Comprehensive Ultra Performance Liquid Chromatographic Separation and Mass Spectrometric Analysis of Eicosanoid Metabolites Suitable for Human Materials

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Reagents

All eicosanoids and deuterated internal standards were purchased from Cayman Chemical. Optima LC-MS grade acetonitrile (ACN), methanol (MeOH), and water were obtained from Fisher Scientific. Isopropanol (IPA) was purchased from Sigma-Aldrich. Formic acid (FA) was obtained from EMD Technologies. Dulbecco's Phosphate Buffered Saline (DPBS) was obtained from Corning Life Sciences.

Sample preparation

Preparation of primary standard and internal standard solutions

For the preparation of calibration curves, stock solutions were prepared in ethanol that contained all eicosanoid standards, each at a concentration of 0.25 ng/uL. Working standard solutions for all eicosanoids were prepared by serial dilution of the stock solutions to create the necessary concentrations. A solution containing 26 internal (deuterated) eicosanoid standards was prepared at 0.01 ng/uL in ethanol. All solutions were stored at -80°C when not in use.

Extraction of metabolites from plasma and tissue

Aliquots of 20 ul control plasma (Human Source Plasma, Gemini Bio-Products) were diluted to 1 mL with phosphate salt buffer spiked with 100 uL of the internal standard solution. The eicosanoids were extracted using Strata-X reversed-phase SPE columns (8B-S100-UBJ, Phenomenex). Columns were washed with 3 mL MeOH and then equilibrated with 3 mL H_2O. After loading the sample, the columns were washed with 10% MeOH to remove impurities, and the metabolites were then eluted with 1 mL of MeOH and stored at -80°C to prevent metabolite degradation. Prior to analysis, the eluant was dried under vacuum and re-dissolved in 50 uL of the UPLC solvent A (water/acetonitrile/acetic acid (60:40:0.02; v/v/v)) for UPLC/MS/MS analysis.

Took tissue sample, then weighed, and transferred to 1 mL PBS buffer containing 10% MeOH tube. The tissue was homogenized using Beadbug Microtube Homogenizer (Benchmark Scientific). The eicosanoids extracted from homogenates with SPE followed the same protocol as plasma sample.

UPLC-MS/MS

An Acquity UPLC system (Waters Corp) was used. Reversed-phase separation was performed on an Acquity UPLC BEH shield RP18 column (2.1×100 mm 1.7 μm; Waters). The mobile phase consisted of (A) ACN/water/acetic acid (60/40/0.02, v/v) and (B) ACN/IPA(50/50, v/v). Gradient elution without splitting was carried out for 5 min at a flow rate of 0.5 mL/min. Gradient conditions were as follows: 0-4.0 min, 0.1-55% B; 4.0-4.5 min, 55-99% B; 4.5-5.0 min, 99% B; A 10 μL aliquot of each sample was injected into the column. The column temperature was kept at 40 °C. All samples were incubated at 4 °C during analysis.

Mass spectrometry was performed on an ABI/Sciex 6500 QTRAP hybrid, triple quadrupole, linear ion trap mass spectrometer equipped with a Turbo V ion source. Eicosanoids were detected in negative electrospray ion mode. Curtain gas (CUR), nebulizer gas (GS1) and turbo-gas (GS2) were set at 10 psi, 30 psi and 30 psi, respectively. The electrospray voltage was -4.5 kV, and the turbo ion spray source temperature was 525 °C. Nitrogen was employed as collision gas and the collisionally-activated dissociation (CID) was set at a high level.

Eicosanoids were analysed using scheduled multiple reaction monitoring (MRM). Mass spectrometer parameters including the declustering potentials and collision energies were optimized for each analyte. Data acquisitions were performed using Analyst 1.6.2 software (Applied Biosystems). Multiquant software (Applied Biosystems) was used to quantify all metabolites.

Analytical validation

Linearity and LOD and LOQ

A typical standard curve was prepared by adding 1 ng of each internal (deuterated) eicosanoid standard to the following amounts of eicosanoid (nondeuterated) primary standards: 0.005, 0.015, 0.025, 0.035, 0.05, 0.15, 0.25, 0.35, 0.5, 1.5, 2.5, 3.5, and 5.0 ng. Quantitation of eicosanoid levels was performed using linear regression of the response ratios (peak area analyte/peak area internal standard) obtained from the calibration curve to calculate the corresponding eicosanoid amount. The limit of detection (LOD) was defined as the concentration that resulted in a peak with a signal-to-noise ratio (S/N) greater than 3:1 (3 S/N) and the limit of quantitation (LOQ) was defined as (S/N) greater than 7:1 (7 S/N).

Recovery rate and matrix effect

Recovery from control plasma was determined by comparison of the analyte peak area of the internal standard spiked into a plasma sample compared with the corresponding peak area of standard solutions extracted in a similar manner. The determination was performed in triplicate. The matrix effect was calculated by dividing the peak area of the internal standard spiked into a plasma sample and extracted into mobile phase A by the area of the deuterated standard in the standard solution. This determination was also performed in triplicate.

