SUPPLEMENTARY DATA

Materials and Methods

Precursor LC/MS/MS of esterified HETEs. Lipid extracts were separated on a C_{18} Luna(2), 3 µm, 150 mm x 2 mm column (Phenomenex) using a gradient of 0 to 100 % B over 10 min, then held at 100 % B for 30 min (A, methanol:acetonitrile:water, 60:20:20 containing 1mM ammonium acetate, B, methanol with 1mM ammonium acetate) at 200 µl/min, then analyzed online using electrospray MS/MS (4000 Q-Trap, Applied Biosystems) for negative ion precursors of m/z 319.2 (HETE), using a collision energy of -45V.

Synthesis and purification of 18:0a/15-HETE-PE. 18:0a/20:4-PE was oxidized using soybean lipoxygenase (5.2 KU/ml) in 10 mM deoxycholate, 0.2 M borate buffer, pH 9. Hydroperoxides were reduced to corresponding hydroxides by 1 mM $SnCl_2$ for 10 min at room temperature. Lipids were extracted and purified using reverse phase HPLC. 18:0a/15-HETE-PE was quantified by absorbance of the purified lipid using $E_{1mm,1cm} = 28.1$, and stored at -80 under N_2 in methanol.

Generation of standards for 12-HETE isomer determination. 10mM brain L-α-phosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL) or 18:0a/20:4-PE (Sigma) was incubated for 30 min at 37 °C in 0.1M KH_2PO_4 buffer, pH 7.4, 0.2% sodium cholate, with 100µl (activity: 98 $nmol.min^{-1}.ul^{-1}$ LA oxidized, at pH 7, 37°) of I428A mutant rabbit 15-lipoxygenase, that oxidized primarily at C12. Samples were then reduced using 1 mM $SnCl_2$ for 10 min, 20 °C, and extracted as before for LC/MS/MS analysis.

HETE positional isomer determination Lipid extracts were separated by normal phase HPLC and the PE-containing fraction (5.5-8 min) collected, saponified, then resuspended in 50µl methanol and HETE isomers analyzed by reverse phase LC/MS/MS. For chiral phase analysis, 12-HETE was collected during elution from reverse phase LC/MS/MS, resuspended in the chiral phase mobile phase (hexane:2-propanol:acetic acid, 100:5:0.1) and injected onto a Chiralcel OD 0.46 x 25 cm column (Chiral Technologies Ltd, Exton, PA, USA) with isocratic separation at 1ml/min with the absorbance monitored at 235 nm. Racemic 12-HETE was obtained from Biomol.

LC/MS/MS quantitation of free acid 12-HETEs. Samples were separated on a C_{18} ODS2, 5 µm, 150 mm x 4.6 mm column (Waters Ltd) using a gradient of 50 % to 90 % B over 20 min (A, water:acetonitrile:acetic acid, 75:25:0.1, B, methanol:acetonitrile:acetic acid, 60:40:0.1) at 1 ml/min. Products were quantitated by monitoring specific multiple reaction monitoring (MRM) transitions (negative mode, $[M-H]^-$) on a Q-Trap (Applied Biosystems 4000 Q-Trap), using m/z 319 → 219 (15-HETE), 319 → 179 (12-HETE), 319 → 115 (5-HETE), 319 → 155 (8-HETE), 319 → 167 (11-HETE), 327 → 184 (12-HETE-d8) with collision energies of –20 to –28 V. Standard curves with purified HETEs *versus* 12-HETE-d8 were constructed for metabolite quantitation, with sensitivities down to 0.1 - 1 pg on-column, allowing for detection of down to approximately 1 - 10 pg per lavage of each eicosanoid.

Results

Formation of novel 12/15-LOX products in the murine peritoneal cavity. Precursor scanning LC/MS/MS for molecules containing a HETE functional group (m/z 319) showed the presence of 4 ions at m/z 738, 764, 766 and 782, eluting between 20 and 28 min (Fig S1 A, B). LC/MS/MS analysis with online acquisition of product ion spectra was conducted for all 4 ions. These were monitored by the parent-to-daughter transition arising from collision-induced dissociation to give a

