Supporting Information (SI)

Title: Optimization of Metabolite Extraction of Human Vein Tissue for Ultra Performance Liquid Chromatography-Mass Spectrometry and Nuclear Magnetic Resonance-Based Untargeted Metabolic Profiling

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Supporting information (SI) contains detailed parameters used in the experiments. There are five supplementary figures: the first three (S 1, S 2, S 3) are figures illustrating principal component analysis scores and loadings plots for data acquired using ultra performance liquid chromatography-mass spectrometry (UPLC-MS) in the ***stage 1*** optimization (selection of optimal solvents) of the study. Features in the periphery of loadings plots are the metabolites driving the variation of the model and were further structurally assigned. Figures S 4 and S 5 demonstrate aqueous and organic nuclear magnetic resonance spectroscopy (NMR) spectra and UPLC-MS chromatograms. There are three supplementary tables: Tables S 1 demonstrates UPLC-MS gradient settings, Table S 2 demonstrates the parameters used in data processing using the MassLynx V4.1 software and Table S 3 provides the details of features structurally assigned, as presented in supplementary figures.

Chemicals Used

The organic solvents used were chloroform, acetonitrile, DCM, ISP, hexane and MTBE (Sigma-Aldrich Gillingham, UK). Methanol (Fisher) and water were (Fluka) both LC-MS grade. Additionally, formic acid, leucine enkephalin, ammonium formate (HPLC grade) and sodium formate, were obtained from Sigma-Aldrich, Gillingham, UK. Deuteriated chloroform with 0.05% tetramethylsilane (TMS) was obtained from Goss Scientific, UK.

Preparation of aqueous extracts for NMR spectroscopy

Two aliquots of the dried aqueous extracts were combined after sequential reconstitution in 600 µL sodium phosphate buffer solution (0.2 M, 0.05% of sodium 3-trimethylsilyl-1-[2,2,3,3,-$^{2}H_4$] propionate (TSP), 70% D_2O, pH 7.4). The aliquots were vortexed for 1 min, sonicated for 5 min and vortexed for additional 1 min, followed by centrifugation for 30 s at 17945 x *g* at 4 ^{0}C. The supernatant was transferred to the second aliquot and the reconstitution procedure was repeated. The supernatant (500 uL) was then transferred into an NMR tube with an outer diameter of 5 mm.

Safety considerations

Biological tissue was handled in Class II biological cabinet and local laboratory protocols were strictly followed. Chloroform was handled in safety cabinet due to hazardous risks involved with chloroform. Local laboratory safety precautions were followed while handling liquid nitrogen.

Preparation of organic extracts for NMR spectroscopy

Two aliquots of the organic extracts from each of the extraction procedures (chloroform/methanol, DCM/methanol, ISP/methanol, hexane/ISP/methanol, MTBE/methanol) were combined for NMR spectroscopy analysis. A total of 600µL deuterated chloroform with 0.05% tetramethylsilane (TMS) was added into the first aliquot, followed by vortexing for 1 min. The content was then transferred into the second aliquot and vortexed for 1 min before transferring into an NMR tube with an outer diameter of 5 mm.

Preparation of aqueous extracts for UPLC-MS

Dried aqueous extracts were reconstituted in 200 µL of acetonitrile/water (95:5) for UPLC-MS hydrophilic interaction liquid chromatography (HILIC) analysis, and 200 µL acetonitrile/water

(v:v, 1:1) for reversed phase (RP) analysis. Samples were vortexed for 1 min, sonicated for 5 min, vortexed again for 1 min and then centrifuged at 17949 x *g* at 4 °C for 8 min. Contents were then transferred into LC-MS grade glass total recovery vials (Waters, USA). A total of 50 µl from each sample was added together to make a quality control (QC) sample.

Preparation of organic extracts for UPLC-MS

Dried organic extracts were reconstituted in 250 µL of ISP/acetonitrile/water (2:1:1). The same procedure of sample vortexing and sonication was followed as detailed above for the reconstitution of aqueous extracts. Following centrifugation at 17949 x *g* at 4 °C for 8 min, contents were transferred into LC-MS grade glass vials with inserts (Waters, USA). 50 µl from each sample was added to a pool to make a representative QC sample.

Instrument Settings for UPLC-MS analysis

HILIC-UPLC-MS: The composition of the mobile phases was: 0.1% (v/v) formic acid (FA) and 10 mM ammonium acetate in acetonitrile/H_2O (95:5) (A), and 0.1% (v/v) FA and 10 mM ammonium acetate in acetonitrile/H_2O (50:50) (B). The source temperature was set at 120 ^oC and desolvation gas temperature at 400 ^oC. The electrospray ionization (ESI) conditions for aqueous analysis on UPLC-MS were: cone gas flow of 25 L/hr, desolvation gas flow 800 L/hr, capillary voltage 3000 V for ESI positive (ESI+) and 2000 V for ESI negative (ESI-) modes, and cone voltage 25 V. The instrument was set to acquire in a mass-to-charge ratio (m/z) range of 50-1000 with scan time of 0.2 s and inter-scan delay of 0.01 s.

