Supporting Information:

Thermal Degradation of Small Molecules: A Global Metabolomic Investigation

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Text S1 Chemical Materials.

Pyridine (Acros Organics, cat. No. 131980010) was purchased from Thermo Fisher Scientific. N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TCMS) mixture (99:1) and methoxyamine hydrochloride (98%) were purchased from Sigma-Aldrich (St. Louis, MO, US). A chemical mixture of 64 small molecule metabolite standards (~20 μM each) was prepared in this study. The full list of those small molecules was detailed in Table S-1 in the supporting information (SI). Generally, several groups of small molecules include amino acids, organic acids, sugars and sugar phosphates, and several free fatty acids. These metabolites are involved in several central carbon pathways; glycolysis, purine metabolism, pyrimidine metabolism, citric acid cycle (TCA cycle) and biosynthesis of amino acids, and as such are important to include in the study.

Text S2 LC/MS Analysis.

Analyses of the underivatized and derivatized plasma samples were performed using an HPLC system (1200 series, Agilent Technologies) coupled to a 6538 UHD Q-TOF (Agilent Technologies, Santa Clara, CA). Samples were analyzed using a XBridge C18 column (3.5 μm, 150 mm × 1.0 mm I.D., Waters, Milford, MA) for RPLC/MS analysis in ESI positive mode (RP-ESI (+)), and a Luna Aminopropyl column (3 μm, 150 mm × 1.0 mm I.D., Phenomenex, Torrance, California) for HILIC/MS analysis in ESI negative mode (HILIC-ESI (-)). For the RPLC/MS, the linear gradient elution from 100% in water with 0.1% formic acid (0–5 min) to 100% in acetonitrile with 0.1% formic acid (50–55 min) was applied. For the HILIC/MS, the mobile phase was composed of A = 20 mM ammonium acetate and 40 mM ammonium hydroxide in 95% water and 5 % acetonitrile and B = 95% acetonitrile, 5 % water[1]. The linear gradient elution started from 100% B (0–5 min) to 100% A (50–55 min) was applied. A 10 min post-run was applied for both RPLC and HILIC to ensure the column re-equilibration. The flow rate was 20 and 65 μL/min for RPLC and HILIC; respectively. The sample injection volume was 8 μL. ESI source conditions were set as follows: gas temperature 325 °C, drying gas 5 L/min, nebulizer 15 psi, fragmentor 120 V, skimmer 65 V, and capillary voltage 4000 V or −4000 V in ESI (+) or ESI (-); respectively. The instrument was set to acquire over the m/z range 50–1000, with the MS acquisition rate of 4 spectra/s.

To increase the sensitivity of metabolite detection, the underivatized and derivatized small molecule mixture were analyzed by HPLC system (1200 series, Agilent Technologies) coupled to 6550 iFunnel QTOF mass spectrometer (Agilent Technologies, Santa Clara, CA). The mobile phase was identical with the above-mentioned method. ESI source conditions were set as follows: dry gas temperature 200°C, fragmentor 380 V, sheath gas temperature 300°C and flow 11 L/min, nozzle voltage 500 V, and capillary voltage 2,500 V and −2,500 V in ESI (+) or ESI (-); respectively. The instrument was set to acquire over the m/z range 50–1,000, with the MS acquisition rate of 2 spectra/s. For the MS/MS of selected precursors, the default isolation width was set as narrow (~1.3 m/z), with a MS acquisition rate at 2 spectra/s and MS/MS acquisition at 2 spectra/s to acquire over the m/z range 50–1000 and 25–1000; respectively. MS/MS data were acquired at the collision energy of 20 V.

Text S3 Data Analysis.

LC/MS data were converted to mzXML files using Masshunter Acquisition Software (Agilent Masshunter 6.0B). The mzXML files were uploaded to XCMS Online web platform for data processing (https://xcmsonline.scripps.edu)[2-5] including peak detection, retention time correction, profile alignment, and isotope annotation. Data were processed using both pair-wise and multigroup comparison, and the parameter settings were as follows: centWave for feature detection (Δ m/z = 15 ppm, minimum peak width = 10 s, and maximum peak width = 60 s); obiwarp settings for retention time correction (profStep = 0.5); and parameters for chromatogram alignment, including mzwid = 0.015, minfrac = 0.5, and bw = 5. The relative quantification of metabolite features was based on extracted ion chromatogram (EIC) areas. Paired parametric *t*-test and one-way *ANOVA* (post hoc Tukey test) were used to test the variation pattern of metabolite features between and across samples treated with different heating temperatures and time. To estimate the heating effect on the global molecular profiling, pairwise comparisons between each tested temperature at individual time and its 25°C control were conducted. Furthermore, multiple group analysis including each temperature with different heating periods and their 25°C control was carried out to observe time course dependence. The results output, including EICs, pairwise/multigroup cloud plot, multidimensional scaling plots, and principle components were exported directly from XCMS Online. Generally, the numbers of total pairwise comparison features and significantly altered features (statistically defined as $p < 0.01$, including both upregulated and downregulated features) were reported in this study. The percentage of altered features compared to the total features was calculated to quantitatively describe the heating effect on the global metabolic profiling.

