**SUPPLEMENTARY MATERIAL**

**Determination of ketone bodies in biological samples via rapid UPLC-MS/MS**

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**Figure S1. UPLC separation and MS/MS identification of co-eluting βOHB isomers and AcAc.**
(A) Upper left: XIC of m/z 103.0401 (±10 ppm) in Full MS scan mode (black) and XIC of m/z transitions of 103.0401 to either m/z 59.0133, 57.0340, 73.0290 that were putatively assigned to βOHB (red XIC), 2-OHB or 4-OHB (green XIC), and 3-HIB (blue XIC) using LC Atlantis T3 column. Yellow XIC represents m/z 107.0646 that pertains to internal standard [3,4,4,4-D$_4$]βOHB. Upper right: representative MS/MS spectrum of m/z 103.0401 signal at RT 3.46 min, using N(CE) 30.00 with fragments assigned to βOHB (red), 2-OHB or 4-OHB (green), and 3-HIB (blue). (B) Definitive identification of βOHB and (C) 3-HIB in serum extract was based on RT using Cortecs UPLC T3 column, m/z and MS/MS spectra of 100 µM standards with serum extract using N(CE) 30.00. Figure continues on next page.
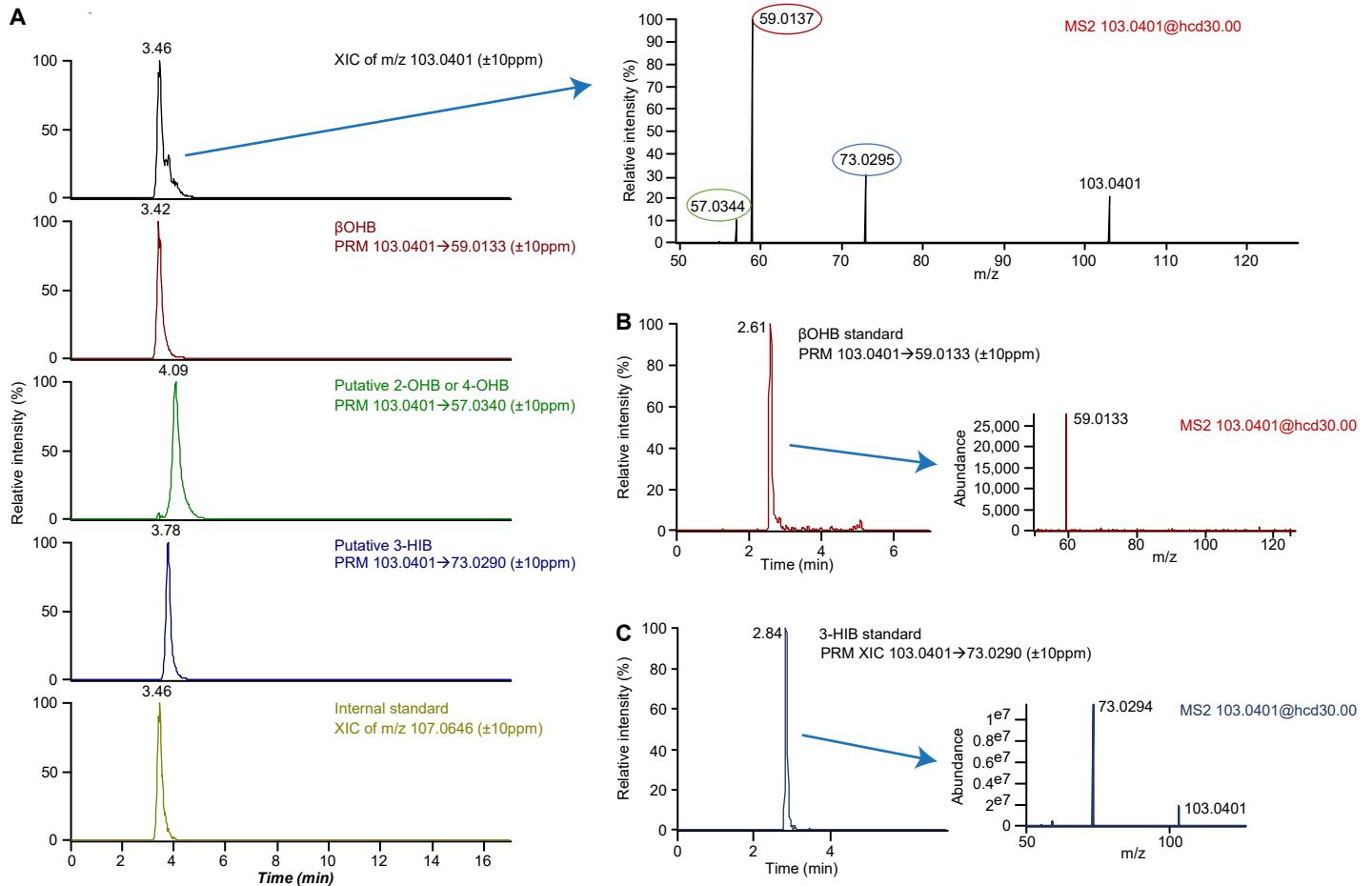
