

Supplementary Material

A high throughput method for the analysis of erythrocyte fatty acids and the omega-3 index

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The amount of each phospholipid (PL) standard stock solutions added to create the five synthetic PL mixtures.

Table S1: Amount of each synthetic PL stock solutions added to create 2% synthetic PL mixtures.

Synthetic PL standards	uMolar	% total phospholipids
PtdEtn 16:0/18:1	80.8	8.1
PtdCho 16:0/18:1	363.8	36.4
PtdCho 16:0/18:2	363.8	36.4
PtdEtn 18:0/20:4	50.5	5.1
PtdCho 16:0/20:4	101.1	10.1
PtdEtn 18:0/22:6	8.0	0.8
PtdCho 16:0/22:6	32.0	3.2

Table S2: Amount of each synthetic PL stock solutions added to create 4% synthetic PL mixtures.

Synthetic PL standards	uMolar	% total phospholipids
PtdEtn 16:0/18:1	77.5	7.7
PtdCho 16:0/18:1	348.6	34.9
PtdCho 16:0/18:2	348.6	34.9
PtdEtn 18:0/20:4	48.4	4.8
PtdCho 16:0/20:4	96.8	9.7
PtdEtn 18:0/22:6	16.0	1.6
PtdCho 16:0/22:6	64.0	6.4

Table S3: Amount of each synthetic PL stock solutions added to create 6% synthetic PL mixtures.

Synthetic PL standards	uMolar	% total phospholipids
PtdEtn 16:0/18:1	74.1	7.4
PtdCho 16:0/18:1	333.5	33.3
PtdCho 16:0/18:2	333.5	33.3
PtdEtn 18:0/20:4	46.3	4.6
PtdCho 16:0/20:4	92.6	9.3
PtdEtn 18:0/22:6	24.0	2.4
PtdCho 16:0/22:6	96.0	9.6

Table S4: Amount of each synthetic PL stock solutions added to create 8% synthetic PL mixtures.

Synthetic PL standards	uMolar	% total phospholipids
PtdEtn 16:0/18:1	70.7	7.1
PtdCho 16:0/18:1	318.3	31.8
PtdCho 16:0/18:2	318.3	31.8
PtdEtn 18:0/20:4	44.2	4.4
PtdCho 16:0/20:4	88.4	8.8
PtdEtn 18:0/22:6	32.0	3.2
PtdCho 16:0/22:6	128.0	12.8

Table S5: Amount of each synthetic PL stock solutions added to create 10% synthetic PL mixtures.

Synthetic PL standards	uMolar	% total phospholipids
PtdEtn 16:0/18:1	67.4	6.7
PtdCho 16:0/18:1	303.2	30.3
PtdCho 16:0/18:2	303.2	30.3
PtdEtn 18:0/20:4	42.1	4.2
PtdCho 16:0/20:4	84.2	8.4
PtdEtn 18:0/22:6	40.0	4.0
PtdCho 16:0/22:6	160.0	16.0

Example of the correction applied to 16:0, 18:0 and 18:1 concentration determined by GC. The example provided here is for 18:0 in a single sample. This procedure was repeated for 16:0, 18:0 and 18:1 in every sample.

1) Determine the proportion of CerPCho 18:0 from MS data.

$$[\text{CerPCho 18:0 (MS)}] / [\text{total 18:0 (MS)}] = \text{proportion of CerPCho 18:0 (MS)}$$

$$(0.95 \text{ nmol/ml}) / (6.91 \text{ nmol/ml}) = 0.14$$

2) Estimate the concentration of CerPCho 18:0 obtained by GC.

$$0.14 \times [\text{18:0 (GC)}] = [\text{CerPCho 18:0 (GC)}]$$

$$0.14 \times 213.8 \text{ nmol/ml} = 29.9 \text{ nmol/ml}$$

3) Correct the concentration of CerPCho 18:0 obtained by GC

$$29.9 \text{ nmol/ml} \times \text{correction factor} = \text{corrected } [\text{CerPCho 18:0 (GC)}]$$

$$29.9 \text{ nmol/ml} \times 2.48 = 74.2 \text{ nmol/ml}$$

4) Add corrected CerPCho 18:0 concentration to the PL 18:0 concentration to determine corrected total 18:0 concentration by GC.

$$([\text{18:0 (GC)}] - [\text{CerPCho 18:0 (GC)}]) + \text{corrected } [\text{CerPCho 18:0 (GC)}] = \text{corrected } [\text{18:0 (GC)}]$$

$$(213.8 - 29.9) + 74.2 = 258.1$$

Sphingomyelin fatty acids transesterification experiment

An experiment was conducted to examine the extraction recovery of sphingomyelin fatty acids using two methods; namely the Lepage and Roy transesterification as used in the GC-FID method and the new high throughput ESI-MS method. CerPCho 24:1 was selected and added to the synthetic PL mixture shown in table S5. The results are shown in table S6 and it clearly shows that 24:1 (sphingomyelin fatty acids) are not completely transesterified using the Lepage and Roy method as there was only a 45% recovery.