prominent daughter ion for free HETE at m/z 319 and 179, the latter being characteristic for the 12-HETE positional isomer (Fig S2). Each eluted as a distinct ion on reverse phase LC, with well defined peaks. Product ion spectra obtained at the apex of each LC elution peak (containing both 319 and 179) shows the fragmentation for all four 12-HETE-containing species (Fig S3). Ions at m/z 319 and 179 are seen for all 4 lipids (Fig S3, A,C,E,G). For m/z 782, a daughter ion at 283, corresponding to stearic acid (18:0) is also observed (Fig S3 A). Based on their m/z values and MS/MS fragmentation patterns, the structures are proposed as phosphatidylethanoamines (PE), specifically 18:0/12-HETE-PE (m/z 782), 18:0p/12-HETE-PE (m/z 766), 18:1p/12-HETE-PE (m/z 764) and 16:0p/12-HETE-PE (m/z 738) (Scheme 1). The largest with m/z 782 is a diacyl species with the remaining 3 being plasmalogens. To further confirm, brain PE or purified 18:0a/20:4-PE was oxidized by a rabbit 15-LOX mutant (I418A), which is primarily a 12-lipoxygenating enzyme(15). LC retention times and MS/MS fragmentation patterns for the in vitro synthesized 12-HETE-PEs were identical to murine products (Fig S3 B,D,F,H). The small peak that elutes immediately prior to the 12-HETE-PEs in the parent to 319 transition in the murine samples, in most instances as a shoulder and does not appear in the parent to 179 transition, represents the 15-HETE containing PE isomer, generated at far lower rates by the mouse 12/15-LOX (Fig S2 A,C,E,G). In the case of the m/z 782 ion, the smaller ion at 22 min appearing in both 319 and 179 daughter ion channels corresponds to 16:0/12-HETE-PC, also formed at low levels in these cells (Fig S2 A).

*Further confirmation of HETE isomer composition of 12-HETE-PEs***.** To further confirm that 12-HETE-PEs contained 12-HETE and determine the enantiomer composition, the PE fraction was isolated using normal phase HPLC, then hydrolyzed to release HETE. LC/MS/MS reveals a predominance of 12-HETE versus other positional isomers (Fig S4 A-D). Chiral phase analysis of the purified 12-HETE reveals almost exclusive generation of the S-isomer, as expected for the leukocyte type 12/15-LOX (Fig S4 F).

Figure Legends

Figure S1. Murine lavage contains four distinct esterified 12-HETEs. *Panel A*. Precursor scanning of lipids eluting from a reverse phase LC separation shows a series of compounds eluting at 20-28 min. *Panel B*: Spectra acquired during this time period were averaged (in region shown by * above trace) and show 4 prominent ions at *m/z* 738, 764, 766 and 782.

Figure S2. LC/MS/MS of the four 12-HETE-containing ions contained in murine lavage. Lipids were extracted from control murine lavage as described in Materials and Methods, then separated using reverse phase HPLC with monitoring for 12-HETE-containing lipids using parent to daughter transitions, with HETE (m/z 319 or 179) as daughter ion.

Figure S3. MS/MS of murine 12-HETE-containing lipids, compared with synthetically-generated 12-HETE-PEs. *Panels A,C,E,G.* MS/MS spectra obtained using ion trap mode for murine 12-HETE-containing lipids generated by platelets, for lipids with m/z 782, 766, 764 and 738, respectively. Spectra are obtained at the apex of elution from the LC chromatograms, as shown in Fig 2. *Panels B,D,F,H.* MS/MS spectra obtained from lipids eluting with the same retention time, generated by I418A rabbit LOX oxidation of either 18:0/20:4-PE (panel B) or brain PE (panels D,F,H), for lipids with m/z 782, 766, 764 and 738, respectively, as described in Materials and Methods.

Figure S4. 12S-HETE is the predominant enantiomer in the PE fraction. *Panels A-E.* After isolation of PE using normal phase chromatography, HETEs were released by saponification and analyzed using LC/MS/MS as described in Methods. *Panel F*. Purified murine 12-HETE, hydrolyzed

from normal phase LC purified PE, and isolated by reverse phase HPLC, was chromatographed using chiral phase LC/UV. Dotted line: murine 12-HETE. Solid line: racemic 12-HETE standards.