RP-UPLC-MS: The composition of mobile phases for RP analysis of aqueous extracts was: (A) water with 0.1% (v/v) FA, and (B) methanol with 0.1% (v/v) FA. The ESI source settings for RP analysis of aqueous extracts was as follows: source temperature of 120 ^oC, desolvation temperature at 350 ^oC, cone gas flow of 50 L/hr, desolvation gas flow 900 L/hr, capillary voltage 3000 V for ESI+ and 2400 V for ESI– modes, and cone voltage 30 V. The instrument was set to acquire the m/z range of 50-1000 with scan time of 0.2 s and inter-scan delay of 0.01sec.

Organic extracts-RP-UPLC-MS: Mobile phases consisted of acetonitrile/water (60:40) with 10 mM ammonium formate and 0.1%FA (A), and ISP/acetonitrile (90:10) with 10 mM ammonium formate and 0.1% FA. ESI conditions were set with the source temperature 120 ^oC, desolvation temperature 400 ^oC, cone gas flow 25 L/hr, desolvation gas flow 800 L/hr, capillary

voltage 3000 V for ESI+ and 2500 V for ESI-, and cone voltage of 30 V. The instrument was set to acquire over the m/z range 50-1200 in V mode with scan time of 0.2 s and an inter-scan delay of 0.02 s.

For all UPLC-MS experiments, samples were maintained at 4 ^{o}C during analyses. Leucine enkephalin (200 pg/µL, in acetonitrile/water 50:50, 0.1% FA) was used as for lock mass correction with double scan acquisition every 30s. The instrument was calibrated before analyses using 0.5mM sodium formate solution.