Text S4 QA/QC.

Triplicates and one heated blank control were prepared for each sample at one temperature and sampling time. The result showed that no obvious difference between the heated blank control and the solvent blank (room temperature), suggesting that negligible background contamination from the septum cap or the vial during the heating process. During the sample analysis on the LC/MS, the triplicates of each sample were run separately in the worklist. To correct the mass, retention time and response drift, an underivatized plasma metabolites and the mixture of 64 small molecules were run every five samples to ensure the result consistent and comparable. Metabolite identification was based on accurate mass (within 10 ppm), retention time comparison with our previous running, and MS/MS data. All samples especially the derivatized samples were analyzed within 48 hours after the sample collection.

Table S1. The calculated derivatization efficiency and response ratio (average±std) between 250°C 60s/300s and 25°C of the underivatized 64 tested compounds as well as their KEGG IDs.

KEGG ID	Compounds	Derivatization efficiency (%)	Response Ratio (250°C, 60s vs.25°C)	Response Ratio (250°C, 300s vs.25°C)
		Pyrimidine metabolism		
C00299	Uridine	67%	136.4±10.8%	94.4±7.6%
C00105	UMP	<5%	235.2±15.3%	198.2±25.0%
C00015	UDP	<10%	27.0±6.5%	9.8±2.6%
C00075	UTP	98%	2.4±0.9%	0.7±0.5%
C00063	CTP	96%	19.2±4.9%	7.5±1.2%
C00112	CDP	42%	25.7±7.2%	0.5±0.7%
C05822	CMP	<5%	121.9±2.4%	34.8±8.4%
C00475	Cytidine	69%	92.6±10.7%	6.5±2.2%
		Purine metabolism		
C00002	ATP	100%	1.9±0.9%	0.1±0.1%
C00008	ADP	58%	14.4±6.7%	0.2±0.1%
C00020	AMP	<5%	158.6±9.5%	11.6±3.6%
C00212	Adenosine	47%	47.4±8.5%	5.6±2.8%
C00147	Adenine	60%	20.6±5.8%	9.7±1.2%
C00130	IMP	<5%	100.4±19.3%	30.7±9.2%
C00294	Inosine	27%	30.5±8.5%	2.9±0.5%
C00262	Hypoxanthine	<5%	246.4±39.0%	275.2±24.4%
C06193	GMP	<5%	196.6±17.7%	25.5±5.1%
C00035	GDP	66%	7.7±5.1%	0.0±0.1%
C00044	GTP	100%	2.8±1.4%	0.2±0.2%
C00387	Guanosine	NA	88.8±6.7%	3.6±0.9%
C00366	Uric acid	<5%	144.4±11.0%	144.7±4.5%
C00385	Xanthine	<5%	90.5±4.6%	101.3±1.2%
		Pentose phosphate pathway		
C00117	Ribose-1-phosphate	100%	0.4±0.2%	0.0±0.0%
C00117	Ribose-5-phosphate	100%	0.4±0.2%	0.0±0.0%
C00119	PRPP	NA	NA	NA
		Glycolysis		
C00092	Glucose-6-phosphate	100%	0.2±0.1%	0.0±0.0%