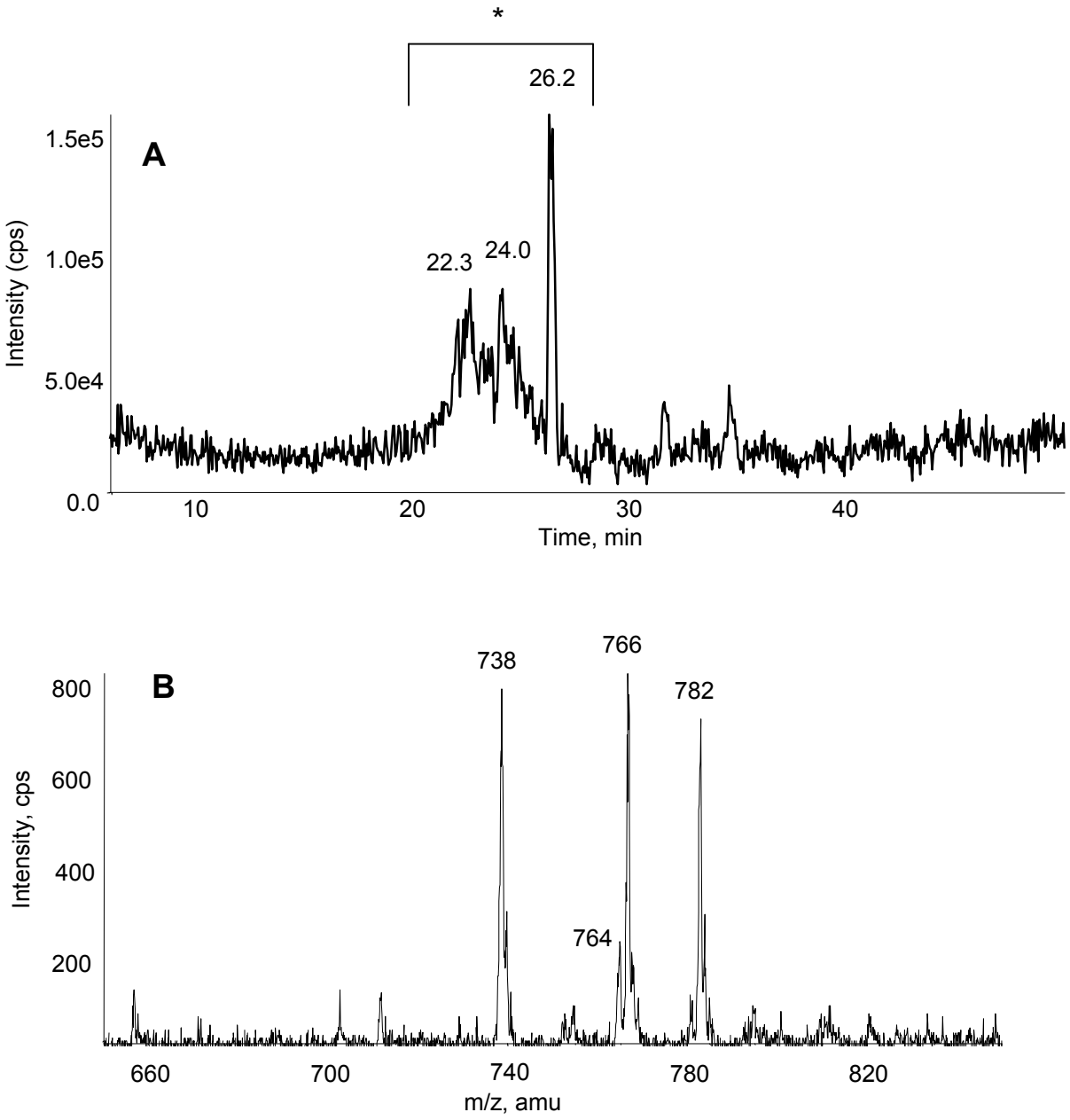
*
26.2
A
1.5e5
1.0e5
5.0e4
0.0
Intensity (cps)
22.3
24.0
10
20
30
40
Time, min
B
800
600
400
200
Intensity, cps
738
766
782
764
660
700
740
780
820
m/z, amu