Figures

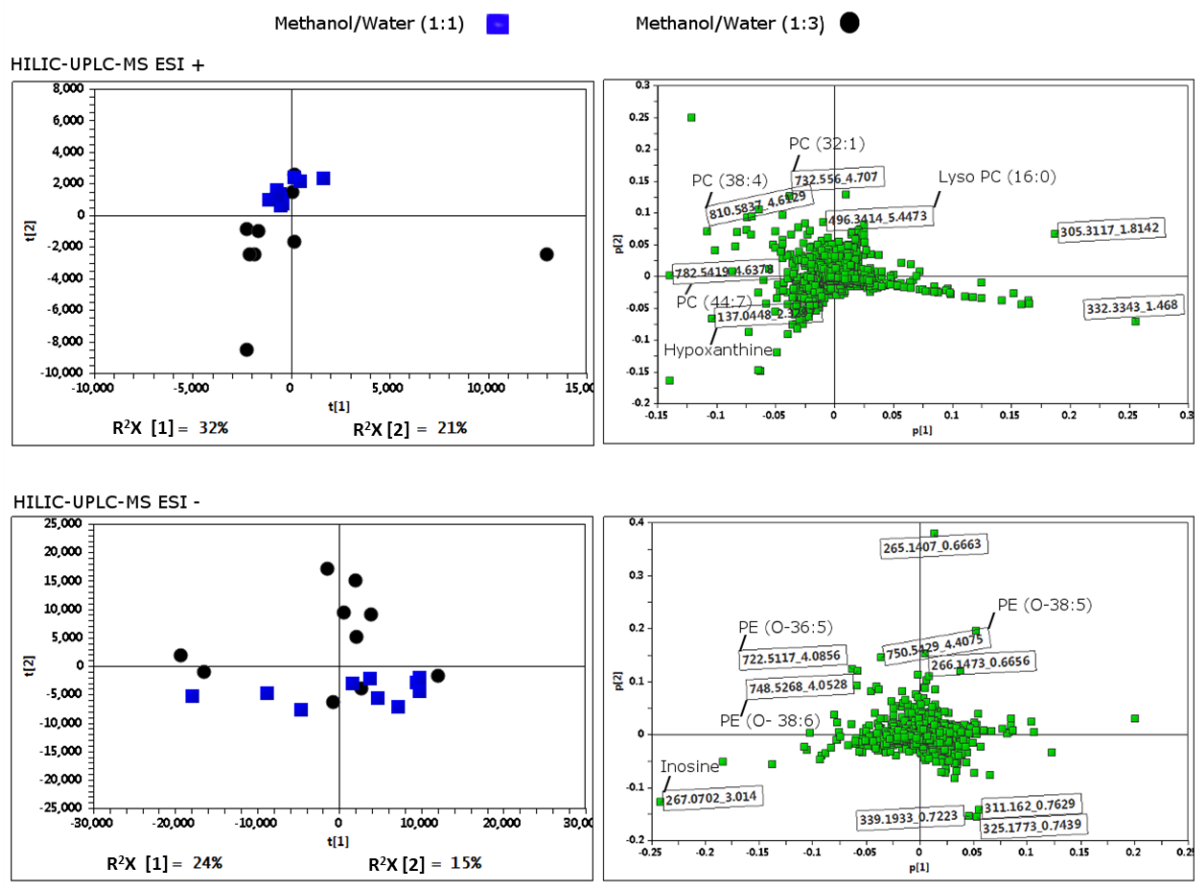

Figure S 1. Principal component analysis scores (left) and loadings (right) plots for HILIC-UPLC-MS-based metabolic profiling of aqueous extracts (for 1st stage of the study). Features located at the periphery of the loadings plots are driving the variation and were further structurally assigned. HILIC; Hydrophilic interaction liquid chromatography, UPLC-MS; Ultra performance liquid chromatography – mass spectrometry, ESI; Electrospray ionization, PC; Phosphatidycholine, PE; Phosphatidylethanolamine.

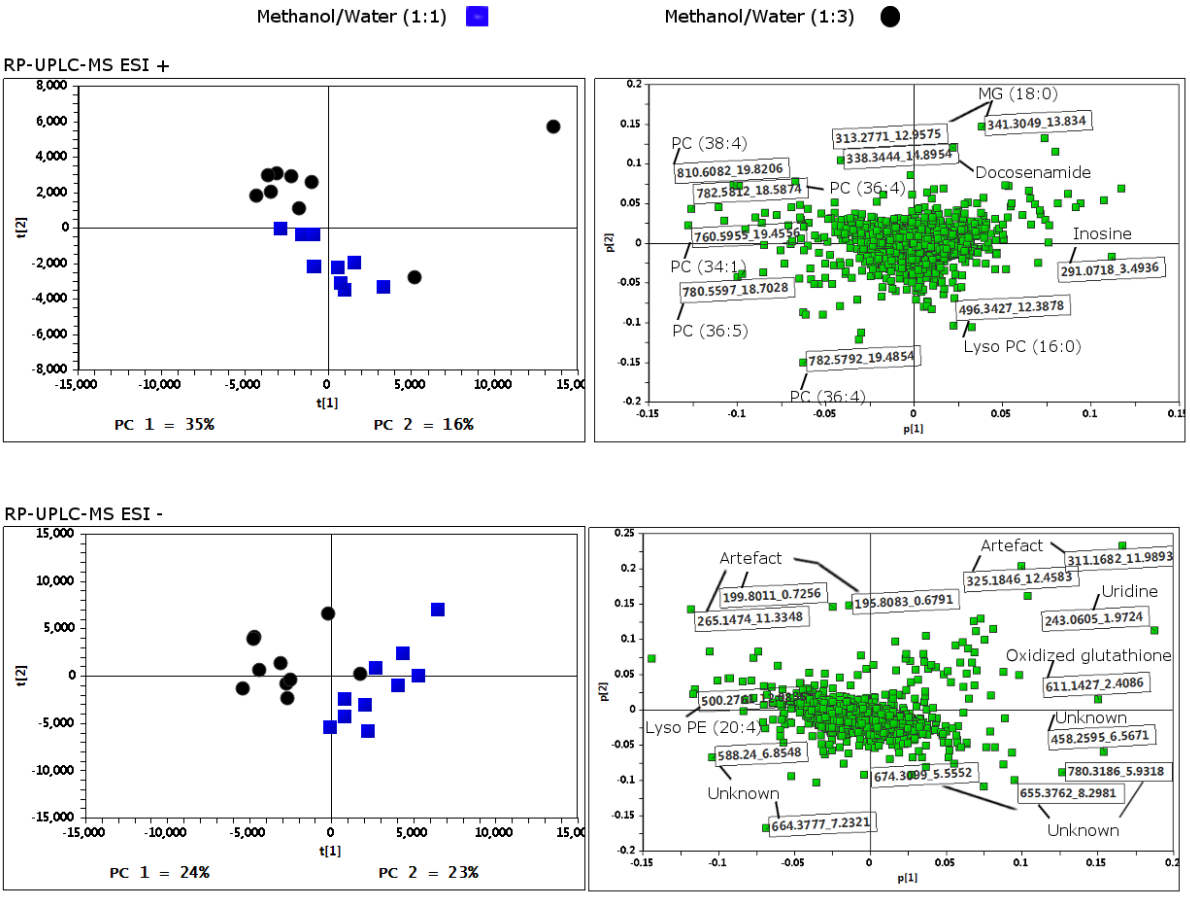

Figure S 2. Principal component analysis scores (left) and loadings (right) plots for RP-UPLC-MS-based metabolic profiling of aqueous extracts (for 1st stage of the study). Features located at the periphery of the loadings plots are driving the variation and were further structurally assigned. RP; Reversed phase, UPLC-MS; Ultra performance liquid chromatography – mass spectrometry, ESI; Electrospray ionization, PC; Phosphatidycholine, PE; Phosphatidylethanolamine; MG; Monoacylglycerol.

