Online supplement to: Downregulation of Tryptophan-related Metabolomic Profile in Rheumatoid Arthritis Synovial Fluid, *The Journal of Rheumatology,* doi:10.3899/jrheum.141505

Method validation

A quality control (QC) sample was prepared by mixing equal aliquots of each sample[1,2,3] and treated by the same method as sample preparation. The QC sample was used for column conditioning and method validation[2,3]. One QC sample was run every 10 samples. The test mixture consisted of commercially available standards: caffeine (1 μg/ml), acetaminocphen (1 μg/ml), and reserpine (1 μg/ml) for the positive ionization mode and hippuric acid (1 μg/ml), glycocholic acid (1 μg/ml), and adipic acid (1 μg/ml) for the negative ionization mode. The mixture was injected 3 times at the beginning, middle, and end of the analysis.

Results

Although acceptable criteria for repeatability of nontargeted metabolite profiling are still being investigated, Want, *et al*[4] suggested that the dataset including more than 70% of the variables with less than 30% coefficient of variation (CV) values are acceptable. Those guidelines were applied to method validation in this study.

Overlaid chromatography of all QC samples was shown in positive and negative ionized modes (Supplementary Figure 1). Repeatability of the datasets was overviewed by a principal component analysis (PCA) of the full dataset and was examined with the 14 ions selected from extracted ion chromatographic peaks (XIC) of the QC sample. As shown in the score plot from the PCA of the dataset with all of the QC injection, 4 replicates of QC samples within the analytical run were tightly clustered in the center (Supplementary Figure 2). Because the closer the QC samples cluster on the score plot, the more stable the analysis should be[5], it was accepted that the analytical system was stable and that the variable difference was meaningful. In score plot of PCA for the present study set, 3 samples did not cluster together, comparing relations among the other samples (Supplementary Figure 3). When the variability of peak shape, retention time (RT) of each peak, and peak area from XIC of QC samples were examined, no significant difference was found during all runs (Supplementary Table 1). The RT variation was negligible with a lower than 1% CV value in every case. Peak area repeatability for the selected ions was satisfactory with 1–12% CV values (Supplementary Table 1). When all of the variables from the MarkerLynx processing for the QC samples were examined, 78.5% of the ions in the positive ionization mode gave CV values < 30%, and 79.9% of the ions in the negative ionization mode presented CV values < 30% (Supplementary Table 2). Because the values satisfied the suggested criteria[4], the analytic runs were determined to be stable and repeatable.

Test mixtures were used for checking mass accuracy as well as peak shape, RT stability, and detector response. When both mass accuracy from mass spectrometry and the variability of peak shape, RT, and peak area from XIC of the test mixtures were examined, no statistical difference was shown (Supplementary Table 3). Especially the mass accuracy of the ions in the test mixture was good, with a variability < 5 mDa. Therefore, these runs were also identified to give a precise mass value for each ion.

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Supplementary Table 1. Repeatability of retention time and ion intensity in QC samples. Dataset derived from the QC only from an analytical run.

XIC from QC Samples		RT			Ion Intensity*		
RT	m/z	Average	SD	CV %	Average	SD	CV %
Positive mode							
1.45	166.08	1.47	0.01	0.50	78.00	1.71	3.12
2.12	271.15	2.12	0.00	0.00	31.67	0.49	3.72
3.87	568.27	3.85	0.00	0.00	1.21	0.28	11.58
4.12	384.12	4.13	0.01	0.27	47.80	3.34	3.95
5.71	221.12	5.71	0.00	0.00	160.27	1.78	1.62
7.13	410.18	7.12	0.00	0.00	152.30	5.01	3.34
12.11	300.30	12.12	0.00	0.00	0.85	0.06	9.42
Negative mode							
1.58	231.10	1.57	0.00	0.00	20.28	0.64	4.84
3.00	215.10	3.00	0.00	0.00	27.62	0.33	0.85
4.73	188.04	4.74	0.00	0.02	18.35	1.75	11.10
5.46	429.08	5.48	0.01	0.12	36.30	1.78	5.60
8.75	141.13	8.75	0.00	0.00	8.32	0.73	7.62
3.76	388.14	3.77	0.01	0.38	4.75	0.14	1.41
10.88	221.15	10.89	0.00	0.00	1.85	0.19	5.94

*Ion intensity is calculated by the ion peak area on chromatography. RT: retention time; QC: quality control; XIC: extracted ion chromatographic peaks; m/z: mass-to-charge ratio; CV: coefficient of variation.

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Supplementary Table 2. QC data from the replicates of QC samples showing the percentage of total ions that achieved 15%, 20%, and 30% CV.

QC Considered	% CV < 15%	% CV < 20%	% CV < 30%
Positive mode	57.6	69.1	78.5
Negative mode	65.7	73.8	79.9

QC: quality control; CV: coefficient of variation.