Tables S6: Recovery results of Sphingomyelin fatty acids transesterification experiment (n=3).

Fatty acids	Added concentration	GC-FID method		The high-throughput ESI-MS method	
	nmol/ml	Mean \pm SEM	(%) recovery	Mean \pm SEM	(%) recovery
16:0	917.9	870.4 \pm 17.3	94.8%	880.2 \pm 11.9	95.9%
18:0	82.1 [#]	85.4 \pm 0.5	104.0%	45.7 \pm 1.0	55.7%
18:1	370.5	361.9 \pm 6.4	97.7%	360.2 \pm 3.7	97.2%
18:2	303.2	278.6 \pm 4.6	91.9%	297.7 \pm 6.2	98.2%
20:4	126.3	111.9 \pm 5.4	88.4%	116.5 \pm 1.9	92.2%
22:6	200 [*]	154.6 \pm 17.3	77.3%	150.0 \pm 5.8	75.0%
24:1	200	89.6 \pm 6.7	44.8%	195.5 \pm 2.4	97.8%

Footnote of abbreviations; * due to oxidation of PtdEtn (18:0/22:6) as described the expected concentration was 160 nmol/ml; [#]Due to oxidation of PtdEtn (18:0/22:6) the expect ESI-MS value was 42.1 nmol/ml.

Table S7: Targeted ion scans used to acquired phospholipid data.

Lipid	Ion	Scan	DP	EP	CE	CXP	Mass range (Da)
Head group scans							
PtdCho/CerPCho	[M+H] ⁺	PI 184.1	100	10	47	8	640-1000
PtdEtn	[M+H] ⁺	NL141.0	100	10	30	8	685-950
PtdSer	[M+H] ⁺	NL 185.0	100	10	30	8	755-965
Fatty acyl chain scans							
16:1	[M-H] ⁻	PI 253.2	-100	-10	-55	-11	600-900
16:0	[M-H] ⁻	PI 255.2	-100	-10	-55	-11	600-900
17:0	[M-H] ⁻	PI 269.3	-100	-10	-55	-11	560-900
18:2	[M-H] ⁻	PI 279.2	-100	-10	-40	-11	600-900
18:1	[M-H] ⁻	PI 281.3	-100	-10	-55	-11	600-900
18:0	[M-H] ⁻	PI 283.3	-100	-10	-55	-11	600-900
19:0	[M-H] ⁻	PI 297.3	-100	-10	-55	-11	600-900
20:5	[M-H] ⁻	PI 301.2	-100	-10	-40	-11	500-1000
20:4	[M-H] ⁻	PI 303.2	-100	-10	-40	-11	600-1000
20:3	[M-H] ⁻	PI 305.2	-100	-10	-40	-11	600-1000
22:6	[M-H] ⁻	PI 327.2	-100	-10	-40	-11	700-1000
22:5	[M-H] ⁻	PI 329.2	-100	-10	-40	-11	700-1000
22:4	[M-H] ⁻	PI 331.2	-100	-10	-40	-11	700-1000
24:0	[M-H] ⁻	PI 365.3	-100	-10	-40	-11	740-1040
24:1	[M-H] ⁻	PI 363.3	-100	-10	-40	-11	740-1040

Scan rate for positive ion mode was 200 Da/s, for negative ion mode 1000 Da/s. Mass shifting was prevented in negative ion mode by increasing number of summed scans. PI Precursor ion, NL neutral loss, DP declustering potential, EP entrance potential, CE collision energy, CXP collision cell exit potential.

Table S8: Justification for Cost per sample for GC-FID method and ESI-MS method.

Items	GC-FID method	ESI-MS method
Materials and consumables	\$ 28.0	\$ 10.0
Maintenance contributions and initial cost	\$ 22.0	\$ 10.0
Total cost per sample	\$ 50.0	\$ 20.0

Table S9: Fatty acids concentrations (nmol/ml blood) in QC samples (n=57). Values are shown as mean \pm SEM (%CV) from 19 runs.