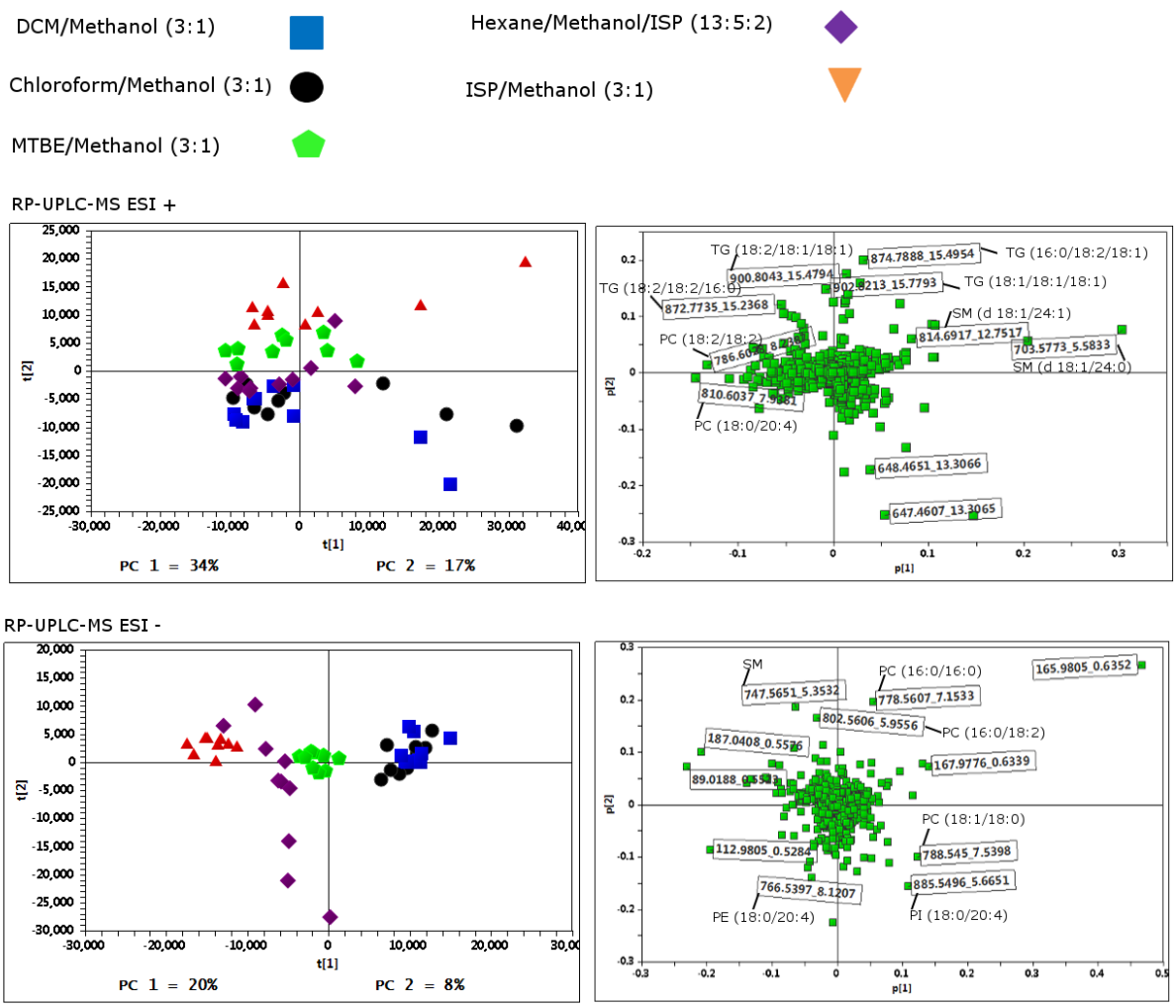

Figure S 3. Principal component analysis scores (left) and loadings (right) plots for RP-UPLC-MS-based metabolic (lipid) profiling of organic extracts (for 1st stage of the study). Features located at the periphery of the loadings plots are driving the variation and were further structurally assigned. DCM; dichloromethane, ISP; isopropanol, MTBE; methyl *tert*-butyl ether, RP; Reversed phase, UPLC-MS; Ultra performance liquid chromatography – mass spectrometry, ESI; Electrospray ionization, PC; Phosphatidycholine, PE; Phosphatidylethanolamine, PI; Phosphatidylinositol, SM: Sphingomyelin, TG; Triacylglycerol.