Supplementary Table 3. Repeatability of retention time and ion intensity in the test mixture.

Standard Chemical in Test Mixture	RT			Ion Intensity		
	Average	SD	CV %	Average	SD	CV %
Positive mode						
Acetaminophen	4.25	0.00	0.00	5959.00	753.50	11.29
Caffeine	5.00	0.01	0.09	28916.00	3298.07	12.87
Reserpine	7.98	0.00	0.00	2835.00	367.9	11.99
Negative mode						
Adipic acid	4.34	0.00	0.00	636.67	28.01	5.24
Hippuric acid	5.11	0.00	0.00	8511.00	315.18	4.59
Glycocholic acid	8.25	0.00	0.00	19612.00	958.30	4.02

Dataset derived from the QC only from an analytical run. Ion intensity is calculated by the ion peak area on chromatography. RT: retention time; CV: coefficient of variation.

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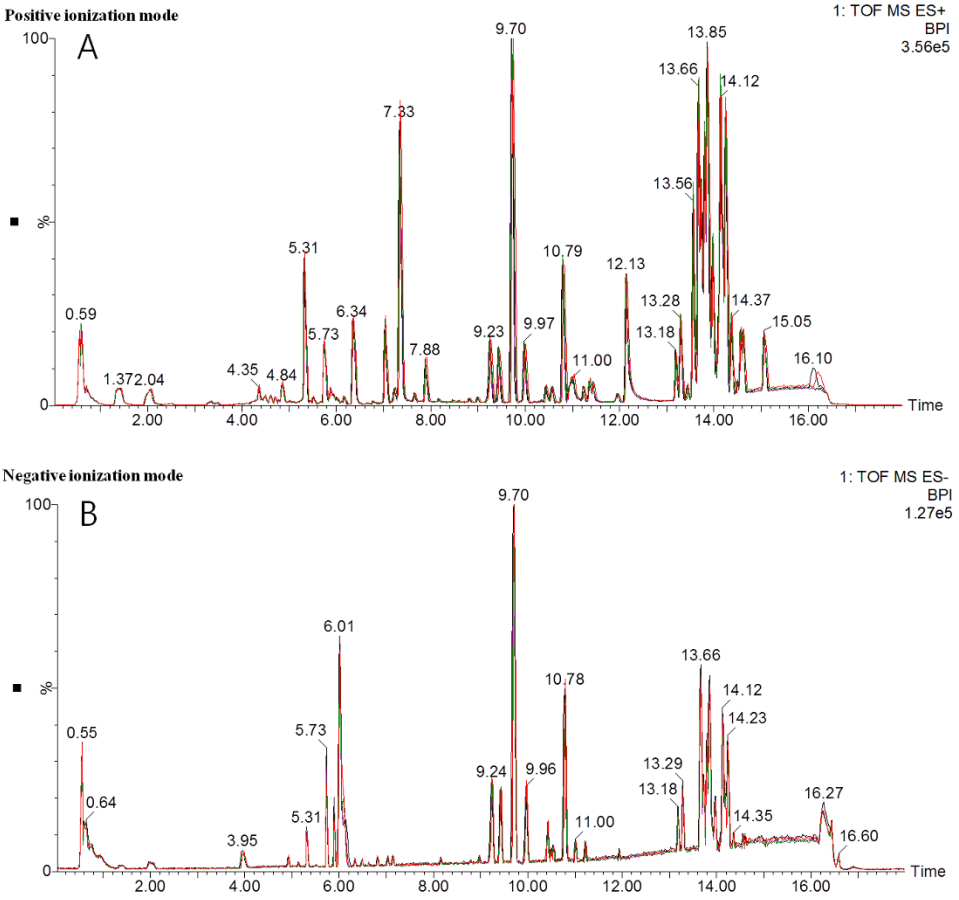
Supplementary Figure 1.



Overlaid chromatography of all QC samples. A. Chromatograms detected in positive ionization modes. B. Chromatograms detected in negative ionization modes. QC: quality control; TOF MS: time-of-flight mass spectrometer; BPI: base peak ion.