Fatty acids	Mean \pm SEM (%CV)
16:0	454.3 \pm 12.5 (13.4%)
17:0	17.6 \pm 1.1 (13.7%)
18:0	257.7 \pm 1.7 (8.3%)
24:0	83.9 \pm 1.3 (19.7%)
16:1	4.4 \pm 0.1 (14.9%)
18:1	206.9 \pm 10.9 (13.8%)
24:1	104.0 \pm 0.5 (7.5%)
18:2	146.5 \pm 3.3 (13.3)
20:3	22.2 \pm 0.3 (17.2%)
20:4	187.9 \pm 2.3 (10.0%)
20:5	5.7 \pm 0.1 (15.5%)
22:4	21.7 \pm 2.2 (17.9%)
22:5	29.8 \pm 1.2 (10.7%)
22:6	73.9 \pm 2.5 (6.9 %)

PL species	Sample #1	Sample #2	Sample #3
PtdCho 16:0 -16:0	0.3067	0.4257	0.4061
PtdCho 16:0 -16:1	0.0529	0.0559	0.0840
PtdCho 16:0 -17:0	0.0223	0.0440	0.0404
PtdCho 16:0 -18:0	0.0670	0.1240	0.0692
PtdCho 16:0 -18:1	1.4029	1.9089	1.7042
PtdCho 16:0 -18:2	2.4135	2.5645	2.4609
PtdCho 17:0 -18:1	0.0577	0.0829	0.0991
PtdCho 17:0 -18:2	0.0571	0.1042	0.1010
PtdCho 18:0 -18:0	0.0218	0.0307	0.0192
PtdCho 18:0 -18:1	0.2411	0.3723	0.4016
PtdCho 18:0 -18:2	0.5075	0.7984	0.8687
PtdCho 18:1 -18:1	0.1741	0.2029	0.2515
PtdCho 18:1 -18:2	0.2553	0.3664	0.4422
PtdCho 16:0 -20:3	0.3944	0.2256	0.2142
PtdCho 16:0 -20:4	0.7541	0.9201	0.6681
PtdCho 16:0 -20:5	0.0323	0.0865	0.0774
PtdCho 18:0 -20:4	0.3001	0.4022	0.3045
PtdCho 18:1 -20:4	0.1172	0.1923	0.1533
PtdCho 16:0 -22:6	0.2240	0.3027	0.2285
PtdCho 18:0 -22:6	0.0488	0.0681	0.0758
PtdEtn 16:0 -18:1	0.9952	1.6980	1.1877
PtdEtn 16:0 -18:2	0.4094	0.5437	0.4861
PtdEtn 18:0 -18:0	0.0107	0.0206	0.0135
PtdEtn 18:0 -18:1	0.2436	0.2933	0.2676
PtdEtn 18:0 -18:2	0.2253	0.2728	0.2325
PtdEtn 18:1 -18:1	0.2107	0.3383	0.3201
PtdEtn 18:1 -18:2	0.2692	0.4544	0.4678
PtdEtn 16:0 -20:3	0.0805	0.0813	0.0891
PtdEtn 16:0 -20:4	0.6756	0.9784	0.6669
PtdEtn 16:0 -20:5	0.0391	0.0648	0.0584
PtdEtn 17:0 -20:4	0.1052	0.1373	0.1044
PtdEtn 17:0 -20:5	0.1183	0.1675	0.1294
PtdEtn 18:0 -20:4	0.4480	0.5038	0.4570
PtdEtn 16:0 -22:4	0.2259	0.2528	0.1547
PtdEtn 16:0 -22:5	0.1216	0.2065	0.1147
PtdEtn 18:1 -20:4	0.3976	0.5620	0.4405
PtdEtn 16:0 -22:6	0.3658	0.4774	0.3093
PtdEtn 17:0 -22:5	0.0595	0.0761	0.0590
PtdEtn 18:0 -22:6	0.1188	0.1370	0.1001
PtdSer 18:0 -18:0	0.0047	0.0056	0.0067
PtdSer 18:0 -18:1	0.2237	0.1789	0.2599
PtdSer 18:0 -20:4	1.3874	1.6430	1.6171
PtdSer 18:0 -22:5	0.2739	0.3720	0.2826
PtdSer 18:0 -22:6	0.4162	0.6404	0.4840
CerPCho 16:0	2.5708	3.6073	3.3175
CerPCho 18:0	0.3409	0.5706	0.4763
CerPCho 18:1	0.1172	0.2077	0.1788
CerPCho 24:0	2.5009	2.9584	2.0206
CerPCho 24:1	3.1676	4.1307	3.6350
Fatty acids	Sample #1	Sample #2	Sample #3
16:0	11.5	15.0	12.7
16:1	0.1	0.1	0.1
17:0	0.4	0.6	0.5
18:0	4.9	6.5	6.0
18:1	4.7	6.8	6.3
18:2	4.1	5.1	5.1
20:3	0.5	0.3	0.3
20:4	4.2	5.3	4.4
20:5	0.2	0.3	0.3
22:4	0.2	0.3	0.2
22:5	0.5	0.7	0.5
22:6	1.2	1.6	1.2
24:0	2.5	3.0	2.0
24:1	3.2	4.1	3.6
Fatty acids	Sample #1	Sample #2	Sample #3
16:0	458.4	599.7	509.7
16:1	2.1	2.2	3.4
17:0	16.8	24.5	21.3
18:0	196.7	259.6	239.0
18:1	187.7	273.5	252.2
18:2	165.5	204.2	202.4
20:3	19.0	12.3	12.1
20:4	167.4	213.6	176.5
20:5	7.6	12.8	10.6
22:4	9.0	10.1	6.2
22:5	18.2	26.2	18.3
22:6	46.9	65.0	47.9
24:0	100.0	118.3	80.8
24:1	126.7	165.2	145.4