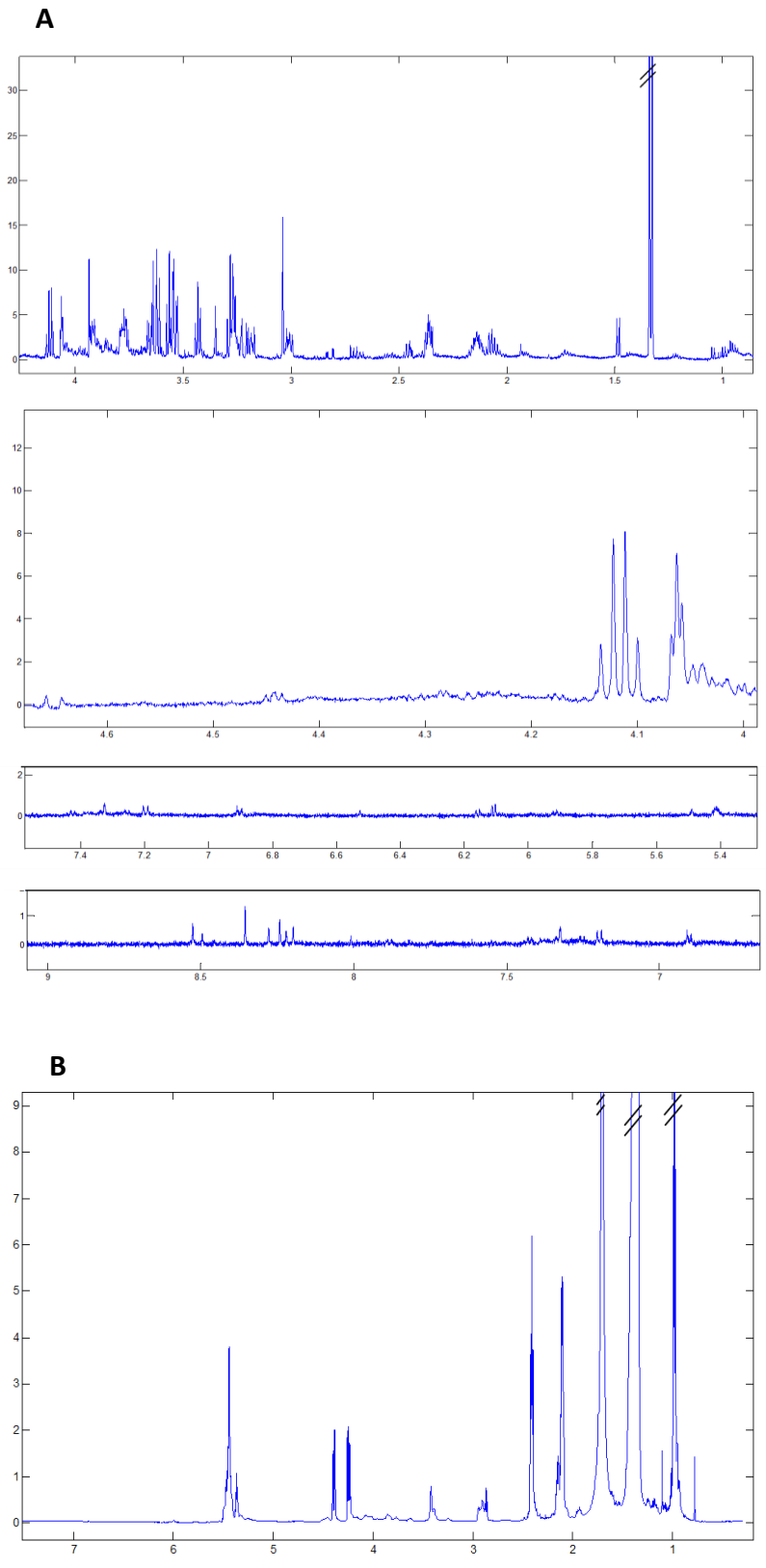

Figure S 4. H^1 NMR 600 MHz spectra of (A) aqueous extracts using Methanol/Water (1:1) extraction and (B) organic extracts obtained using MTBE/Methanol (3:1) extraction.