Figure S1

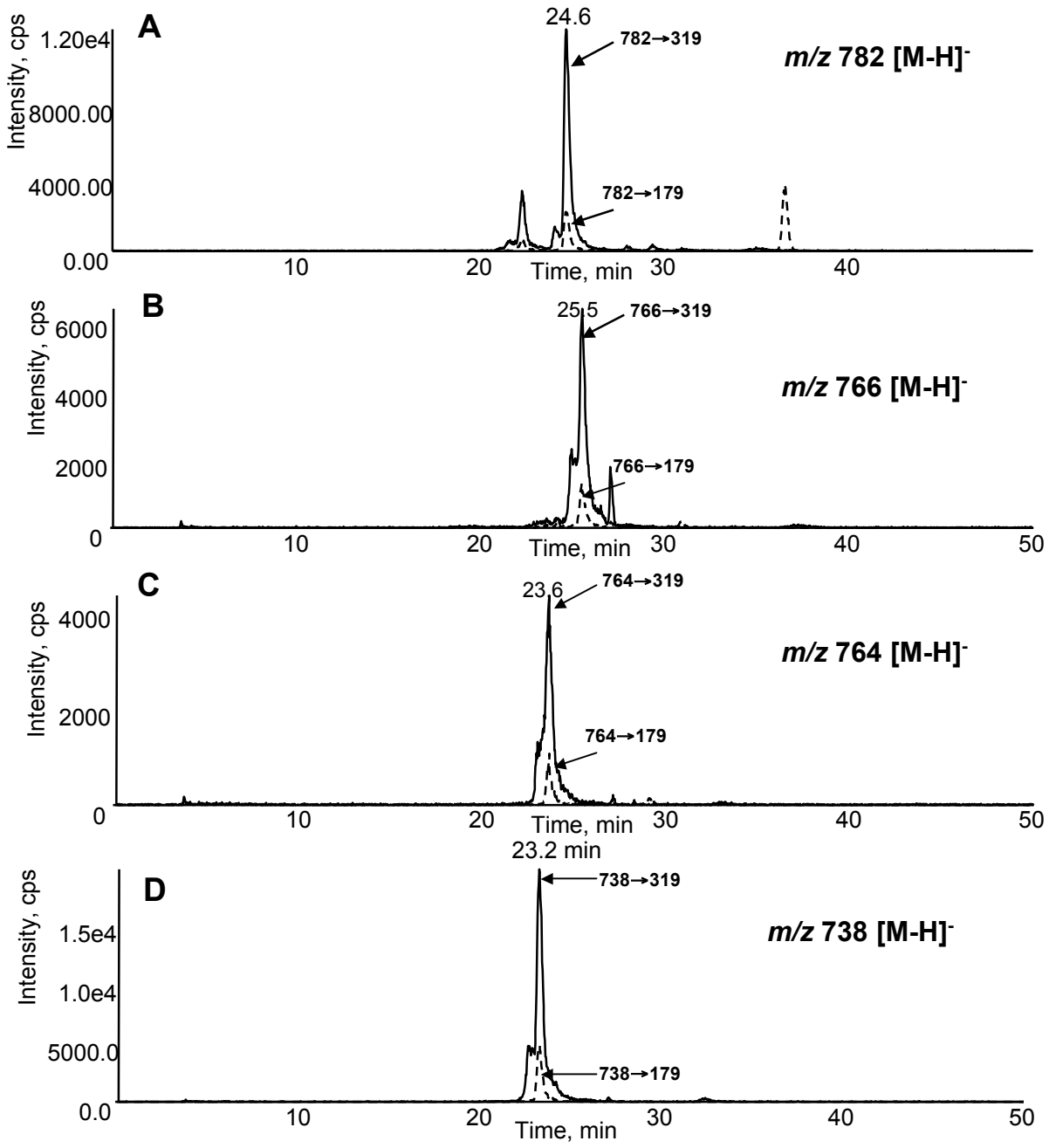
A
24.6
782→319
m/z 782 [M-H]-
782→179
Intensity, cps
1.20e4
8000.00
4000.00
0.00
10
20
Time, min
30
40
B
25.5
766→319
m/z 766 [M-H]-
766→179
Intensity, cps
6000
4000
2000
0
10
20
Time, min
30
40
50
C
23.6
764→319
m/z 764 [M-H]-
764→179
Intensity, cps
4000
2000
0
10
20
Time, min
30
40
50
23.2 min
D
738→319
m/z 738 [M-H]-
738→179
Intensity, cps
1.5e4
1.0e4
5000.0
0.0
10
20
Time, min
30
40
50