**Figure S1, continued. UPLC separation and MS/MS identification of co-eluting βOHB isomers and AcAc.**
(D) Identification of 2-OHB (XIC of m/z 103.0401→57.0340) was based on spiking of serum extract with either water (top), 5 µM 4-OHB (middle) or 3 µM 2-OHB (bottom). Confirmation of AcAc identity by PRM XIC of m/z 101.0244→ 57.0343 and representative MS/MS of 101.0244 using N(CE) 30 in (E) 100µM AcAc standard and (F) AcAc in serum extract.
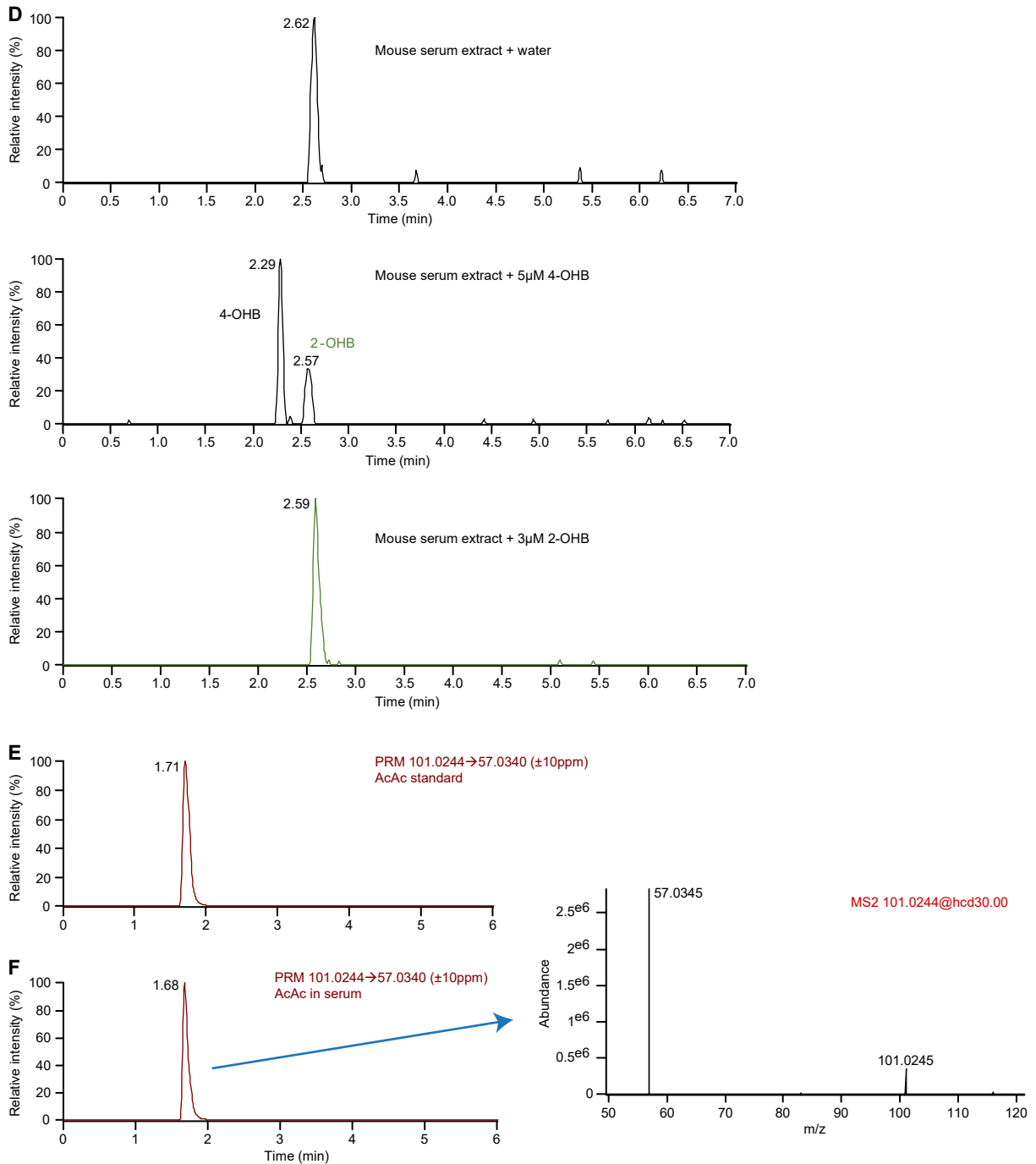
**Figure S2. Stability of AcAc stored at 4ºC after extraction from serum.**
Quantified by the AcAc/ [3,4,4,4-D$_4$]βOHB signal ratio over time (n=3/group). Data expressed as the mean standard error of the mean (SEM).
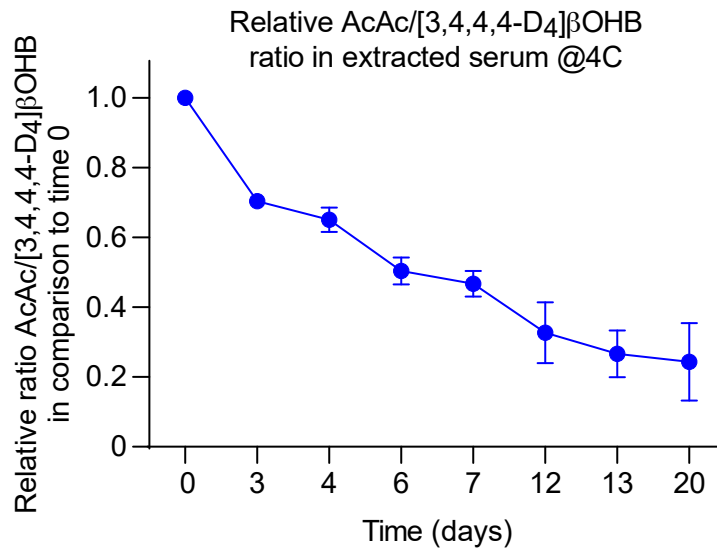
**Figure S3. Recovery of [U-$^{13}$C$_4$]AcAc I.S.**
[U-$^{13}$C$_4$]AcAc directly added to extraction buffer alone (direct analysis, left); extraction buffer that went through three cycles of freeze/thaw (middle), and [U-$^{13}$C$_4$]AcAc added to tissue extract. Comparisons are made to direct analysis condition. Data expressed as the mean ± standard error of the mean (SEM). No statistical differences were found.
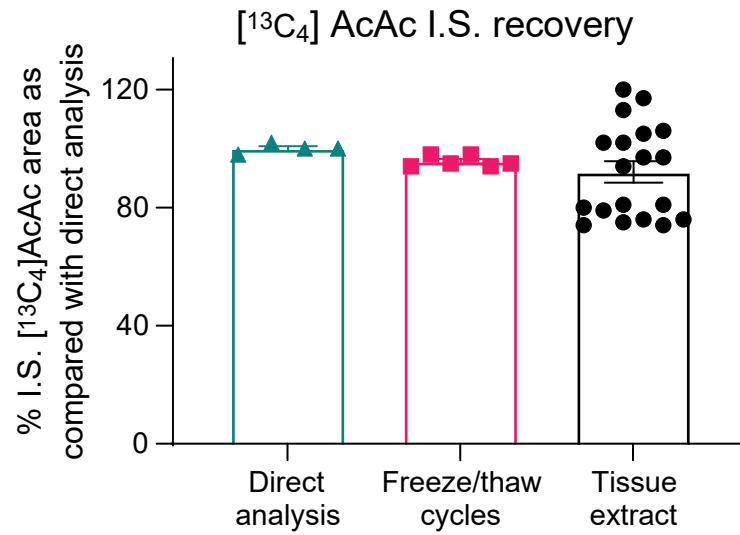


