Supplementary Material for:

Isobaric 6-plex and tosyl dual tagging for the determination of positional isomers and quantitation of monounsaturated fatty acids using rapid UHPLC-MS/MS

Michael R. Armbruster, Mahmoud Elhusseiny Mostafa, Rhea N. Caldwell, Scott F. Grady, Christopher K. Arnatt, and James L. Edwards *

Department of Chemistry and Biochemistry, Saint Louis University, 3501 Laclede Ave, St Louis, MO 63103, United States

Contents:

Supplemental Methods:

Capillary LC:

Capillary columns (50 µM inner diameter) with nano tip orifices were fabricated using a trap-end frit, laser-pulled method. Briefly, a window was generated in a 30 cm long fused silica capillary using an electrical arc to remove the polyimide coating. Photopolymerized frits were generated using a monomer mix of 350 µL trimethylolpropane trimethacrylate and 150 µL of glycidyl methacrylate with 7.9 mg of benzoin methyl ether. The porogenic solvent was prepared by mixing 250 µL toluene and 750 µL isooctane. The monomer solution (300 µL) was added to the porogen solution and sonicated for 15 min. The frit mixture was loaded into the capillary column and polymerization was initiated under a UV lamp (UVP, Cambridge, UK). The reaction took 30 min at ambient temperature with an exposure wavelength of 365 nm.

Nanospray tips were generated using a laser micropipette puller model P-2000 (Sutter Instruments, Novato, CA, USA) with heating time: 420 msec, velocity: 80 msec, delay time: 150 msec, pulling time: 225 msec. The nano emitter fritted capillary was etched in hydrofluoric acid (51%) to open the fine tip for nanospray capLC-MS. The capillary column was packed in-house with EVO 3-micron superficially porous particles using a pressure cell. Final capillary column lengths were trimmed to 16.5 cm.

A pH of 8 was maintained using 20 mM ammonium acetate mixed with 8 mM ammonium phosphate and ammonium hydroxide for analysis in negative mode. The gradient for the capillary LC method was as follows (minute, %B): 0 min, 45%; 0.25 min, 45%; 10 min, 98%; 15 to 18 min, 98%; 19 to 25 min, 45%. 2 µL sample volume was used for all capillary LC experiments split by 1:2500 resulting in 800 pL mass injected on column (0.6% column volume). The LC was pumping 250 µL/min splits to 100 nL/min flowing through the capillary column. A linear velocity of 2.1 mm/sec was used to isolate fatty acids in a 10-minute gradient method.

The PRM method operated at a resolution of 35K, max injection time of 100ms, and AGC of $5e^5$ for 4 scans before returning to a full scan. Ionization was achieved with a spray voltage of 1.75 kV for the capillary LC method with a capillary temperature of 200 °C using the nanospray flex ion source.

Tag synthesis:

Isotopic variants of 1,2-dibromoethane, potassium cyanide, formaldehyde, and methyl iodide were purchased from Cambridge Isotope Labs (Andover, MA, USA).

General procedure for succinonitrile isotopologue synthesis

The corresponding 1,2-dibromoethane starting material was dissolved in MeOH (or MeOD for d_4-dibromoethane) in a microwave reaction vial. The corresponding potassium cyanide (KCN, $K^{13}CN$, $KC^{15}N$ or $K^{13}C^{15}N$) was added (2 eq) to the solution. The suspension was heated to 140°C and stirred for 20 minutes in a microwave synthesizer. The resulting solution was filtered to and washed with acetone (10mL x 3). The filtrate was concentrated to yield succinonitrile as a dark red waxy solid.

General procedure for di-tert-butyl butane-1,4-diyldicarbamate isotopologue synthesis

The corresponding succinonitrile isotopologue was dissolved in dry MeOH and cooled to 0°C in a 1L round bottom flask under nitrogen atmosphere. Di-*tert*-butyl decarbonate (2 eq) and $NiCl_2$ hexahydrate (20 mol%) were added to the stirring solution. $NaBH_4$ (14 eq) added in small portions over 30 minutes, adding ice as needed. The reaction mixture was allowed to warm to room temperature and stirred overnight. Diethylene triamine (8 eq) added and the solution was stirred for 30 minutes. The reaction mixture was concentrated, dissolved in 50 mL EtOAc, and washed with $NaHCO_3$ (50 mL). The aqueous layer was extracted with EtOAc twice more (50 mL x2). The

combined organic layer was dried with $MgSO_4$ and concentrated to yield di-tert-butyl butane-1,4-diyldicarbamate as a yellow solid.