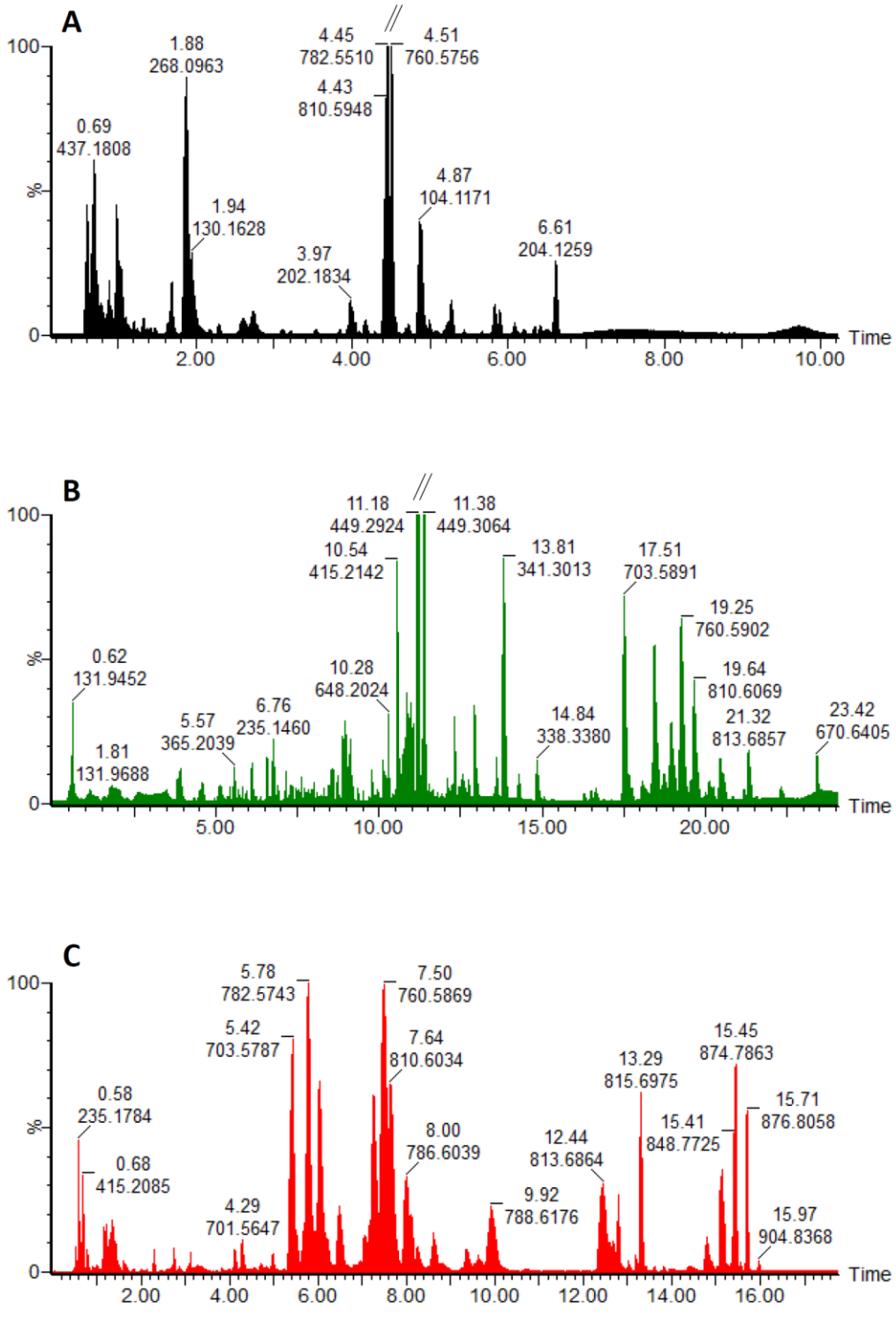

Figure S 5. BPI chromatograms from (A) HILIC-UPLC-MS ESI + analysis of aqueous extracts of vein tissue, (B) RP-UPLC-MS ESI + analysis of aqueous extracts and (C) RP-UPLC-MS ESI + analysis of organic extracts. Aqueous extracts were obtained using the Methanol/Water (1:1) and organic extracts using the MTBE/Methanol (3:1) extraction protocols.

Tables

Table S 1. Gradient programs used for chromatographic separation in HILIC-UPLC-MS and RP-UPLC-MS of aqueous and organic extracts**.**

a. HILIC-UPLC-MS

Time (min)	Flow rate (ml/min)	% A	% B	curve
Initial	0.40	99.0	1.0	-
2.00	0.40	99.0	1.0	6
8.00	0.40	45.0	55.0	6
9.00	0.40	1.0	99.0	6
9.10	0.60	1.0	99.0	6
11.00	0.60	99.0	1.0	6
11.10	0.60	99.0	1.0	6
19.00	0.60	99.0	1.0	6
19.10	0.40	99.0	1.0	6
23.00	0.40	99.0	1.0	6