Accuracy and precision

Accuracy and precision of the method was determined using quality control (QC) samples spiked at three levels of eicosanoids: low (LQC) 0.15 ng, medium (MQC) 1.5 ng, and high QC (HQC) 5 ng. QC samples were prepared by spiking blank plasma with three levels of eicosanoids and internal standards prior to plasma extraction as described. Intra-batch and inter-batch (three different batches) accuracy and precision were determined by analysing five QC samples covering the calibration range. The precision of the quantitation was expressed as percent coefficient of variance (CV %), calculated by dividing the standard deviation by the mean and then multiplied by 100.

These QC amounts included the known fortified level added to the control plasma plus the endogenous amount of analytes. The endogenous amounts of analytes in plasma were determined in five replicate measurements. The accuracy of the analytic method was denoted by the relative error (RE %), calculated as percent of the mean deviation from the known amount plus endogenous amount, RE % = [(amount found − (known amount + endogenous amount) × 100/(known amount + endogenous amount)].

Stability

To determine the stability of the processed samples, they were kept at 4°C in the autosampler and injected three times at 0, 4 and 8 h with three levels of quality control samples, respectively. The peak area of the analytes at the initial point (0 h) was used as the reference to determine the relative stability at subsequent points.

Method Development

SPE techniques were used to extract eicosanoid metabolites from plasma, which is more suitable for processing a large number of samples than a more efficient liquid/liquid extraction (LLE) technique. The extraction efficiency of LLE is generally higher than SPE, but this method also extracts many endogenous impurities that can affect the separation and quantitation of target analytes. The ability of SPE to eliminate impurities is better than that of LLE, which improves the detection of eicosanoids in biological matrices, especially when present at low levels.

A crucial aspect of our method is the inclusion of 26 deuterated internal standards. All samples are spiked with a mixture of deuterated internal standards prior to lipid extraction. An internal standard is used to correct for run-to-run variation in extraction efficiency, for monitoring the chromatographic response, and for normalization purpose which allows for accurate quantification. Also, eicosanoid quantitation was performed by the stable isotope dilution method. For each eicosanoid to be quantify, an internal standard was selected that had a different precursor ion mass than the target analyte but was chemically and structurally similar to the target analyte as possible. This is ideally achieved by using a deuterated analogue of the analyte. We employed these standards whenever they were commercially available. For example, (d4) PGE_2 was employed as the internal standard for PGE_2. In other cases, we employed a deuterated analogue that was the closest to the desired analogue in characteristics. For example, (d4) 15d $PGJ_2$was employed as the internal standard for PGJ_2, 15d- PGJ_2, and 15d-PGD_2. The 26 deuterated internal standards, which presently used to quantify, assigned to each of the 158 eicosanoids analysed are listed in Table 1.

Table 1. **Internal Standard Assigned for Analytes**

No.	Internal Standard	Analytes Assigned	No.	Internal Standard	Analytes Assigned
1	**(d4) 6k PGF1α**	6	**14**	**(d6) 20-HETE**	10
2	**(d4) TXB2**	5	**15**	**(d4) 9-HODE**	2
3	**(d4) PGF2α**	12	**16**	**(d4) 13-HODE**	4
4	**(d4) PGE2**	10	**17**	**(d7) 5-oxoETE**	6
5	**(d4) PGD2**	11	**18**	**(d4) Resolvin E1**	6
6	**(d4) 15d PGJ2**	6	**19**	**(d11) 8,9 EET**	1
7	**(d4) dhk PGF2α**	1	**20**	**(d11) 11,12 EET**	5
8	**(d4) dhk PGD2**	2	**21**	**(d11) 14,15 EET**	1
9	**(d11) 5-iso PGF2αVI**	4	**22**	**(d4) 9,10 diHOME**	1
10	**(d4) LTB4**	18	**23**	**(d4) 12,13 diHOME**	1
11	**(d8) 5-HETE**	13	**24**	**(d5) LTC4**	1
12	**(d8) 12-HETE**	13	**25**	**(d5) LTE4**	2
13	**(d8) 15-HETE**	9	**26**	**(d8) Arachidonic Acid**	8

A targeted approach was used to identify and quantify lipids using mass spectrometry (MS) coupled with ultra-high performance liquid chromatography (UPLC-MS). A targeted MRM approach provided for higher sensitivity than an unbiased full scan MS analysis. UPLC also provided enhanced chromatographic resolution, sensitivity, reproducibility and fast separation. Additionally, a QTRAP 6500 was used in the present study. The advantages of the QTRAP 6500 are scan speeds of up to 20,000 Da/second for optimized UPLC strategies, a twenty-fold increase in the detector dynamic range, and LOQ improvement of up to five fold. In summary, the UPLC-QTRAP 6500 system provides a sensitive, accurate and fast separation and monitoring platform.