Deprotection of diboc-protected amines to yield putrescine isotopologues

The diboc-protected diamine intermediates were deprotected at RT in a stirred 4M HCl/Dioxane solution overnight. The solutions were concentrated and recrystallized in 9:1 EtOH/H_2O, filtered, and isolated as the HCl salt of the corresponding putrescine isotopologue.

General procedure for tert-butyl N-(4-aminobutyl)carbamate isotopologue synthesis

1,4-diaminobutane (3 mmol) was dissolved in 10mL EtOH and triethylamine (3 eq) was added. The reaction mixture was stirred while tert-butyl phenyl carbonate (3 mmol) was added slowly. The reaction mixture was heated to 160°C and stirred for 20 minutes in a microwave synthesizer. The pH was adjusted to 3 with 2M HCl and washed with DCM (3x 16mL). The aqueous layer was basified to pH 10 with 2M NaOH and extracted with DCM (5x 20 mL), dried with K_2CO_3, and concentrated to afford tert-butyl N-(4-aminobutyl)carbamate as a dark red oil.

General procedure for methylation of tert-butyl N-(4-aminobutyl)carbamate with formaldehyde

Tert-butyl N-(4-aminobutyl)carbamate was dissolved in 5 mL dry ACN. To the stirred solution, the corresponding isotopic variant of formaldehyde solution (20%) (3 eq) was added, followed by $NaBH_3CN$ (2.6 eq). The reaction mixture was stirred for 15 minutes before adjusting the pH to 7 with acetic acid, several 3Å molecular sieves were added, and the solution was stirred for an additional 2 hours. The reaction mixture was evaporated and reconstituted in 2M KOH (10 mL), extracted with ether (20 mL x 3), and washed with 40 mL 0.5M KOH. The organic layer was dried with K_2CO_3 and concentrated to yield the dimethylated product, tert-butyl N-(4-N',N'-dimethylaminobutyl)carbamate, as a colorless oil.

General procedure for methylation of tert-butyl N-(4-aminobutyl)carbamate with methyl iodide

In a sealable microwave reaction vial, tert-butyl N-(4-aminobutyl)carbamate or tert-butyl N-(4-N',N'-dimethylaminobutyl)carbamate was dissolved in 5 mL ACN and K_2CO_3 (4 eq) was added. The solution was stirred and methyl iodide (5 eq) was added dropwise to the stirred solution. The vial was heated to 130°C and stirred for 20 minutes in a microwave reactor. The vial cap was then removed and two mL water was added to the vial. The top layer was extracted by pipette, dried and suspended in dichloromethane. The resulting solution was filtered by 0.45µm syringe tip filter and concentrated to yield a dark red oil.

Deprotection of boc-protected amines to yield isotopic tags

The boc-protected quaternary ammonium ion intermediates were deprotected at RT in a stirred 4M HCl/Dioxane solution overnight. The solutions were concentrated and purified via normal phase column chromatography (DCM/MeOH gradient over 10 minutes with an isocratic hold at 100% MeOH for 5 minutes) to yield the isotopic tags as HCl salts. All isotope labelled tags were characterized by and high resolution MS and NMR (Figure S1, S2).