C05345	Fructose-6-phosphate	100%	0.2±0.1%	0.0±0.0%
C00354	Fructose-1,6-bisphosphate	NA	0.9±1.3%	0.0±0.0%
C00118	Glyceraldehyde-3P	NA	NA	NA
C00197	Glycerate-3P	41%	4.4±2.6%	0.3±0.0%
C00074	Phosphoenol pyruvate	100%	0.0±0.0%	0.0±0.0%
C00022	Pyruvate	NA	NA	NA
C00024	Acetyl-CoA	100%	0.7±0.4%	0.0±0.0%
C01432	Lactate	100%	95.0±34.4%	148.4±33.4%
		TCA cycle		
C00158	Citric acid	<10%	48.3±5.2%	4.7±%
C00158	Isocitric acid	<10%	48.3±5.2%	4.7±%
C00026	alpha-ketoglutarate	100%	32.2±3.3%	29.7±20.2%
C00042	Succinate	<5%	27.8±3.9%	0.7±0.6%
C00122	Fumarate	NA	NA	NA
C00149	Malate	<5%	287.5±80%	324.1±21%
		Biosynthesis of amino acids		
C00073	Methionine	NA	NA	NA
C00078	Tryptophan	60%	22.4±13.0%	2.9±0.7%
C00082	Tyrosine	25%	38.0±8.3%	0.4±0.8%
C00956	Aminoadipic acid	61%	1.3±1.6%	0.0±0.0%
C00449	Saccharopine	72%	0.1±0.1%	0.1±0.2%
C00047	Lysine	NA	NA	NA
C00025	Glutamate	<5%	5.5±4.5%	0.1±0.1%
C00624	Acetyl-glutamic acid	<5%	82.7±4.4%	32.2±9.6%
C0064	Glutamine	NA	NA	NA
C01602	Ornithine	NA	NA	NA
C00062	Arginine	<5%	NA	NA
C05824	Cysteine sulfate	36%	3.2±1.4%	0.0±0.0%
		Oxidative phosphorylation		
C00003	NAD+	100%	0.8±0.3%	0.1±0.0%
C00004	NADH	100%	2.3±0.4%	0.6±0.0%
		Amino sugar and nucleotide sugar metabolism		
C00052	UDP-galactose	100%	25.9±2.6%	23.8±3.8%
C00043	UDP-N-acetyl-glucosamine	100%	1.5±0.3%	1.0±0.0%
C00096	GDP-mannose	100%	0.3±0.2%	0.1±0.0%

		Glutathione metabolism		
C00127	Oxidized glutathione	61%	4.7±1.7%	0.1±0.0%
C00006	NADP+	100%	0.5±0.2%	0.0±0.0%
		Taurine & Hypotaurine metabolism		
C14179	Sulfoacetic acid	41%	92.2±5.1%	83.0±2.6%
		C5-branched dibasic acid metabolism		
C01732	Mesaconic acid	NA	NA	NA
		Lipid metabolism		
C01595	Linoleic acid	85%	90.9±2.9%	57.9±10.2%
C00219	Arachidonic acid	NA	NA	NA
C00712	Oleic acid	85%	92.6±28.1%	69.4±16.7%
C01530	Stearic acid	NA	NA	NA
C04102	PC(16:0/0:0)	83%	95.8±13.5%	77.9±19.3%

NA: not detected in the LC-MS with the spiked concentration or too high background ions.

Table S2. The number of total features, significantly altered features, upregulated and downregulated features of heated raw and derivatized plasma metabolites analyzed using both RP-ESI (+) and HILIC-ESI (-). All the data were from the output of XCMS Online software.

Pairwise comparison	Total number of features (RPLC/HILIC)	Number of altered features (RPLC/HILIC)*	Number of down-regulated features (RPLC/HILIC)	Number of the up-regulated features (RPLC/HILIC)	Percentage of dysregulated features in total features (RPLC/HILIC)	Percentage of upregulation in total dysregulated features (RPLC/HILIC)
25°C vs. 60°C at 30s	5937/1807	51/8	43/1	8/7	0.9%/0.4%	15.7%/87.5%
25°C vs. 60°C at 60s	6092/1807	79/13	54/0	25/13	1.3%/0.4%	31.6%/100%
25°C vs. 60°C at 300s	6014/1796	153/13	133/0	20/13	2.5%/0.7%	13.1%/100%
25°C vs. 100°C at 30s	5957/1816	309/228	254/111	55/117	5.2%/0.3%	17.8%/83.3%
25°C vs. 100°C at 60s	5964/2437	156/10	138/7	18/3	2.6%/0.4%	11.5%/30.0%
25°C vs. 100°C at 300s	6443/2121	503/220	327/23	176/197	7.8%/10.4%	35.0%/89.5%
25°C vs. 250°C at 30s	6381/1761	745/31	584/12	161/19	11.7%/1.8%	21.6%/61.3%
25°C vs. 250°C at 60s	6818/1693	1105/81	831/48	274/33	16.2%//4.8%	24.8%/40.7%
25°C vs. 250°C at 300s	9757/1832	3830/228	1494/111	2336/117	39.3%/12.4%	61.0%/51.3%
Derivatized 25°C vs. 250°C at 30s	8355/1306	1160/169	931/167	229/2	13.9%/12.9%	19.7%/1.25
Derivatized 25°C vs. 250°C at 60s	8636/1306	2747/126	2424/109	323/17	31.8%/9.6%	11.8%/13.5%
Derivatized 25°C vs. 250°C at 300s	11885/1306	6147/169	3984/167	2188/2	51.7%/12.9%	35.6%/1.2%

*****The altered features were filtered with a $p < 0.01$ and fold change > 1.5.

Table S3. The number of total features, significantly altered features ($p < 0.01$), upregulated and downregulated features of heated underivatized and derivatized metabolite mixtures with both RP-ESI (+) and HILIC-ESI (-). All the data was from the output of XCMS Online software.