All eicosanoids are detected in the negative ion mode to take advantage of their conserved terminal carboxyl moiety. **Figure 1** shows a representative extracted ion chromatogram for the separation of all 184 eicosanoids including the 26 internal standards, which are monitored in a single 5 min LC-MS/MS analytical run. The optimal

declustering potential (DP) and the collision energy (CE) has been determined for each MRM pair (**Table 2**). These values were optimized by directly infusing commercial standards into the mass spectrometer.

Figure 1. **Extracted Ion Chromatograms of a mixture 187 Eicosanoid Standard (A) and a Magnified View of the Chromatograms of HETE Metabolites**

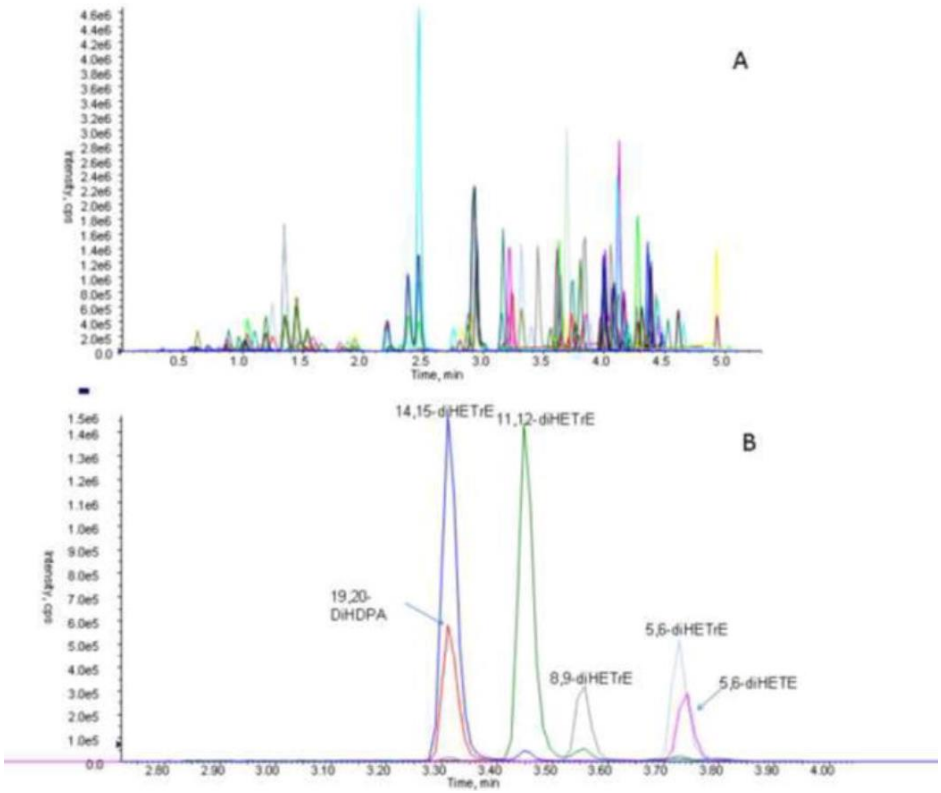


Table 2. **Optimized MRM Pairs and parameters for Eicosanoids**

No.	Common name	Retention Time (min)	m/z		DP (V)	CE (V)	LOQ (pg)
			Parent	Daughter			
1	(d4) 6k PGF1α	0.76	373	167	-60	-34	-
2	(d4) TXB2	0.98	373	173	-50	-22	-
3	(d4) PGF2α	1.14	357	197	-50	-35	-
4	(d4) PGE2	1.29	355	275	-50	-23	-
5	(d4) PGD2	1.47	355	275	-50	-23	-
6	(d4) 15d PGJ2	3.54	319	203	-30	-20	-
7	(d4) dhk PGF2α	1.74	357	295	-80	-28	-
8	(d4) dhk PGD2	2.13	355	297	-40	-26	-
9	(d4) 8-iso PGF2α VI	0.99	357	197	-20	-22	-
10	(d4) LTB4	2.85	339	197	-45	-23	-
11	(d8) 5-HETE	4.17	327	116	-40	-20	-