$[^{13}$C$_4]$ AcAc I.S. recovery

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**Figure S4. Separation of βOHB enantiomers, and the enantiomers of βOHB structural isomers.**
Full MS Scan of (A) blank for the derivatization reaction and (B) 20 μM derivatized DL-βOHB-PMP standard in positive mode. Precursor ion m/z 241.1910 in red circle matches to the DL-βOHB-PMP molecular mass ion in positive mode. XIC in Full MS scan in positive mode of 20 μM derivatized (C) D-βOHB-PMP, (D) L- βOHB, (E) L-3-HIB, and (F) L-2-OHB standards with m/z 241.1910
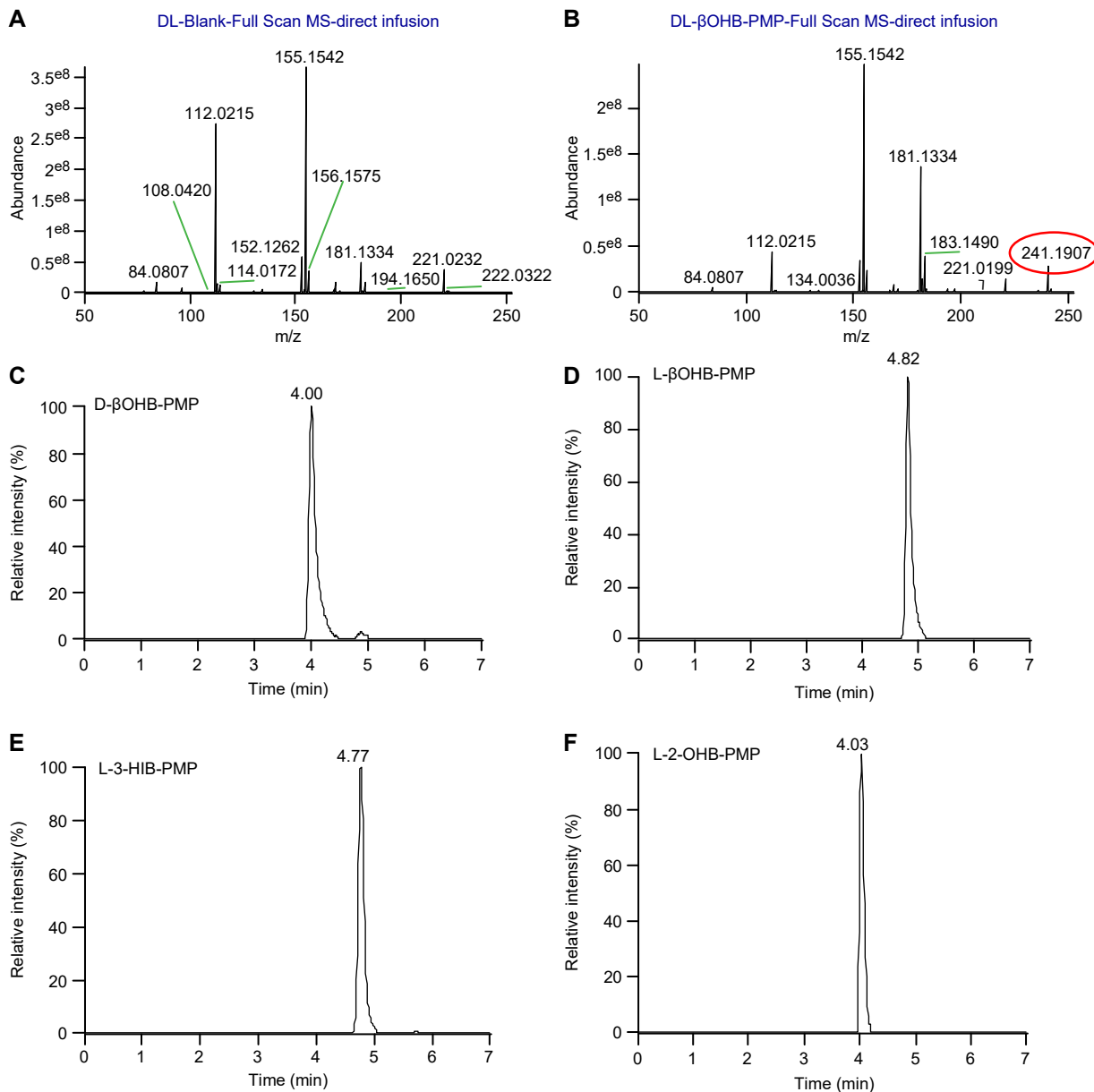
**Figure S5. MS/MS of m/z 241.1910 (±10 ppm) derivatized structural isomers of hydroxybutyrate precursor ions.**