Pairwise comparison	Total numbers of features (RPLC/HILIC)	Number of altered features (RPLC/HILIC)*	Numbers of down-regulated features (RPLC/HILIC)	Numbers of the upregulated features (RPLC/HILIC)	Percentage of dysregulated features in total features (RPLC/HILIC)	Percentage of upregulation in total dysregulated features (RPLC/HILIC)
25°C vs. 250°C at 60s	17371/16054	4370/4279	2099/1519	2341/2760	26.7%/25.2%	64.5%/53.6%
25°C vs. 250°C at 300s	16415/23546	4934/6416	2623/1576	2311/4840	27.2%/30.1%	75.4%/46.8%
Derivatized 25°C vs. 250°C at 60s	14076/8886	1065/251	908/55	157/196	2.8%/7.6%	78.1%/14.7%
Derivatized 25°C vs. 250°C at 300s	14489/9479	2884/760	2388/531	496/229	8.0%/19.9%	30.1%/17.2%

*****The altered features were filtered with a $p < 0.01$.

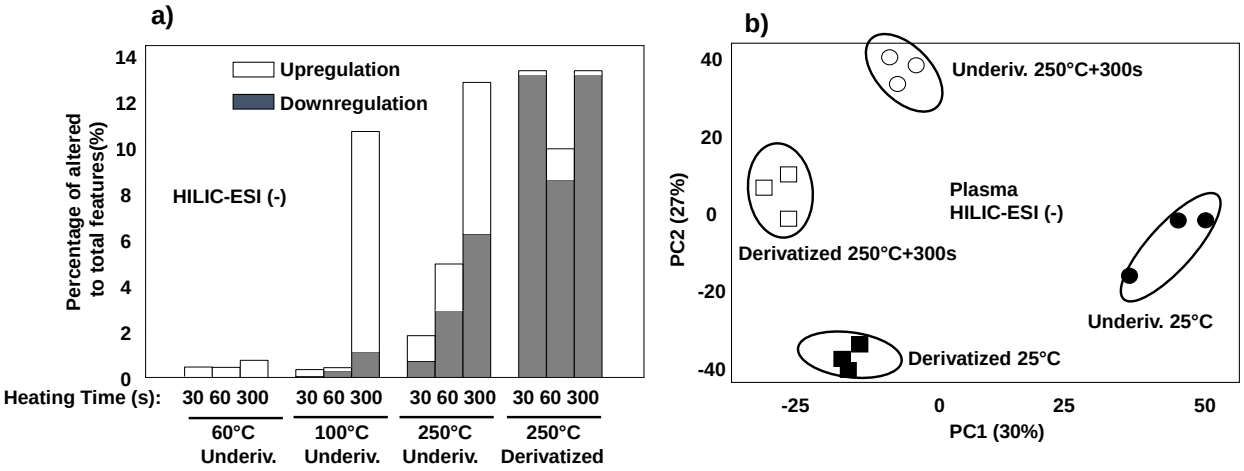

Figure S1. The percentage of altered (both upregulated and downregulated) features compared to the total features in the underivatized and derivatized plasma metabolites heated at 60°C, 100°C, and 250°C for 30s, 60s, and 300s. The subsequent analyses were performed using: a) HILIC-ESI (-) and overall results displayed with b) principal component analysis (PCA) scores plots of 25°C and 250°C for 300s heated plasma. All the upregulated and downregulated features were from pairwise comparison of each tested condition with room temperature (25°C) and filtered using $p < 0.01$.