Individual PL species concentration (nmol/10 uL of packed RBCs) determined by mass spectrometry as described in methods.

Individual fatty acid concentration (nmol/10 uL of packed RBCs) calculated from the sum of each individual PL species that contains that FA. If the PL contained 2 moles of the FA, e.g. PtdCho 16:0-16:0 it was added twice.

To convert FA concentration from nmol/10 uL of packed RBCs to nmol/mL of blood the FA concentration obtained for 10 uL of packed RBCs was multiplied by 40 (x 100 to obtain mL of RBCs then x 0.4 to convert to whole blood, assuming an hematocrit of 40%).

Figure S1: An example of spread sheet used to calculate the fatty acids levels from PL individual species detected from ESI-MS method.

Testing solvents for lysing erythrocyte membranes

a) The TRIS-Aliquot method

In 2 mL glass vials, 10 μ L of packed erythrocytes were added to 240 μ L of TRIS-HCl. Following this, the tubes were vortexed for two minutes before a 25 μ L aliquot was taken. The aliquot was dispensed into a new tube to which 75 μ L of 200 mM ammonium acetate and 90 μ L of methanol were added prior to a further 2 minutes of vortexing. Following this, 300 μ L of MTBE was added and tubes were vortexed again for 2 minutes after which they were left to stand for 1 minutes to allow phase separation. After phase separation had occurred, 300 μ L of the upper lipid containing phase was removed and dried under nitrogen at 37°C. The dried lipid were then reconstituted to 150 μ L using 2:1 methanol: chloroform and were stored at -20°C until ready to be analysed. Prior to mass spectrometric analysis, extracts were diluted using 2:1 methanol: chloroform solution containing 5 mM ammonium acetate as spray solvent.

b) Adding ammonium acetate first

In 2 mL eppendorf tube, 10 μ L of packed erythrocytes were added to 100 μ L of an aqueous mixture containing 150 mM ammonium acetate and 2 mM EDTA before being vortexed. Following this, 100 μ L of methanol was added and vortexed for 2 minutes. After that 400 μ L of MTBE was added and vortexed for 2 minutes. 400 μ L of the supernatant was removed and dried under nitrogen at 37°C and then reconstituted with 200 μ L of 2:1 methanol: chloroform solution containing 5 mM ammonium acetate. Lipid extracts were stored at -20°C until ready for analysis.

c) TRIS-buffered ammonium chloride

TRIS-buffered ammonium chloride was firstly optimised to PH 7.4 by the addition hydrochloric acid. As per methods above, 10 μ L of packed erythrocytes were added to 100 μ L of the TRIS-buffered ammonium chloride and mixed by repeated aspirating/dispensing. The tubes were then vortexed for 2 minutes after which 120 μ L of methanol was added

followed by further 2 minutes of vortexing. To each tube, 400 μ L of MTBE was then added and each tube was again vortexed for 2 minutes. The upper phase was extracted, placed in a new glass vial and dried under nitrogen at 37°C and then reconstituted with 300 μ L of 2:1 methanol: chloroform and then stored at -20 °C until ready for analysis.

d) *Adding methanol first*

The method carried out was analogous to those previously described with some minor adjustments to reagent order and times for vortexing. Briefly, 10 μ L of packed erythrocytes were added to 120 μ L of methanol containing 0.01% BHT in eppendorf tubes and vortexed for 10 minutes. Following this, 400 μ L of MTBE was added and the tubes underwent another 10 minutes vortex before phase separation was initiated by addition 100 μ L of 5 mM ammonium acetate. Lipid extracts were stored at -20 °C until ready for analysis.

This study demonstrated that adding ammonium acetate as first reagent to packed erythrocytes produced quality mass spectra when analysed using ESI-MS. Having the erythrocytes suspended in aqueous mixture containing ammonium acetate and EDTA enable the methanol to interface more readily with the packed erythrocytes than if they were not in suspension. Further to this, the uniform solution created by suspending the packed erythrocytes in ammonium acetate and EDTA makes their analysis considerably more amenable to the robotic platform as the propensity of packed erythrocytes to clot is ameliorated.