b. RP-UPLC-MS

Time (min)	Flow rate (ml/min)	% A	% B	curve
Initial	0.40	99.9	0.1	-
2.00	0.40	99.9	0.1	6
6.00	0.40	75.0	25.0	6
10.00	0.40	20.0	80.0	6
12.00	0.40	10.0	90.0	6
21.00	0.40	0.1	99.9	6
23.00	0.40	0.1	99.9	6
23.01	0.80	0.1	99.9	6
29.00	0.80	0.1	99.9	6
29.01	0.40	99.9	0.1	6
32.00	0.40	99.9	0.1	6

c. Organic extracts-RP-UPLC-MS

Time (min)	Flow rate (ml/min)	% A	% B	curve
Initial	0.40	60.00	40.00	-
2.00	0.40	57.00	43.00	6
2.10	0.40	50.00	50.00	1
12.00	0.40	46.00	54.00	6
12.10	0.40	30.00	70.00	1
18.00	0.40	1.00	99.00	6
18.10	0.40	60.00	40.00	6
20.0	0.40	60.00	40.00	-

Table S 2. Table demonstrating the different parameters used in MassLynx V4.1 software for deconvoluting the data acquired from the UPLC-MS experiments.

Experiment type	HILIC-UPLC-MS ESI+	HILIC-UPLC-MS ESI-	RP-UPLC-MS ESI+	RP-UPLC-MS ESI-	RP-UPLC-MS Org ESI+	RP-UPLC-MS Org ESI-
Initial retention time	0.40	0.50	0.30	0.30	0.40	0.40
Final retention time	16.00	9.00	23.00	22.00	16.20	17.00
Low mass	50	50	50	50	150	100
High Mass	1000	1000	1000	1000	1200	1200
Peak width at 5% height (s)	25	25	15	15	25	25
Marker intensity threshold (counts)	640	500	2500	460	200	800
Mass window	0.10	0.10	0.10	0.10	0.10	0.10
Retention time window	0.50	0.50	0.50	0.50	0.50	0.50
Noise elimination level	6	6	6	6	6	6

Table S 3. Structurally assigned metabolites observed at the periphery of the loadings plots.

A= Methanol/Water (1:3) B= Methanol/Water (1:1)

C= DCM/Methanol (3:1) D= Chloroform/Methanol (3:1)

E= MTBE/Methanol (3:1) F=Hexane/Methanol/ISP (13:5:2)

G= Isopropanol/Methanol (3:1)

TG;Triacylglycerol, PC;Phosphatidylcholine, SM;Sphingomyelin, PE;Phosphatidylethanolamine, MG;Monoacylglycerol, PI;Phosphatidylinositol

a. HILIC-UPLC-MS ESI+

Met Name	**Mol Formula as Detected**	**Retention time (min)**	**m/z (found)**	**m/z (theor)**	**Δppm**	**CV%**
PC(32:1)	C40H79NO8P+ [M+H]+	4.71	732.5558	732.5543	-2	A=26%, B=6%, QC=5%
PC(38:4)	C46H85NO8P+ [M+H]+	4.61	810.5834	810.6013	22	A=12%, B=3%, QC=5%
Hypoxanthine	C5H5N4O+ [M+H]+	2.33	137.0449	137.0463	10	A=19%, B=5%, QC=4%
LysoPC	C24H51NO7P+ [M+H]+	5.45	496.3416	496.3403	-3	A=32%, B=12%, QC=8%

b. HILIC-UPLC-MS ESI-

Met Name	Mol Formula as Detected	Retention time (min)	m/z (found)	m/z (theor)	Δppm	CV%
PE(O-38:5)	C43H77NO7P-[M-H]-	4.41	750.5429	750.5438	1	A=61% B=14% QC=6%
PE(O-36:5)	C41H73NO7P-[M-H]-	4.09	722.5117	722.5125	1	A=25% B=17% QC=5%
PE(O-38:6)	C43H75NO7P-[M-H]-	4.05	748.5268	748.5281	-2	A=28% B=17% QC=3%
Inosine	C10H11N4O5-[M-H]-	3.01	267.0702	267.0729	10	A=14% B=10% QC=3%