12	(d8) 12-HETE	4.05	327	184	-50	-19	-
13	(d8) 15-HETE	3.92	327	226	-40	-19	-
14	(d6) 20-HETE	3.66	325	281	-50	-24	-
15	(d4) 9-HODE	3.89	299	172	-60	-23	-
16	(d4) 13-HODE	3.89	299	198	-60	-23	-
17	(d7) 5-oxoETE	4.31	323	279	-60	-22	-
18	(d4) Resolvin E1	0.8	353	197	-40	-20	-
19	(d11) 8,9 EET	4.37	330	155	-50	-19	-
20	(d11) 11,12 EET	4.34	330	167	-50	-19	-
21	(d11) 14,15 EET	4.23	330	175	-40	-19	-
22	(d4) 9,10 diHOME	3.12	317	203	-50	-29	-
23	(d4) 12,13 diHOME	3.07	317	185	-50	-29	-
24	(d5) LTC4	2	629	272	-50	-34	-
25	(d5) LTE4	2.25	443	338	-30	-26	-
26	(d8) Arachiconic Acid	4.87	311	267	-55	-20	-
27	6k PGF1α	0.76	369	245	-60	-34	5
28	TxB2	0.98	369	169	-50	-22	3
29	PGF2α	1.12	353	197	-50	-35	70
30	PGE2	1.27	351	271	-50	-23	3
31	PGD2	1.45	351	27	-50	-23	3
32	11b PGF2α	1.08	353	335	-50	-35	100
33	TXB1	0.95	371	171	-30	-27	5
34	PGF1α	1.18	355	293	-60	-33	7
35	PGE1	1.4	353	235	-40	-29	30
36	PGD1	1.51	353	235	-40	-29	30
37	d17 6k PGF1α	0.66	367	163	-60	-34	7
38	TXB3	0.81	367	169	-40	-27	7
39	PGF3α	0.94	351	193	-50	-30	50
40	PGE3	1.03	349	269	-30	-24	3
41	PGD3	1.14	349	269	-30	-24	7
42	dihomo PGF2α	1.85	381	337	-40	-37	3
43	dihomo PGE2	2.2	379	299	-40	-37	-
44	dihomo PGD2	2.3	379	299	-40	-37	-
45	dihomo PGJ2	3	361	299	-40	-37	-
46	dihomo 15d PGD2	3.3	361	299	-40	-37	-
47	6k PGE1	0.83	367	331	-40	-25	7
48	6,15 dk-,dh- PGF1α	1.01	369	267	-40	-37	300
49	15k PGF1α	1.59	353	221	-50	-38	30
50	15k PGF2α	1.42	351	219	-50	-32	10
51	15k PGE2	1.56	349	235	-30	-26	7
52	15k PGD2	1.65	349	235	-30	-40	70
53	dh PGF2α	1.42	355	311	-60	-29	7
54	dhk PGF2α	1.71	353	291	-80	-28	5
55	dhk PGE2	1.82	351	207	-40	-26	7
56	dhk PGD2	2.1	351	207	-40	-26	3
57	bicyclo PGE2	2.28	333	175	-40	-30	7
58	11b dhk PGF2α	1.5	353	221	-60	-37	50
59	9oh PGF2α	0.55	369	192	-60	-35	300

60	20oh PGF2α	0.54	369	165	-40	-39	7
61	19oh PGE2	0.57	367	243	-20	-31	10
62	20oh PGE2	0.57	367	175	-30	-27	5
63	2,3 dinor 11b PGF2α	0.83	325	227	-30	-22	5
64	PGFM	0.58	329	293	-40	-25	1
65	PGEM	0.6	327	291	-30	-23	7
66	tetranor 12-HETE	3.12	265	109	-20	-18	1
67	11b PGE2	1.37	351	271	-40	-23	3
68	PGK2	2.02	349	249	-40	-31	10
69	12-HHTrE	3.28	279	217	-30	-21	5
70	11-HETE	4	319	167	-40	-23	1
71	11-HEPE	3.58	317	215	-40	-20	3
72	13 HDoHE	3.98	343	221	-30	-19	5
73	PGA2	2.36	333	271	-20	-20	3
74	PGB2	3.13	333	271	-40	-25	3
75	15d PGA2	3.64	315	255	-40	-20	-
76	PGJ2	2.28	333	189	-40	-22	3
77	15d PGD2	2.83	333	271	-30	-22	3
78	15d PGJ2	3.52	315	203	-30	-20	3
79	5-iso PGF2αVI	1.08	353	115	-60	-28	10
80	8-iso PGF2αIII	0.96	353	193	-40	-33	7
81	9-HETE	4.09	319	123	-40	-20	3
82	9-HEPE	3.7	317	149	-40	-20	3
83	8 HDoHE	4.11	343	109	-40	-20	5
84	16 HDoHE	3.94	343	233	-50	-19	3
85	20 HDoHE	3.86	343	241	-30	-18	3
86	LTB4	2.82	335	195	-45	-23	3
87	20oh LTB4	0.83	351	195	-40	-23	5
88	20cooh LTB4	0.84	365	303	-40	-26	30
89	5,6-diHETE	3.64	335	115	-50	-29	3
90	6t LTB4	2.9	335	195	-45	-22	3
91	12epi LTB4	2.83	335	195	-45	-22	3
92	6t,12epi LTB4	2.82	335	195	-45	-22	3
93	12oxo LTB4	2.84	335	253	-50	-22	3
94	LTC4	1.8	624	272	-50	-33	-
95	LTD4	1.47	495	177	-50	-29	5
96	LTE4	2.18	438	333	-30	-25	3
97	11t LTC4	2.3	624	272	-50	-34	-
98	11t LTD4	1.77	495	177	-50	-29	5
99	11t LTE4	2.42	438	333	-50	-33	7
100	5-HETE	4.16	319	115	-40	-20	3
101	5-HEPE	3.77	317	115	-30	-22	3
102	7 HDoHE	4.07	343	141	-40	-19	5
103	4 HDoHE	4.28	343	101	-60	-18	3
104	9-HOTrE	3.48	293	171	-40	-22	0.1
105	5-HETrE	4.56	321	205	-30	-19	3
106	5,15-diHETE	2.73	335	201	-40	-26	3
107	6R-LXA4	1.81	351	167	-20	-21	-