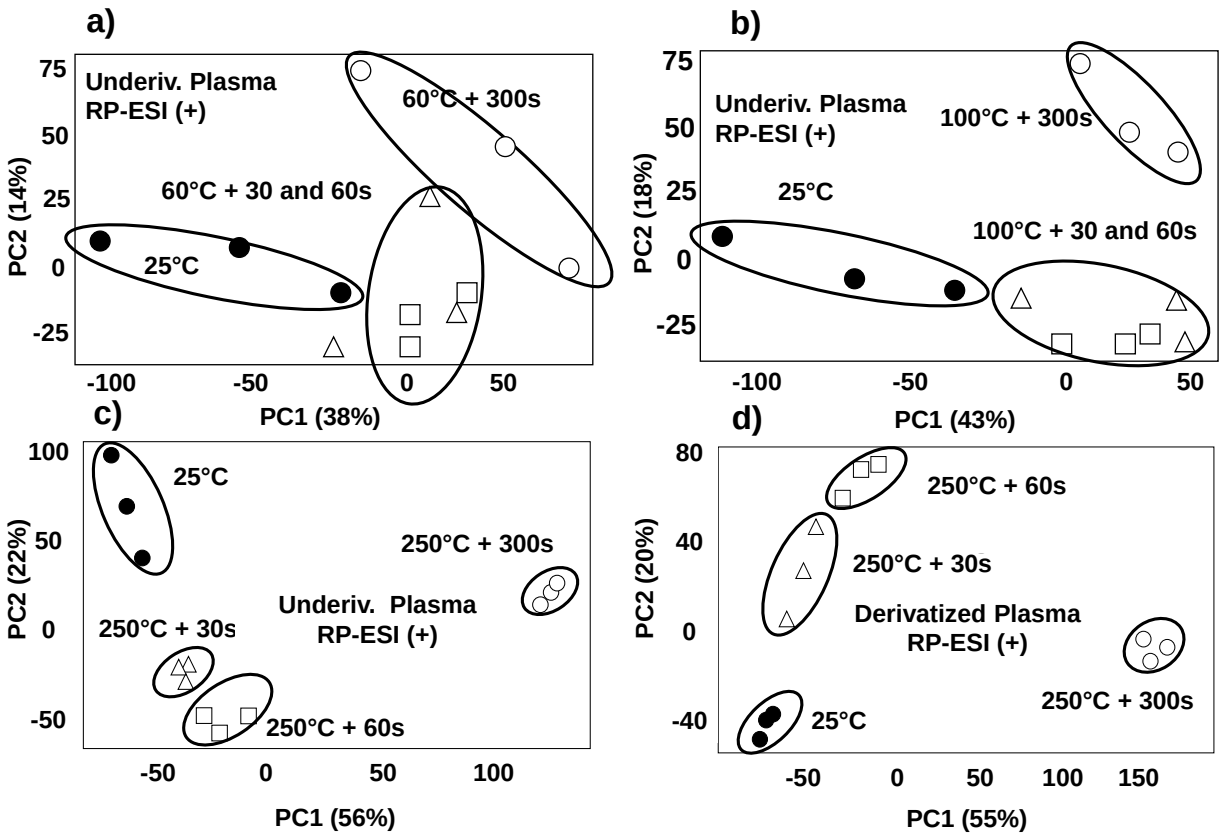

Figure S2. Principal component analysis (PCA) scores plot for group a) underivatized plasma metabolites heated at 25°C, 60°C for 30s, 60s and 300s; b) underivatized plasma metabolites heated at 25°C, 100°C for 30s, 60s and 300s; c) underivatized plasma metabolites heated at 25°C, 250°C for 30s, 60s and 300s; and d) derivatized plasma metabolites heated at 25°C and 250°C for 30s, 60s and 300s analyzed using RP-ESI (+).

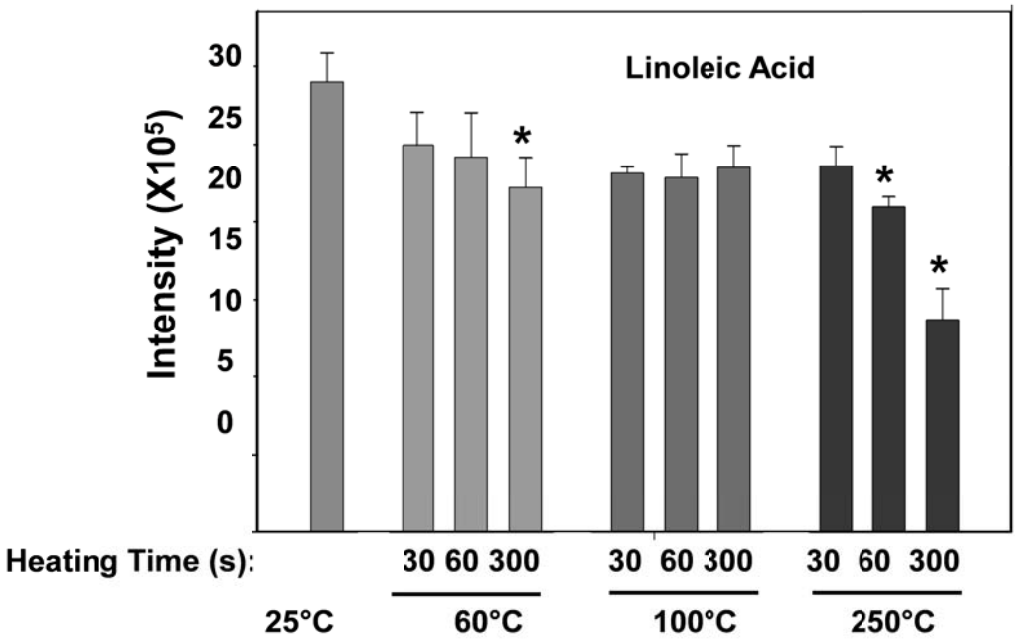

Figure S3. Response change of linoleic acid heated at different temperatures and heating times in the underivatized plasma metabolites. The results and the standard deviation were obtained from triplicate analysis.