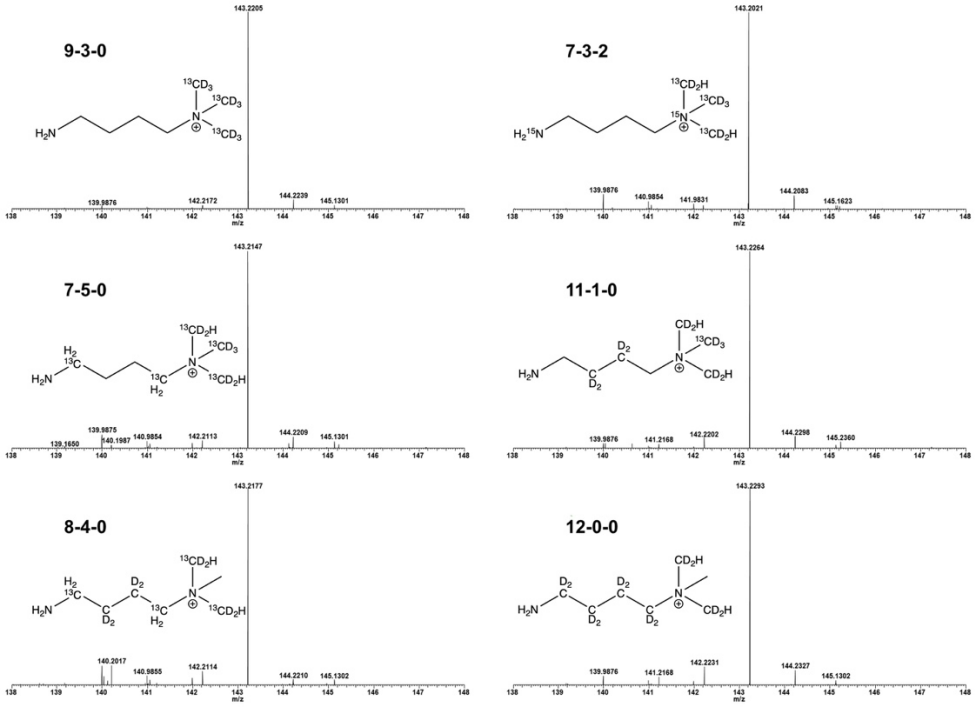


Figure S1: HRMS[1] spectra of each isobaric tag.

Each of the six isobaric tags were direct injected on a Q-Exactive orbitrap operating at a resolution of R = 140K to assess isotopic purity. All tags were within 2 ppm of the theoretical m/z.