c. RP-UPLC-MS ESI+

Met Name	Mol Formula as Detected	Retention time (min)	m/z (found)	m/z (theor)	Δppm	CV%
MG(18:0)	C21H41O3+ [M+H-H2O]+	13.83	341.3054	341.3056	0	A=47% B=15% QC=13%
MG(16:0)	C19H37O3+ [M+H-H2O]	12.96	313.2776	313.2743	-10	A=28% B=10% QC=13%
Docosenamide	C22H44NO+ [M+H]+	14.89	338.3457	338.3423	-10	A=52% B=68% QC=17%
PC(36:2)	C44H85NO8P+ [M+H]+	19.92	786.6107	786.6013	-12	A=55% B=42% QC=30%
PC(38:4)	C46H85NO8P+ [M+H]+	19.82	810.6085	810.6013	-9	A=55% B=43% QC=9%
PC(36:4)	C44H81NO8P+ [M+H]+	18.59	782.5814	782.5700	-14	A=47% B=21% QC=13%
PC(34:1)	C42H83NO8P+ [M+H]+	19.46	760.5959	760.5856	-13	A=50% B=30% QC=24%
PC(38:7)	C46H79NO8P+ [M+H]+	18.58	804.5611	804.5543	-8	A=38% B=19% QC=16%
PC(36:5)	C44H79NO8P+ [M+H]+	18.70	780.5602	780.5543	-7	A=61% B=13% QC=20%
LysoPC(16:0)	C24H50NO7PNa+ $[M+Na]^+$	12.39	518.3252	518.3223	-5	A=53% B=14% QC=7%
Inosine	C10H12N4O5Na+ $[M+Na]^+$	3.49	291.0721	291.0705	-5	A=65% B=30% QC=27%

d. RP-UPLC-MS ESI-

Met Name	**Mol Formula as Detected**	**Retention time (min)**	**m/z (found)**	**m/z (theor)**	**Δppm**	**CV%**
Uridine	C9H11N2O6-[M-H]-	1.97	243.0605	243.0617	5	A=68% B=49% QC=40%
Oxidized glutathione	C20H31N6O12S2-[M-H]-	2.41	611.1427	611.1441	2	A=63% B=33% QC=54%
LysoPE	C25H43NO7P-[M-H]-	12.08	500.2761	500.2777	3	A=49% B=35% QC=7%

e. RP-UPLC-MS ESI+ of organic extracts

Met Name	Mol Formula as Detected	Retention time (min)	m/z (found)	m/z (theor)	Δppm	CV%
TG(16:0/18:2/18:1)	C55H104O6N+ [M+NH4]+	15.49	874.7888	874.7864	-3	C=19% D=21% E=64% F= 40%, G=13% QC=6%
TG(18:2/18:1/18:1)	C57H106O6N+ [M+NH4]+	15.48	900.8043	900.8020	-2	C=15% D=22% E=75% F= 40% G=15% QC=7%
TG(18:1/18:1/18:1)	C57H108O6N+ [M+NH4]+	15.78	902.8213	902.8177	-4	C=21% D=25% E=95% F= 43% G=16% QC=8%
TG(18:2/18:2/16:0)	C55H102O6N+ [M+NH4]+	15.24	872.7736	872.7707	-3	C=21% D=32% E=72% F= 35% G=19% QC=8%
PC(38:4)	C46H85NO8P+ [M+H]+	7.94	810.6036	810.6013	-3	C=38% D=6% E=44% F= 22% G=5% QC=4%
PC(36:2)	C44H85NO8P+ [M+H]+	8.24	786.6028	786.6013	-2	C=31% D=16% E=30% F= 10% G=11% QC=9%
PS(36:1)	C42H81NO10P+ [M+H]+	8.21	790.5623	790.5598	-3	C=50% D=43% E=69% F= 28% G=26% QC=5%

PC(36:1)	C44H87NO8P+ [M+H]+	10.29	788.6191	788.6169	-3	C=38% D=46% E=47% F= 29% G=24% QC=37%
SM(d18:1/24:1)	C47H94N2O6P+ [M+H]+	12.75	813.6876	813.6850	-3	C=19% D=16% E=29% F= 11% G=12% QC=4%
SM(d18:1/16:0)	C39H80N2O6P+ [M+H]+	5.58	703.5773	703.5754	-3	C=42% D=40% E=47% F= 25% G=21% QC=7%

f. RP-UPLC-MS ESI- of organic extracts

Met Name	Mol Formula as Detected	Retention time (min)	m/z (found)	m/z (theor)	Δppm	CV%
PC(32:0)	C41H81NO10P-[M+FA-H]-	7.15	778.5675	778.5598	-9	C=13% D=12% E=53% F= 40% G=4% QC=35%
PC(34:2)	C43H81NO10P-[M+FA-H]-	5.95	802.5606	802.5598	-1	C=14% D=12% E=41% F= 30% G=4% QC=36%
PE(38:4)	C43H77NO8P-[M-H]-	8.12	766.5397	766.5387	-1	C=18% D=35% E=21% F= 20% G=3% QC=38%
PS(36:1)	C42H79NO10P-[M-H]-	7.54	788.5450	788.5442	-1	C=18% D=26% E=69% F= 30% G=22% QC=35%
PC(37:5)	C46H81NO10P-[M+FA-H]-	7.13	838.5623	838.5598	-3	C=71% D=28% E=134% F= 74% G=27% QC=64%
PI(38:4)	C47H82O13P-[M-H]-	5.67	885.5496	885.5493	0	C=9% D=8% E=21% F= 19% G=4% QC=35%