Positive mode ionization PRM obtained from direct infusion of derivatized-PMP 20 µM DL-βOHB, DL-3-HIB, DL-2-OHB, or 4-OHB standards using (A-B) 35 N(CE). Panel B represent zoomed spectrum of panel A. Circles m/z represent selected selective fragment ions for each structural isomer. See Table 1 for a summary. Figure continues on next page.
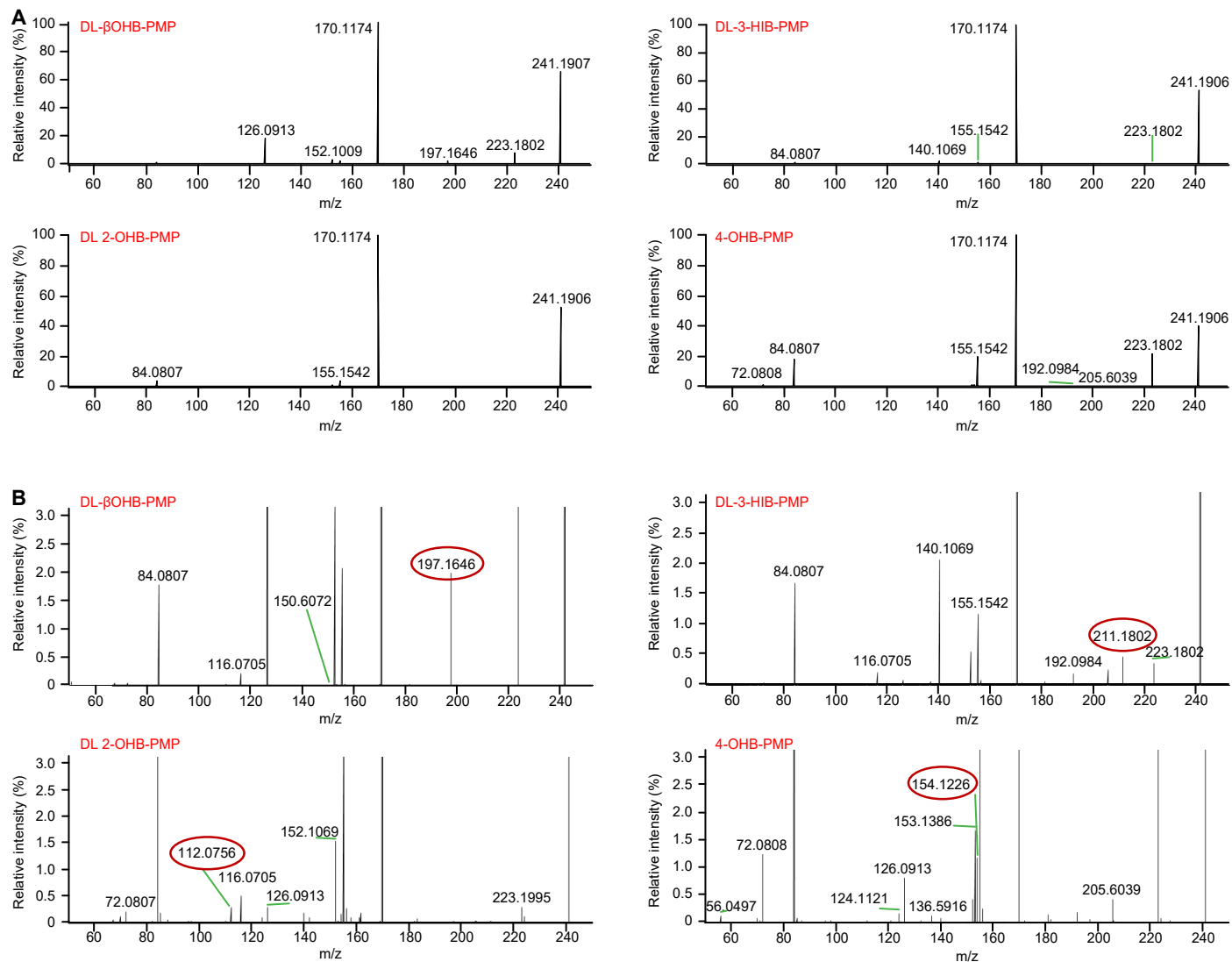
**Figure S5, continued. MS/MS of m/z 241.1910 (± 10 ppm) derivatized structural isomers of hydroxybutyrate precursor ions.**
Positive mode ionization PRM obtained from direct infusion of derivatized-PMP 20 µM DL-βOHB, DL-3-HIB, DL-2-OHB, or 4-OHB standards using (C-D) 50 N(CE). Panel D represents zoomed spectrum of panel C. Circles m/z represent selected selective fragment ions for each structural isomer. See Table 1 for a summary.

**Figure S6. Selectivity of transitions used to selectively detect derivatized structural isomers for β-hydroxybutyrate precursor ions.**

20 µM (A) DL- βOHB- PMP (241.1910→ 197.1654), (B) DL-3-HIB (241.1910→211.1810), (C) DL-2-OHB (241.1910→112.0762) and (D) 4-OHB (241.1910→153.1392), standards using 35 N(CE). See Table 1 for a summary. Figure continues on next page.