108	6S-LXA4	1.89	351	217	-20	-18	-
109	15R-LXA4	1.82	351	165	-20	-23	50
110	LXA5	3.29	349	215	-30	-25	-
111	LXB4	1.44	351	221	-50	-21	5
112	Resolvin E1	0.8	349	195	-40	-20	70
113	Resolvin D1	1.7	375	141	-20	-20	5
114	Protectin D1	1.8	359	153	-20	-20	5
115	15t-Protectin D1	2.75	359	153	-20	-27	5
116	10S-Protectin D1	2.78	359	153	-20	-21	3
117	8,15-diHETE	2.64	335	235	-40	-26	100
118	15-HETE	3.91	319	175	-40	-19	3
119	15-HEPE	3.61	317	219	-40	-18	3
120	17 HDoHE	3.94	343	229	-20	-19	30
121	13-HODE	3.89	295	195	-60	-23	3
122	13-HOTrE	3.65	293	195	-40	-28	10
123	13-HOTrE(y)	3.63	293	193	-40	-19	5
124	15-HETrE	4.15	321	221	-30	-21	3
125	8-HETE	4.05	319	155	-40	-19	5
126	8-HEPE	3.74	317	155	-50	-29	7
127	10 HDoHE	4.06	343	153	-50	-19	3
128	8-HETrE	4.2	321	157	-20	-22	5
129	14,15 LTC4	3.29	624	272	-30	-32	-
130	14,15 LTD4	1.1	495	177	-60	-25	300
131	14,15 LTE4	1.61	438	333	-40	-22	7
132	12-HETE	4.03	319	135	-50	-19	3
133	12-HEPE	3.67	317	179	-30	-19	3
134	14 HDoHE	4	343	205	-30	-18	5
135	11 HDoHE	4.04	343	149	-20	-19	5
136	9-HODE	3.88	295	171	-60	-23	3
137	HXA3	3.48	335	195	-60	-26	70
138	HXB3	3.46	335	183	-40	-21	5
139	5-oxoETE	4.3	317	203	-60	-22	3
140	12-oxoETE	4.06	317	153	-50	-23	5
141	15-oxoETE	3.94	317	113	-20	-25	3
142	9-oxoODE	3.97	293	185	-50	-28	3
143	13-oxoODE	3.91	293	167	-50	-29	10
144	15 oxoEDE	4.49	321	223	-80	-32	3
145	20-HETE	3.64	319	245	-50	-24	3
146	19-HETE	3.57	319	231	-40	-23	30
147	18-HETE	3.67	319	261	-60	-20	3
148	17-HETE	3.71	319	247	-50	-20	3
149	16-HETE	3.73	319	189	-30	-21	3
150	18-HEPE	3.52	317	215	-50	-20	3
151	5,6-EET	4.42	319	191	-30	-17	3
152	8,9-EET	4.37	319	155	-30	-18	7
153	11,12-EET	4.34	319	167	-30	-17	3
154	14,15-EET	4.23	319	175	-30	-17	3
155	14(15) EpETE	3.99	317	207	-30	-19	3

156	17(18) EpETE	3.85	317	259	-40	-18	10
157	16(17) EpDPE	4.27	343	193	-40	-19	10
158	19(20) EpDPE	4.16	343	241	-50	-18	5
159	19,20 DiHDPA	3.2	361	229	-40	-22	5
160	9,10 EpOME	4.25	295	171	-60	-21	3
161	12,13 EpOME	4.23	295	195	-50	-23	3
162	5,6-diHETrE	3.62	337	145	-40	-22	3
163	8,9-diHETrE	3.44	337	127	-30	-27	3
164	11,12-diHETrE	3.33	337	167	-40	-25	3
165	14,15-diHETrE	3.19	337	207	-30	-24	3
166	9,10 diHOME	3.09	313	201	-50	-29	3
167	12,13 diHOME	3.04	313	183	-50	-29	3
168	Arachidonic Acid	4.85	303	259	-55	-20	0.1
169	Adrenic Acid	4.97	331	287	-70	-20	0.1
170	EPA	4.76	301	257	-40	-16	0.1
171	DHA	4.88	327	283	-40	-19	0.1
172	20cooh AA	3.66	333	271	-60	-23	5
173	17k DPA	4.21	343	247	-40	-23	3
174	2,3 dinor TXB2	0.84	341	137	-20	-31	50
175	11d-TXB2	1.38	367	305	-20	-26	7
176	2,3 dinor 8-iso PGF2a	0.77	325	237	-30	-19	5
177	2,3 dinor-6k PGF1a	4.32	363	281	-30	-23	-
178	PGK1	2.02	351	251	-40	-26	5
179	8-iso PGF3a	0.82	351	307	-30	-28	5
180	8-iso-15k PGF2b	1.2	351	219	-50	-22	5
181	9-Nitrooleate	4.74	326	168	-40	-20	3
182	10-Nitrooleate	4.73	326	169	-40	-19	7
183	tetranor-PGDM	0.7	327	247	-20	-20	50
184	7(R) Maresin-1	2.75	359	177	-30	-22	30