Figure S2

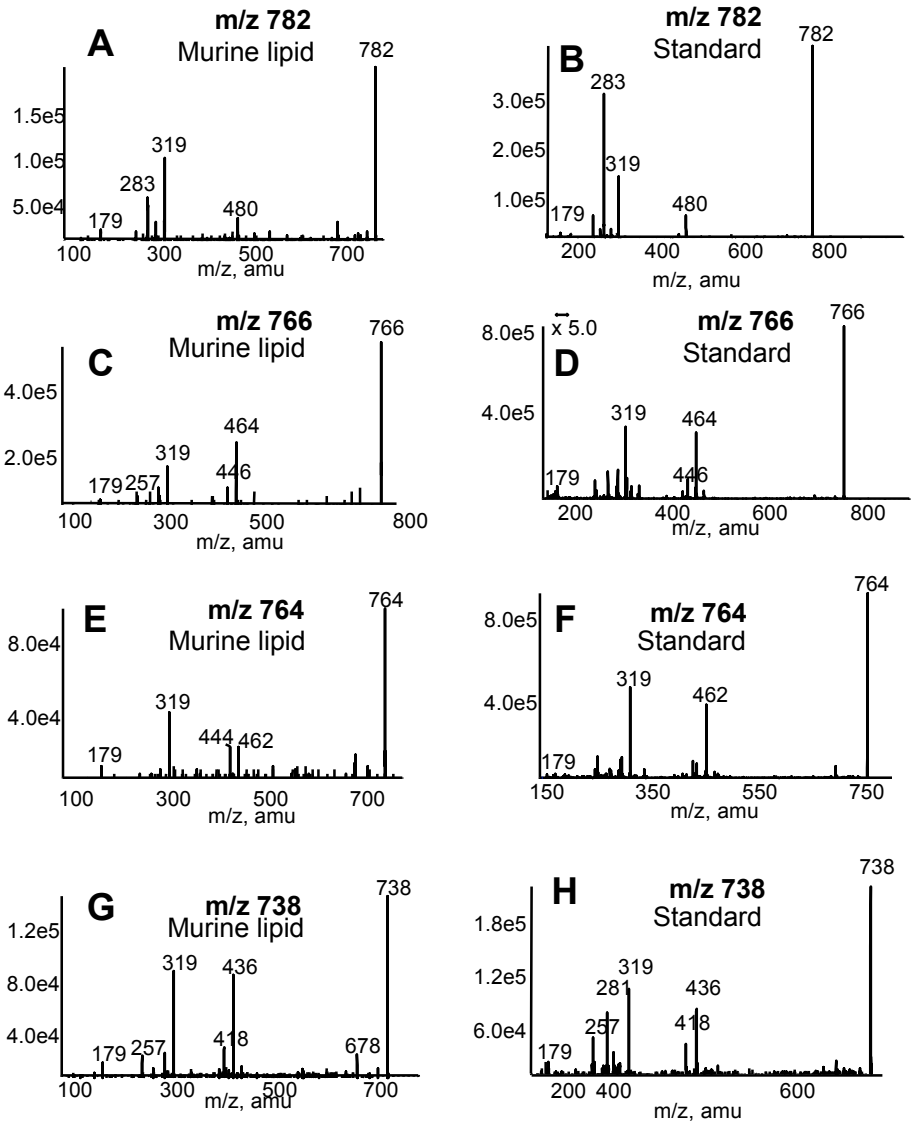
A
m/z 782
Murine lipid
782
319
283
480
179
1.5e5
1.0e5
5.0e4
100
300
500
700
m/z, amu
B
m/z 782
Standard
782
283
319
480
179
3.0e5
2.0e5
1.0e5
200
400
600
800
m/z, amu
C
m/z 766
Murine lipid
766
464
319
446
179
257
4.0e5
2.0e5
100
300
500
800
m/z, amu
D
x 5.0
m/z 766
Standard
766
319
464
446
179
8.0e5
4.0e5
200
400
600
800
m/z, amu
E
m/z 764
Murine lipid
764
319
444
462
179
8.0e4
4.0e4
100
300
500
700
m/z, amu
F
m/z 764
Standard
764
319
462
179
8.0e5
4.0e5
150
350
550
750
m/z, amu
G
m/z 738
Murine lipid
738
319
436
418
179
257
678
1.2e5
8.0e4
4.0e4
100
300
500
700
m/z, amu
H
m/z 738
Standard
738
319
281
436
418
257
179
1.8e5
1.2e5
6.0e4
200
400
600
m/z, amu