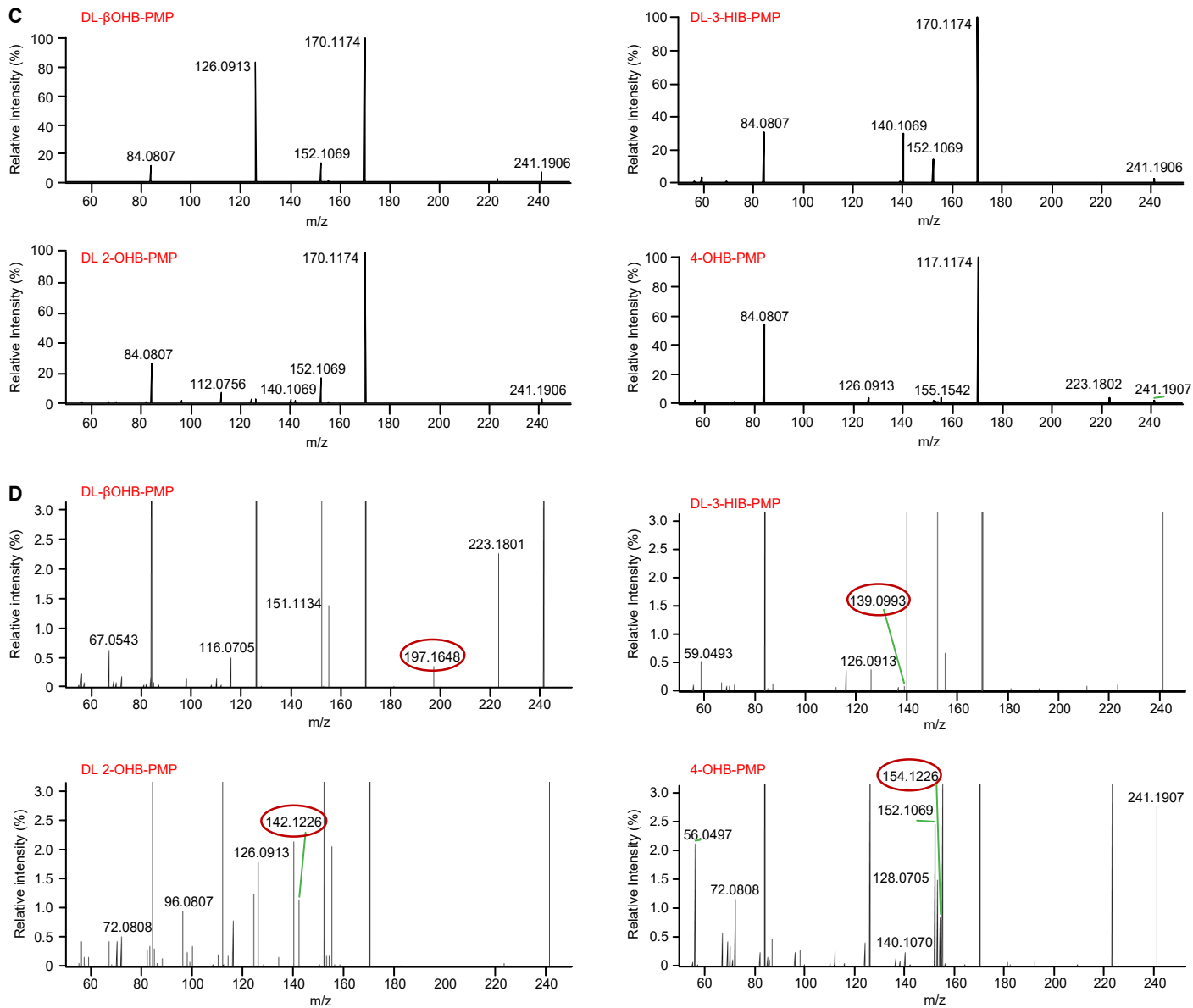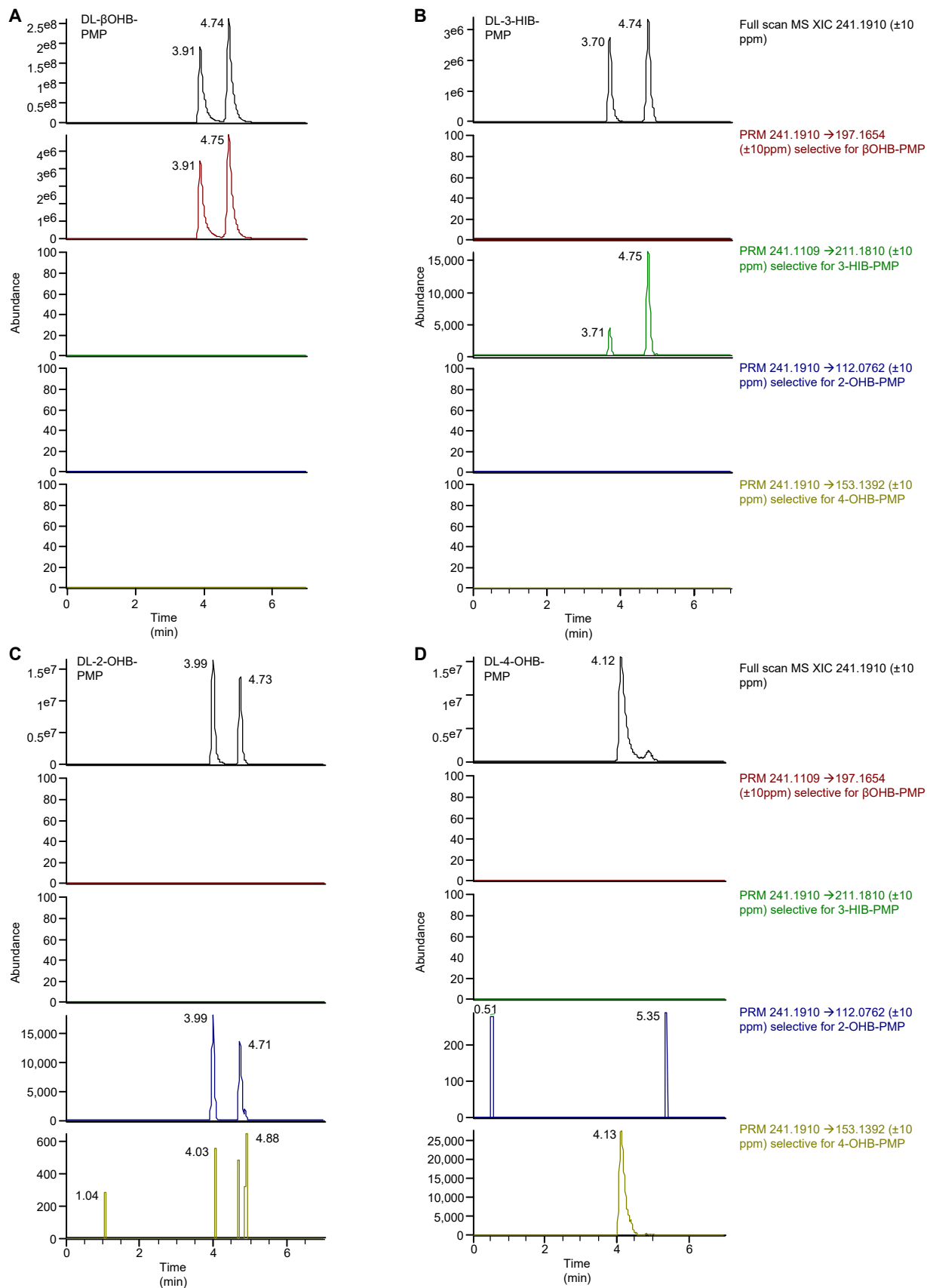**Figure S6, continued. Selectivity of transitions used to selectively detect derivatized structural isomers for β-hydroxybutyrate precursor ions.**
20 µM (E) DL-βOHB-S-PMP (241.1910→197.1654), (F) DL-3-HIB-S-PMP (241.1910→139.0997), (G) DL-2-OHB-S-PMP (241.1910→142.1232), (H) 4-OHB-S-PMP (241.1910→154.1232), standards using 50 N(CE). See Table 1 for a summary.