Peak selectivity was demonstrated by comparison with individual MRM transitions and the retention time of each analyte, as shown in **Table 2**. Scheduled MRM is an improvement over traditional MRM allowing for better data collection and more analytes to be monitored in a single analysis. We found a 30 sec retention time window for each MRM pair to allow for potential small shifts in retention time. In addition to providing good sensitivity, MRM approaches are highly selective, reducing the need for extensive sample clean-up. As a proof of principle, control plasma was analysed and we found no discernible distortions.

The MS/MS spectra of most eicosanoids show numerous product ions. Various product ions from the precursor were also listed In **Table 2** and the product ions for MRM detection was marked with underline. The product ions employed here for the MRM detection were selected to yield the best discrimination from other eicosanoids that co-elute in the vicinity of the analyte and to yield the highest signal. By balancing LC retention time and product ion selection, we were able to successfully distinguish the large majority of the eicosanoids listed. For example, owing to the similar structure, (d4) PGE_2 and PGE_2 have the same retention 7.1 min from LC, but the precursor and product of (d4) PGE_2 and PGE_2 was different, which was 355→275 and 351→271, respectively. In the other case, the precursor and product of PGE_2 and PGD_2 was the same, 351→271, but the retention time of PGE_2 and PGD_2 was different.

While a similar eicosanoid may have the same product ions, their relative intensities usually vary. The ratio of intensities of these product ions can be used to distinguish these species. In this case, multiple MRM transitions can then be analysed, and the ratio of product ions found in the unknown can be compared with either an MS/MS

library spectra or a pure standard run under the same conditions. This would aid in confirming the identity of a chromatographic peak.

Different precursor for eicosanoids

The key precursor polyunsaturated fatty acid (PUFA) for eicosanoids is arachidonic acid (AA, 20:4, *n*-6). Other eicosanoids and related compounds are formed from eicosapentaenoic acid (EPA, 20:5, n-3), docosahexaenoic acid (DHA, 22:6, *n*-3), and dihomo-γ-linolenic acid (DGLA, 20:3, *n*-6). Arachidonic acid is the main precursor for a wide spectrum of unique eicosanoids produced by COX, LOX, and CYP. Biochemical characterizations of EPA and DHA have generally suggested that these fatty acids are less prone to metabolism by eicosanoid pathway enzymes. In the present studies, we probed for 88 eicosanoids derived from AA, 22 from DHA, 17 from EPA, 13 from DGLA, and 18 from other fatty acids, that were identified as outlined in **Fig. 2**. The solid black lines around circles showed how many metabolites from the different PUFA. The comprehensive and simultaneous analysis of all eicosanoids is important because eicosanoids derived from different PUFA sources may have different physiological effects. For example, in neural trauma and neurodegenerative diseases, there is a dramatic rise in the levels of AA-derived eicosanoids and in contrast, DHA-derived metabolytes can prevent neuroinflammation.

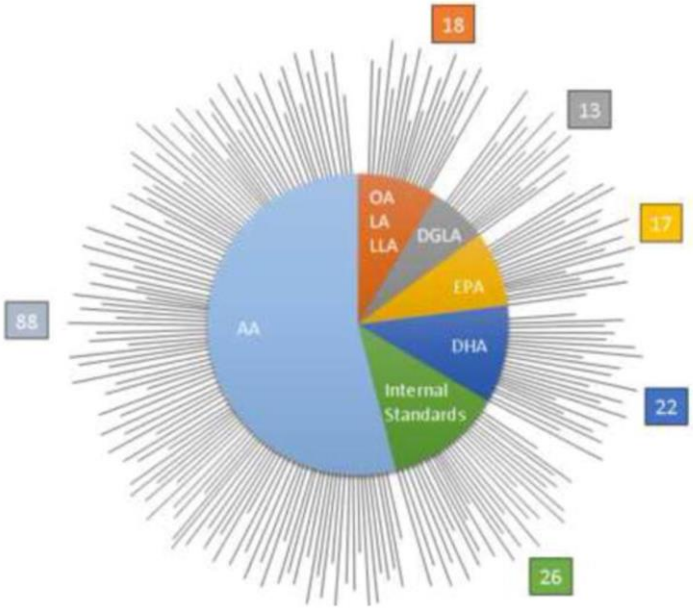


Figure 2. **The Relationship of the Member Eicosanoid Analytes to PUFA Precursors**