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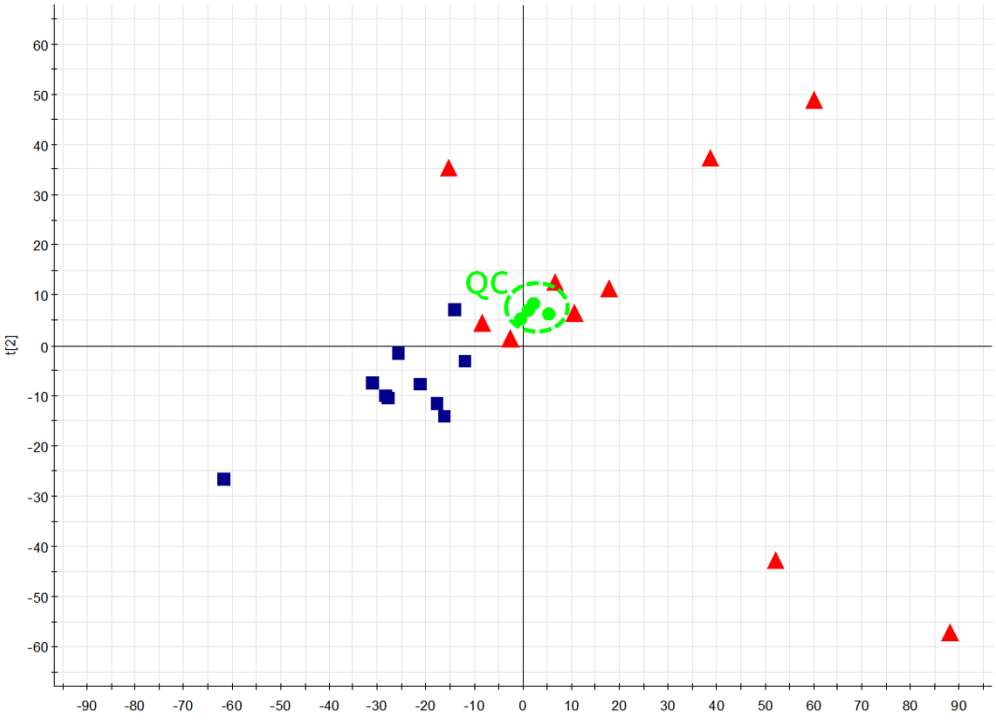
Supplementary Figure 2.



PCA score plot based on the plasma metabolic profiling of OA (boxes) and RA (triangles) groups, including quality control (QC) samples (dots). PCA: principal component analysis; OA: osteoarthritis; RA: rheumatoid arthritis.

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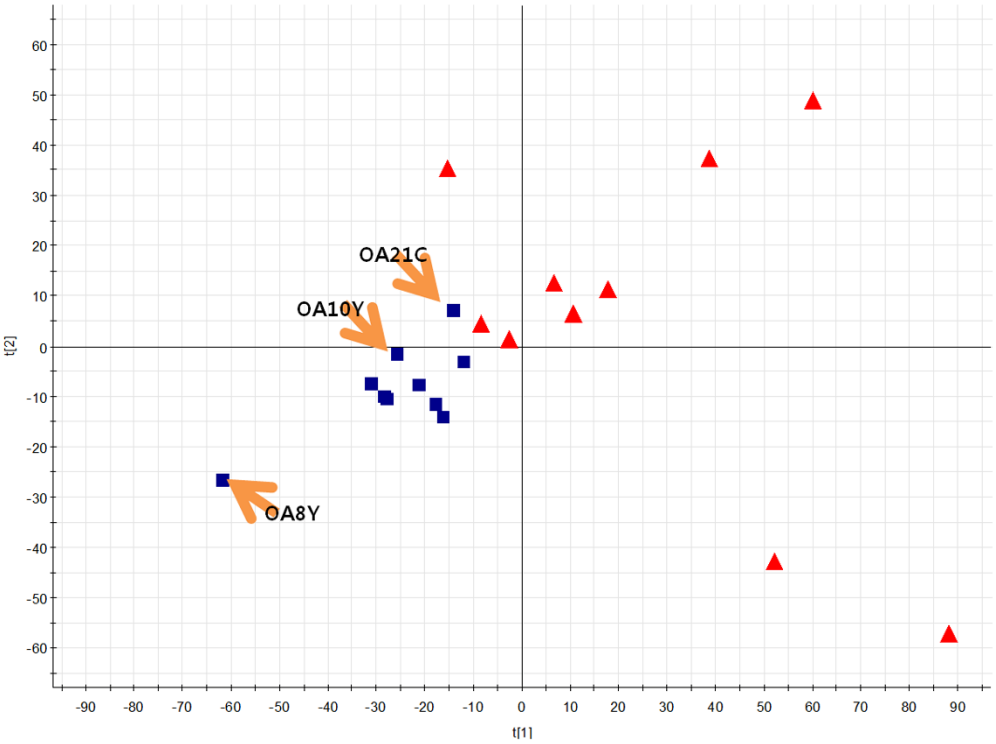
Supplementary Figure 3.



PCA score plots of SF metabolic profiling of the OA (boxes) and RA (triangles) groups. Arrows indicate patients with diabetes. PCA: principal component analysis; OA: osteoarthritis; RA: rheumatoid arthritis; SF: synovial fluid.

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