**Table S1. Optimized UPLC-MS/MS parameters for the detection and differentiation of unlabeled ßOHB isomers in negative and positive FS, or negative PRM scan modes.** RT, retention time in minutes on UPLC Cortecs column using optimal chromatographic separation; FS, Full MS scan mode; PRM, Parallel Reaction Monitoring.

| Analyte | RT (min) | FS (m/z) Positive mode | FS (m/z) Negative Mode | PRM (m/z of precursor and m/z of produced fragment) |
|---|---|---|---|---|
| β-hydroxybutyrate (βOHB) | 2.6 | 105.0546 | 103.0401 | 103.0401→ 59.0133 |
| 3-hydroxyisobutyrate (3-HIB) | 2.8 | 105.0546 | 103.0401 | 103.0401→73.0290 |
| 2-hydroxybutyrate (2-OHB) | 2.6 | 105.0546 | 103.0401 | 103.0401→57.0340 |
| 4-hydroxybutyrate (4-OHB) | 2.3 | 105.0546 | 103.0401 | 103.0401→57.0340 |

**Table S2. Analytical characteristics of the method based on standards**

| | AcAc PRM 101.0244→57.0340 ±10 ppm | βOHB PRM 103.0401→59.0133 ±10 ppm |
|---|---|---|
| **External calibration curve** **Range** **Slope** **Linearity (R$^2$)** | 0.1-250 µmoles/L y=0.0452x-0.0102 0.9991 | 0.1-250 µmoles/L y=0.0378+0.1300 0.9968 |
| **LOD (nmol/L)[1]** | 10.3±3.5 | 11.7±2.1 |
| **LOQ (nmol/L)[2]** | 35.3±12.1 | 39.5±7.6 |
| **Repeatability %RSD (n=5)[3]** At 250 µmol/L At 30 µmol/L At 4 µmol/L | 1.4% 1.6% 1.8% | 3.3% 2.8% 3.1% |

[1] LOD – determined as the minimum concentration, which yielded a signal to noise ratio equal to 3. Quantified based on three independently prepared calibration curves (n=3).