Method validation

The assay was validated for analyte recovery rate, matrix effect, linearity, inter-batch and intra-batch precision and accuracy for the major analytes. This procedure was performed on five replicates and on three consecutive days. Analyte stability was performed on processed samples maintained at 4°C for 4h and 8h.

Recovery rate and matrix effect

Matrix effect occurring between different matrices and adding to the complexity of a measurement and can lead to a bias. In the case of eicosanoid analysis, there is no true “blank” plasma available because of endogenous analytes present in all human plasma at various levels. Since a deuterated internal standard is either an analogues lipid metabolite or a molecule with similar chemical characteristics (chemical structure of the eicosanoids can be found in Ref. 2, 21 and 23 in the original paper), both lipid metabolites and internal standards will have similar ion suppression and extraction efficiencies. Thus, internal standards were used to evaluate the matrix effect and the recovery rate of the method.

The matrix effect was determined by spiking the internal standard mixture into a blank plasma sample after exaction. The results are shown in **Table 3**. Except for (d4) PGF2a, (d8) 5-HETE and (d8) arachidonic acid, the matrix effect of the other 23 internal standards are all above 80 %, indicating that ion suppression affects during analysis are minimal for most analytes. For the three exceptions, it is possible that co-eluting matrix components may reduce the ion intensity of the analytes. Phospholipids, in particular glycerophosphocholines and lysophosphatidylcholines represent the major class of endogenous compounds causing significant matrix effects, which will suppress the signal of other co-eluting lipids. The removal of phospholipids is preferable for analysis of other kinds of lipids in lipidomics study. Because Phospholipids are amphipathic compounds which generally consist of a phosphate-containing polar head group and one or two long hydrophobic fatty acid ester chains. The absorption of phospholipids on the C18 column is much stronger than eicosanoids. If using C18 material, phospholipids generally can be eluted with isopropanol as mobile phase. In our experiment condition, phospholipids generally cannot be eluted from SPE column. Therefore, the interference from phospholipids may be neglected. However, lysophospholipids may be coeluted from SPE in our experiment conditions and part of matrix effect may be caused from lysophospholipids. To minimize the matrix effect, a more efficient and selective extraction method or an efficient chromatographic separation of the analytes from matrix interferences could be developed.

Table 3. **Recovery Rate and Matrix Effect of Internal Standards**

No	Internal Standard	Recovery rate (%)	Matrix effect (%)
1	(d4) 6k PGF1a	94.1	86.6
2	(d4) TXB2	87.2	89
3	(d4) PGF2a	62.1	76.6
4	(d4) PGE2	89.5	84.8
5	(d4) PGD2	82.2	99.5
6	(d4) 15d PGJ2	58.2	91.4
7	(d4) dhk PGF2a	94.7	97.3
8	(d4) dhk PGD2	84.4	96.8
9	(d11) 8-iso PGF2a III	81.3	97
10	(d4) LTB4	82.9	90.8
11	(d8) 5-HETE	67.9	68.1
12	(d8) 12-HETE	80.8	87.4
13	(d8) 15-HETE	82.5	88.4
14	(d6) 20-HETE	71	85.8
15	(d4) 9-HODE	76.2	82.1
16	(d4) 13-HODE	86.71	85.9
17	(d7) 5-oxoETE	92.8	85.2
18	(d4) Resolvin E1	63	85.8
19	(d11) 8,9 EET	78.7	87.5
20	(d11) 11,12 EET	74.4	89.9
21	(d11) 14,15 EET	78.1	93.9
22	(d4) 9,10 diHOME	70.9	91.1
23	(d4) 12,13 diHOME	78	90
24	(d5) LTC4	112.6	119
25	(d5) LTE4	112.7	118.3
26	(d8) Arachiconic Acid	54.3	70.4