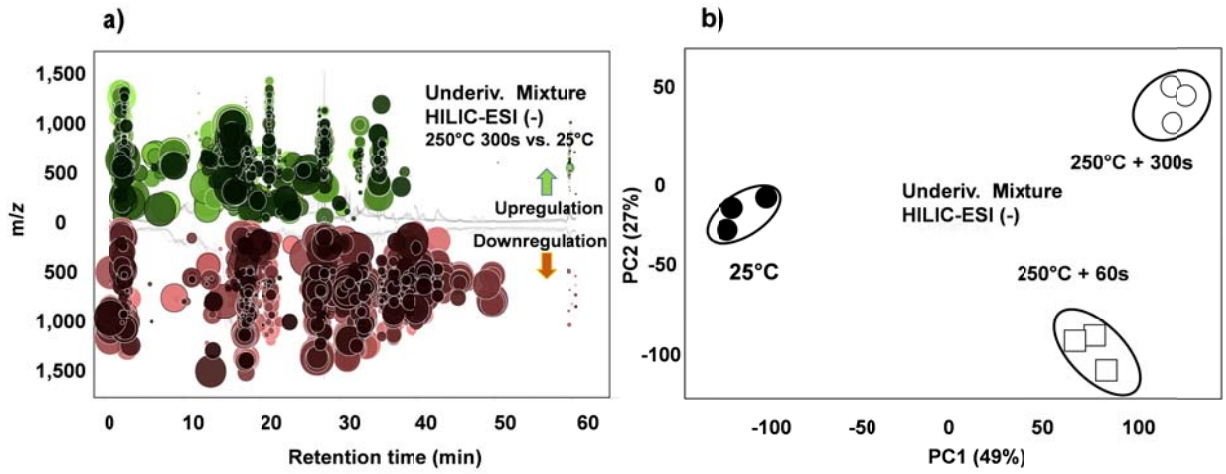

Figure S4. a) Cloud plot showing altered metabolite features (represented by "bubbles") between the 250°C 300s and 25°C heated small molecule mixtures analyzed using HILIC-ESI (-); and b) PCA scores plot of underivatized metabolite mixture at room temperature (25°C) and heated with 250°C at 60s and 300s. The feature in the cloud pot was filtered with fold change > 1.5 and $p < 0.01$.