[2] LOQ – determined ad the minimum concentration, which yielded a signal to noise ratio equal to 10. Quantified based on three independently prepared calibration curves (n=3).

[3] Repeatability determined by five injections of standard at three concentration levels (n=5/group).

**Table S3. Analytical characteristics of the method based on extract from fed and 24 hour fasted wild type mice**

| | | Serum extract FED SERUM | | Serum extract FASTED SERUM | |
|---|---|---|---|---|---|
| | | AcAc PRM 101.0244→57.0340 ±10 ppm | βOHB PRM 103.0401→59.0133 ±10 ppm | AcAc PRM 101.0244→57.0340 ±10 ppm | βOHB PRM 103.0401→59.0133 ±10 ppm |
| Inter-sample precision Day 1[1] | µmoles/L | 25.9 | 84.6 | 415.8 | 1021.8 |
| | Stdv | 0.8 | 2.3 | 23.1 | 52.5 |
| | RSD(%) | 3.1 | 2.7 | 5.6 | 5.1 |
| Inter-sample precision Day 2[1] | µmoles/L | 19.0 | 76.8 | 477.6 | 1198.2 |
| | Stdv | 1.4 | 5.0 | 12.0 | 43.4 |
| | RSD(%) | 7.5 | 6.5 | 2.5 | 3.6 |
| Inter-sample precision Day 3[1] | µmoles/L | 19.1 | 63.1 | 392.7 | 945.1 |
| | Stdv | 1.3 | 3.9 | 23.7 | 47.4 |
| | RSD(%) | 6.8 | 6.1 | 6.0 | 5.0 |
| Inter-day precision[2] | µmoles/L | 26.60 | 84.6 | 472.2 | 1183.5 |
| | Stdv | 0.7 | 1.6 | 12.9 | 39.5 |
| | RSD(%) | 2.5 | 1.9 | 2.7 | 3.3 |

[1]Inter-sample precision determined by three consecutive injections of five individually prepared extracts from 24 hour fasted WT mice (n=15). Each day represent sample obtained from different animal housed in same cage.

[2]Determined by three day injection in triplicate of same extract from 24 hour fasted WT mice (n=9).

**Table S4. Recovery analysis based on extracts from fed and 24 hour fasted wild type mice. Each spike constitutes three individually prepared samples.**

| | | Serum extract FED SERUM | | Serum extract FASTED SERUM | |
|---|---|---|---|---|---|
| | | AcAc PRM 101.0244→57.0340 ±10 ppm | βOHB PRM 103.0401→59.0133 ±10 ppm | AcAc PRM 101.0244→57.0340 ±10 ppm | βOHB PRM 103.0401→59.0133 ±10 ppm |
| Spike 1 (20 µmoles/L) | Recovery(%) | 98.8 | 97.9 | 101.4 | 98.8 |
| | RSD(%) | 8.4 | 7.4 | 0.9 | 10.3 |
| Spike 2 (40 µmoles/L) | Recovery(%) | 96.4 | 96.8 | 102.2 | 105.0 |
| | RSD(%) | 4.5 | 2.1 | 2.4 | 6.5 |
| Spike 3 (60 µmoles/L) | Recovery(%) | 98.3 | 95.1 | 101.8 | 110.9 |
| | RSD(%) | 2.9 | 1.0 | 3.4 | 5.7 |

**Table S5. Recovery analysis based on extracts from 24 hour fasted and 6 hours refed WT mice liver tissue. Each spike constitutes three individually prepared samples.**

| | | Extract of FASTED LIVER | | Extract of 6h Refed LIVER | |
| --- | --- | --- | --- | --- | --- |
| | | AcAc PRM 101.0244→57.0340 ±10 ppm | βOHB PRM 103.0401→59.0133 ±10 ppm | AcAc PRM 101.0244→57.0340 ±10 ppm | βOHB PRM 103.0401→59.0133 ±10 ppm |
| Spike 1 (2 and 10 µmoles/L AcAc and βOHB) | Recovery(%) | 87.2 | 111.9 | 91.5 | 98.2 |
| | RSD(%) | 7.8 | 1.0 | 3.7 | 2.6 |
| Spike 2 (4 and 20 µmoles/L AcAc and βOHB) | Recovery(%) | 84.6 | 86.5 | 103.7 | 102.4 |
| | RSD(%) | 12.0 | 3.5 | 2.6 | 4.4 |