Figure S3

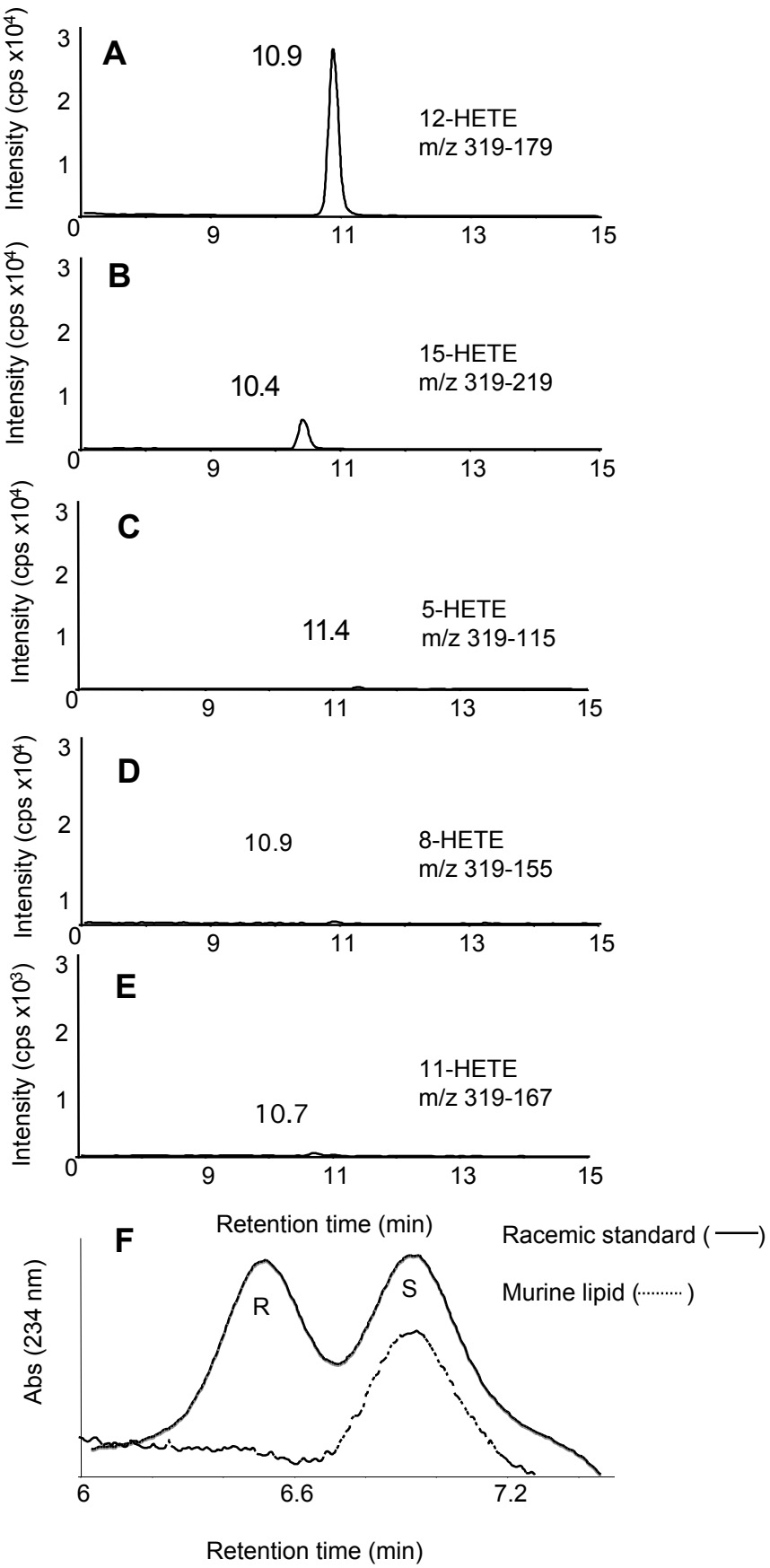
A
10.9
12-HETE
m/z 319-179
B
10.4
15-HETE
m/z 319-219
C
11.4
5-HETE
m/z 319-115
D
10.9
8-HETE
m/z 319-155
E
10.7
11-HETE
m/z 319-167
Intensity (cps x10⁴)
Intensity (cps x10³)
0
1
2
3
9
11
13
15
F
Retention time (min)
Racemic standard (——)
Murine lipid (·····)
R
S
Abs (234 nm)
6
6.6
7.2
Retention time (min)

Figure S4