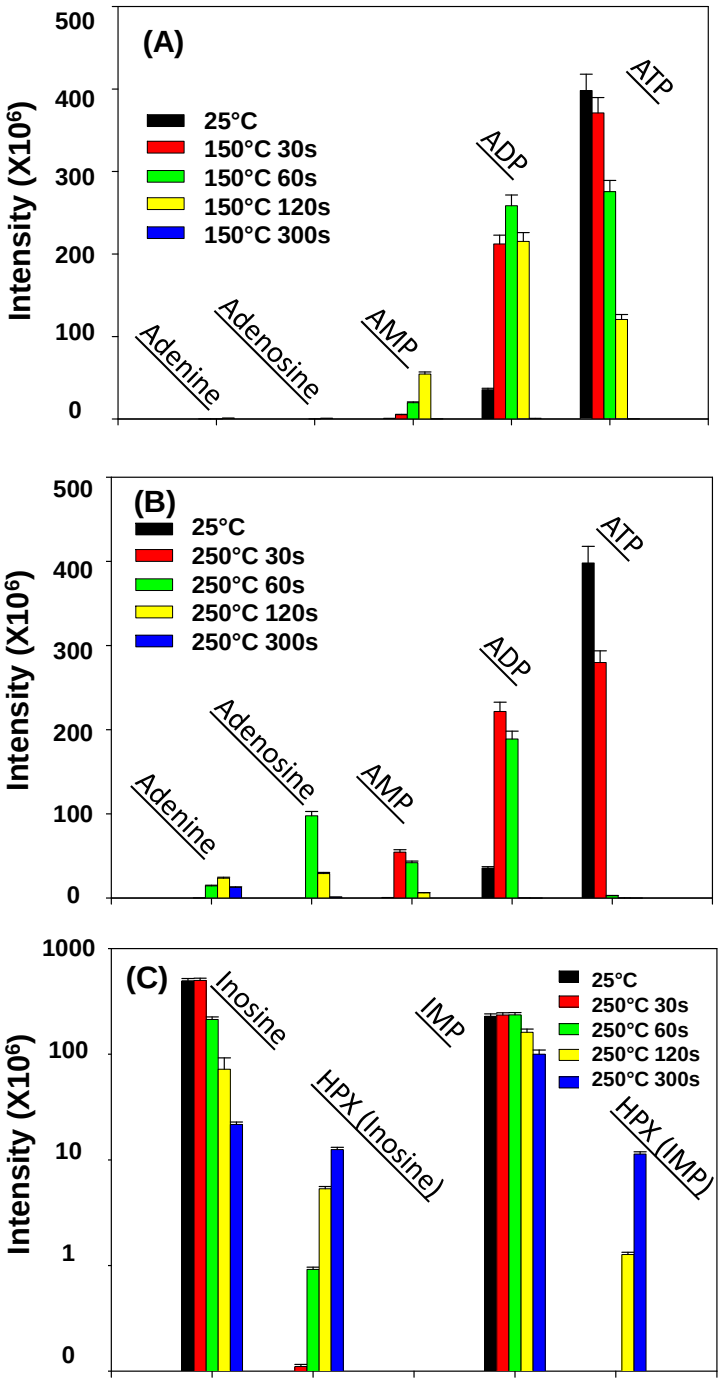

Figure S5. Formation of adenine, adenosine, AMP and ADP from single 10 μM ATP heating at A). 150 °C for 30s, 60s, 120s, and 300s; B). 250 °C for 30s, 60s, 120s, and 300s; and formation of hypoxanthine (HPX) from single 10 μM inosine and IMP heating at C). 250 °C for 30s, 60s, 120s, and 300s. The results and the standard deviation were obtained from triplicate analysis.

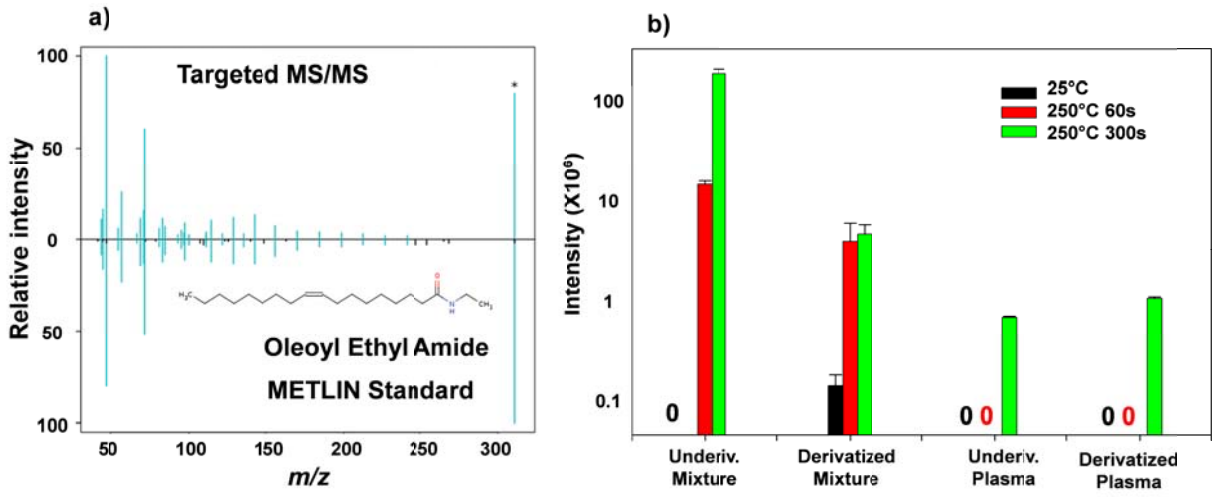

Figure S6. Identification of oleoyl ethyl amide as a possible byproduct during heating. a) MS/MS match with the standard metabolite fragments in METLIN library with a collision energy of 20V; b) the formation of oleoyl ethyl amide in the heated underivatized and derivatized metabolite mixture as well as plasma metabolites at 250°C for 60 and 300s. The result and the error bar were the average and standard deviation of triplicates; respectively.