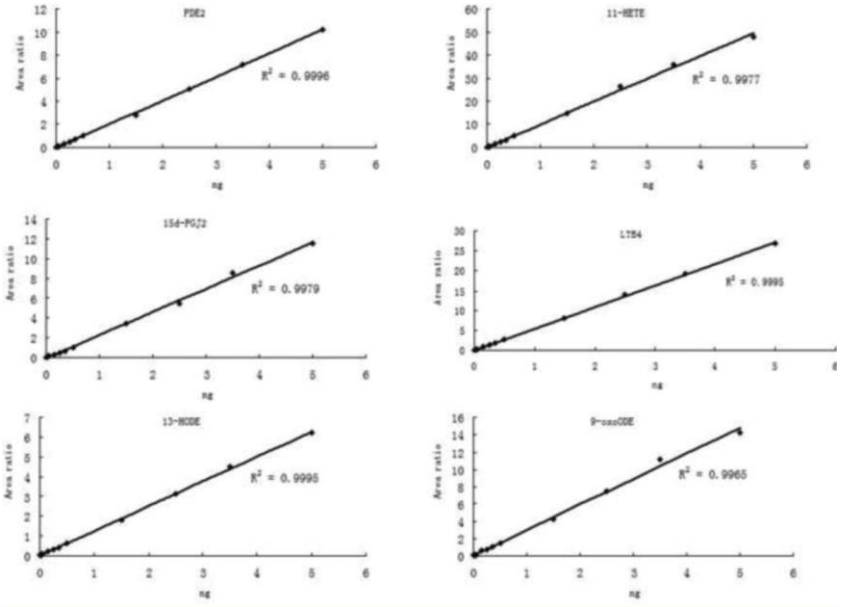
Absolute recovery was determined by spiking the internal standards into plasma samples before exaction, as shown in **Table 3.** Recovery of 21 of the internal standards was above 70%. The lower recovery for (d4) PGF2a, (d8) 5-HETE and (d8) arachidonic acid may be caused by the matrix effect noted above. The lower recovery for (d4) 14d PGJ_2 and (d4) Resolvin E_1 may be due to non-specific binding by the SPE absorbent. The error margin for the matrix effect and absolute recovery rate was ±10%.

Linear and lower limit of quantitation

The limit of quantitation for each analyte was defined as the lowest signal obtained with S/N ≥7. Based on these criteria, the LOQ of 86% of the analytes was less than 10 pg, as shown in **Table 2**. For target analytes, if the concentration of endogenous is much higher and separation interference is much lower, the LOQ is lower. Otherwise, the LOQ for target eicosanoids was much high.

Eicosanoid quantitation was performed by the stable isotope dilution method. For each eicosanoid to be quantified an internal standard was selected and a linear standard curve was generated where the ratio of analyte standard peak area to internal standard peak area was plotted versus the amount of analyte standard. The method offers good linearity for all analytes, the R^2 value is above 0.97 and the typical standard eicosanoid curves are shown in **Figure 3**.

Figure 3. **Linearity of Representative Standards**



Accuracy and Precision

The results of intra- and inter-day precision and accuracy testing using QC standard samples prepared in plasma as a blank matrix are summarized in Table 4. Human plasma contains a number of endogenous eicosanoids and we found that we could identify and quantify 60 endogenous metabolites in 20 ul of standard plasma.

For QC samples, the nominal amount of the QC sample was expressed as the sum of the endogenous amount and the spiked amount. In all, 147 eicosanoids from plasma can be monitored and 121 eicosanoids can be monitored and also quantified using QC samples (Supplementary Table S1, Supporting Information).

Precision (CV %) values for 95% of the analytes were within 20%, 100% of the analytes were within 30%. Accuracy (RE %) for 87% of the analytes was within 20%, 100% analytes were within 30%. These results indicate a good reproducibility for the determination of 121 analytes in human plasma using QC samples.

For the bioanalytical method validation and sample analysis, calibration standards and QC samples should ideally be prepared in the same matrix as the intended sample. This is especially true when electrospray ionization mass spectrometry is used because, as outlined above, components in the biological sample can lead to matrix effects [31]. Typically, suppression or enhancement of analyte response is accompanied by diminished precision and the

accuracy of subsequent measurements [32]. Due to the endogenous presence of eicosanoids at various levels, it is difficult to prepare a calibration curve in plasma; thus, we had to use calibration curves prepared in ethanol that are devoid of interfering matrices and impurities. This may contribute to analytical difficulties in detecting and accurately quantitating some of the eicosanoid molecular species for plasma sample. For example, the matrix effect may contribute to the deviation of the measurements of arachidonic acid for spiked plasma samples. To analyse the free fatty acids themselves, we have found that GC-MS provides an optimal approach [33]

Stability

To test the stability of the processed samples, they were kept at 4°C in the loading tray for various times prior to injection. Post-preparative stability of resuspended extracts showed a precision (CV %) and accuracy (RE %) for 100% of the analytes were within 20% for 0, 4 and 8h of pre-loading. Therefore it is concluded that eicosanoids in resuspended plasma extracts are stable for at least 8h when stored at 4°C.

Application of method to other tissues

The method was also applied to detect eicosanoids in mouse and human tissues, including adipose tissues, liver tissues and muscle tissues. Complete eicosanoid profiles similar to these from plasma were obtained from sample sizes as small as 2 mg of tissue. Of course, homogenization of the tissue samples before SPE extraction may be needed. These results demonstrate that this method is broadly applicable and can be used to measure eicosanoids in various biological sources.