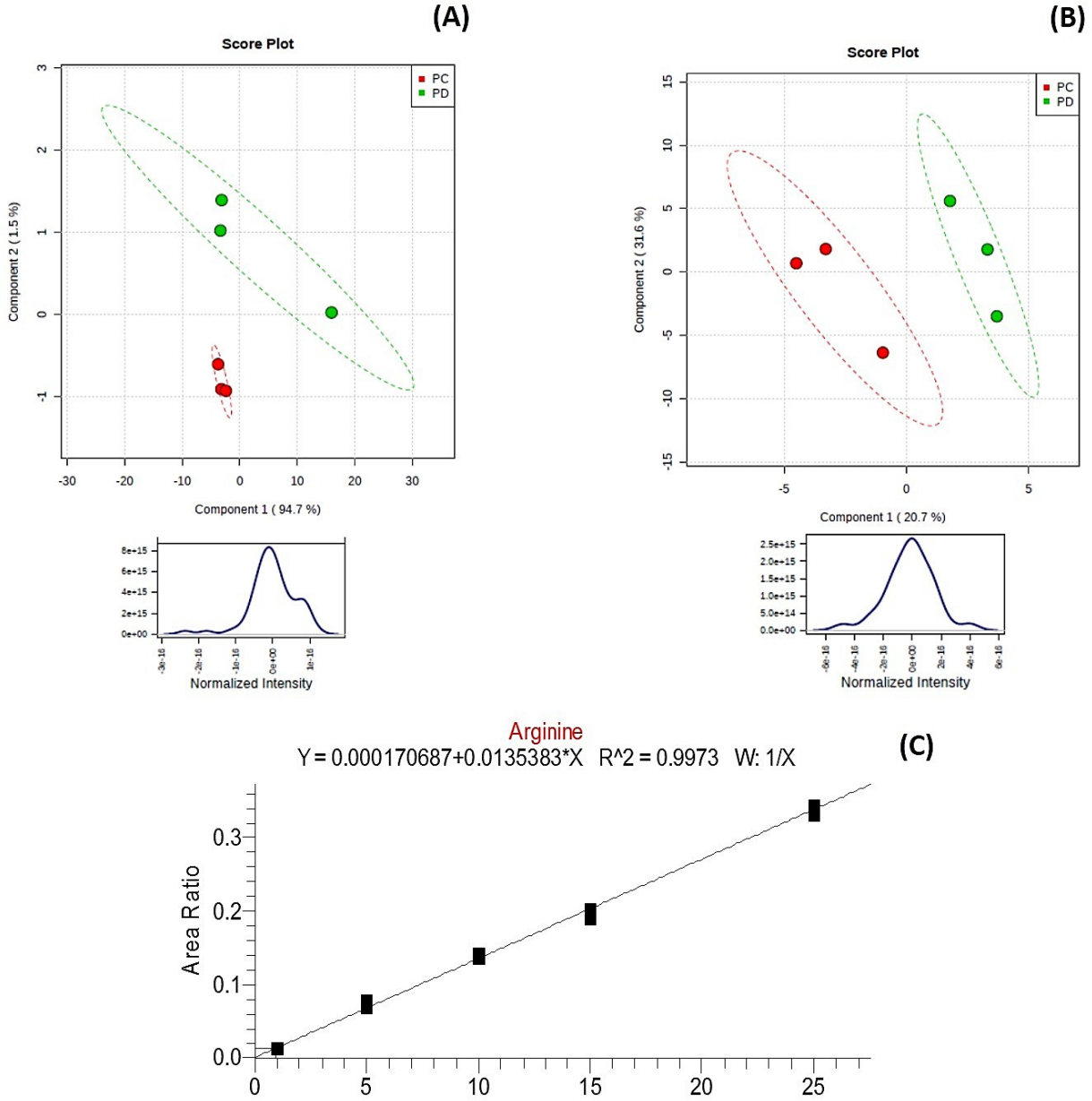

Figure ~~3S~~S3. Data clinical control (CC) and PD samples (three replicates acquired on three different

days) were normalized by **(A)** the stable isotope of arginine (arginine $^{13}C_6$ spiked at 50ppm) and by the **(B)** endogenous arginine, indicating that endogenous arginine could be used for data normalization. Also, **(C)** arginine spiked into artificial CSF showed excellent linear response for quantification in the range 1X - 25X (5 replicates for each concentration level) of the physiological concentration (3.5ppm used as 1X physiological concentration).

Most relevant MRMs indicated by principal component analysis (PCA) were manually combined into equations and ratios using the BioFIND testing set samples. The six ratios/equations presenting AUC>0.68 and the MRM 188->171 (parent->fragment) (**Table S3**) were used for the multivariate ROC. By the MRM profiling method, 67% (40 CSF out of 60 samples) of the BioFIND validation set (presented to the ROC curve as new samples) were correctly assigned.

Table S3. List of MRM, MRM ratios or MRM equations manually selected for the multivariate ROC curve, with correspondent AUC and fold change (HC/PD).

MRM/MRM ratio/MRM equation	***AUC***	***fold change***
(134.1->72.4 + 177->159.1+76.2->59.4 + 184->125.2)/188->171	0.75	0.4
184->125.2/188->171	0.73	0.5
(76.2->59.4 + 134.1->72.4 +184->125.2+386->303.9)/188->171	0.72	0.9
(134.1->72.4 + 177->159.1)/188->171	0.71	0.4
188->171	0.7	-0.4
[(78.2->61.5+ 79.2->61.5 +116.1->88.2+ 188->171)-(76.2->59.4 + 134.1->72.4 +184->125.2+386->303.9)]/188->171	0.69	-6.4
134.1->72.4/188->171	0.68	2.1

References

1 Z. Takáts, J. M. Wiseman, B. Gologan and R. G. Cooks. *Anal Chem*, 2004, **76**, 4050-8.
2 R. Wei, G. Li G and A. B. Seymour. *Anal Chem,* 2010, **82**, 5527-5533.
3 C. H. Hawkes, K. Del Tredici , H. Braak. *Parkinsonism Relat Disord*. 2010, **16**, 79-84.
4 M. A. Kuiper, T. Teerlink, J. J. Visser, P. L. Bergmans, P. Scheltens and E. C. Wolters. *J Neural Transm* (Vienna), 2000, *107*, 183-9.