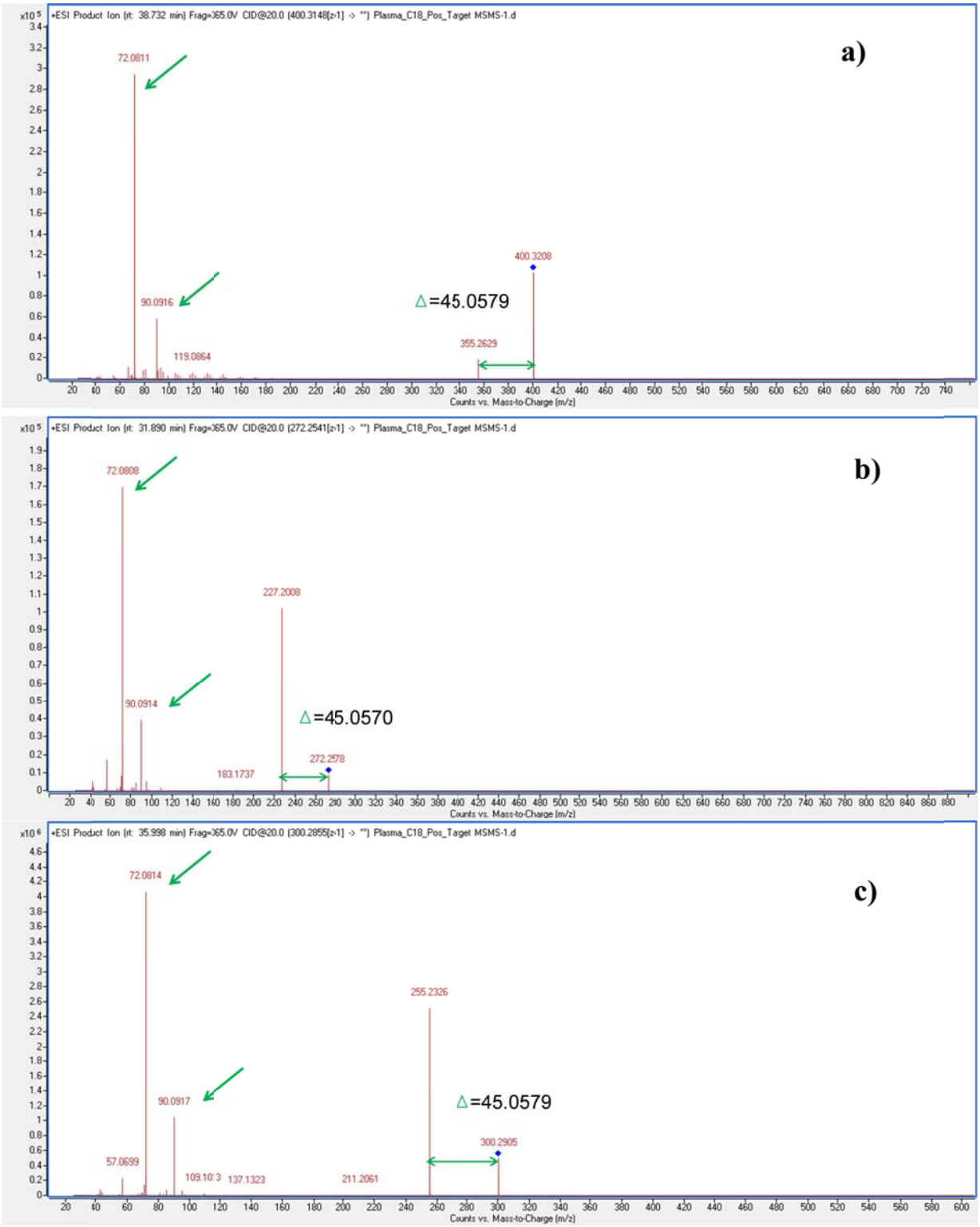

Figure S7. Examples of MS/MS fragments in the targeted MS/MS (*m/z* 400.3148, 272.254, and 300.2855) using RP-ESI (+).

References

(1) Ivanisevic, J.; Zhu, Z. J.; Plate, L.; Tautenhahn, R.; Chen, S.; O'Brien, P. J.; Johnson, C. H.; Marletta, M. A.; Patti, G. J.; Siuzdak, G. *Analytical chemistry* 2013, *85*, 6876-6884.

(2) Nikolskiy, I.; Mahieu, N. G.; Chen, Y. J.; Tautenhahn, R.; Patti, G. J. *Analytical chemistry* 2013, *85*, 7713-7719.

(3) Patti, G. J.; Tautenhahn, R.; Siuzdak, G. *Nature protocols* 2012, *7*, 508-516.

(4) Smith, C. A.; Want, E. J.; O'Maille, G.; Abagyan, R.; Siuzdak, G. *Analytical chemistry* 2006, *78*, 779-787.

(5) Tautenhahn, R.; Patti, G. J.; Rinehart, D.; Siuzdak, G. *Analytical chemistry* 2012, *84*, 5035-5039.