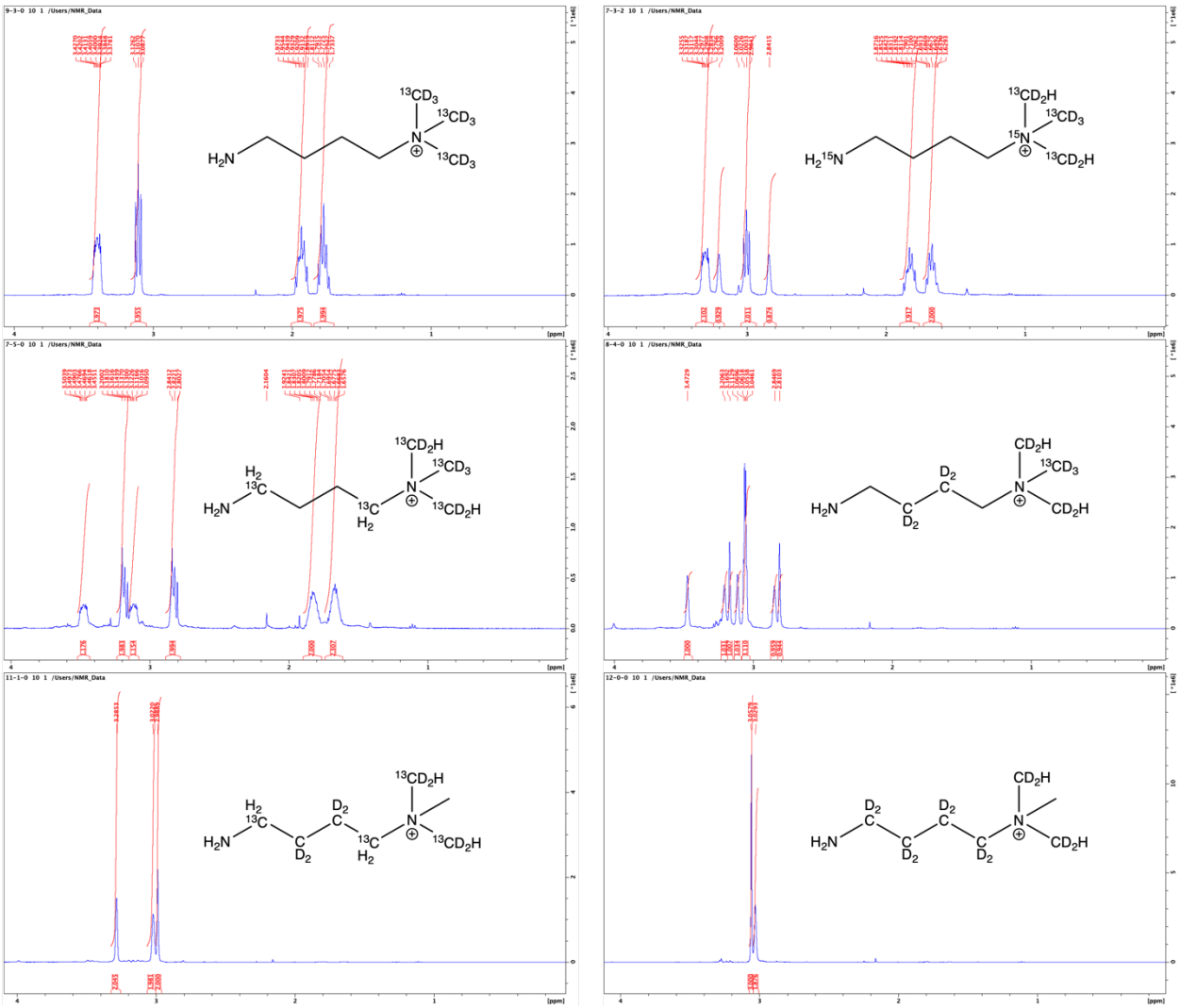


Figure S2: Proton NMR spectrum of each synthesized tag

Spectra were acquired using a 400MHz Bruker NMR with D_2O as the solvent. The tag structure is overlaid for each spectrum.

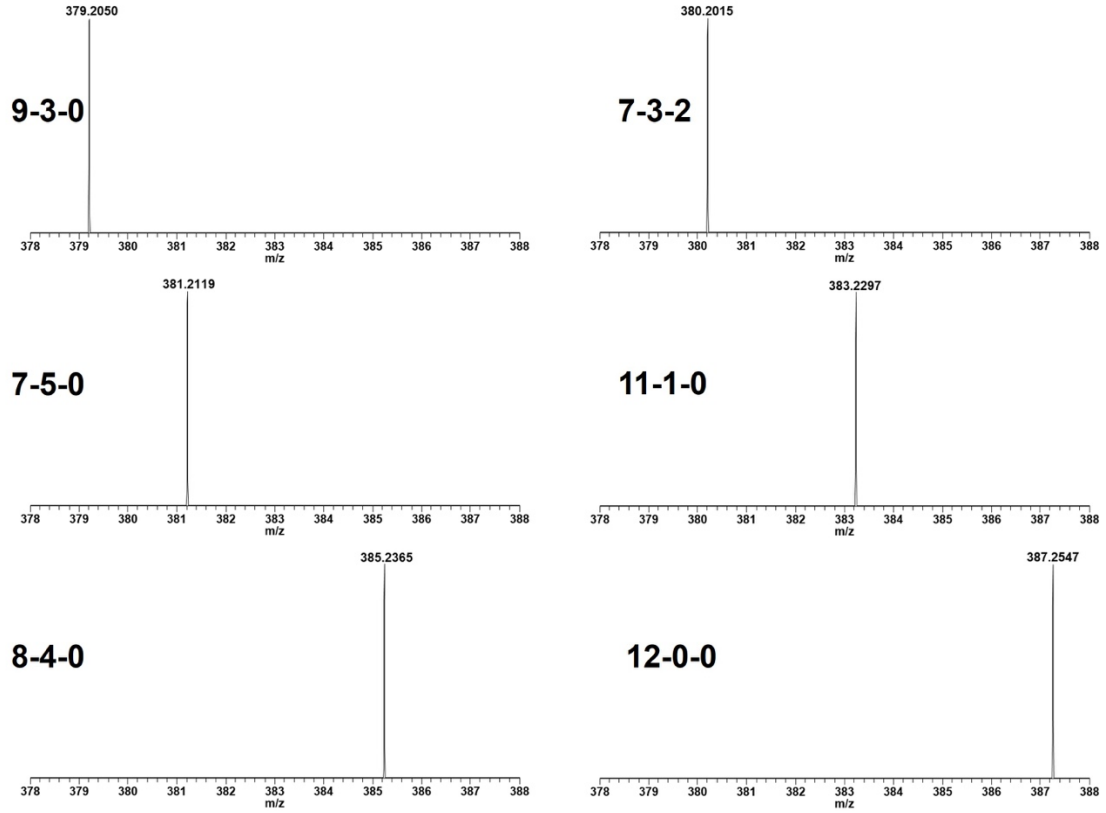


Figure S3: Individually injected, dual tagged palmitoleic acid shows no reporter overlap.

Each isotope tagged variant of palmitoleic acid was injected and fragmented individually to assess possible reporter overlap. The reporter region for Δ^9 acids is shown (m/z 379 – 387). Fragmentation spectra were acquired at R = 35K and each reporter was observed within 2 ppm of the theoretical m/z. Spectra are labeled with the D-^{13}C-^{15}N isotope variant used.

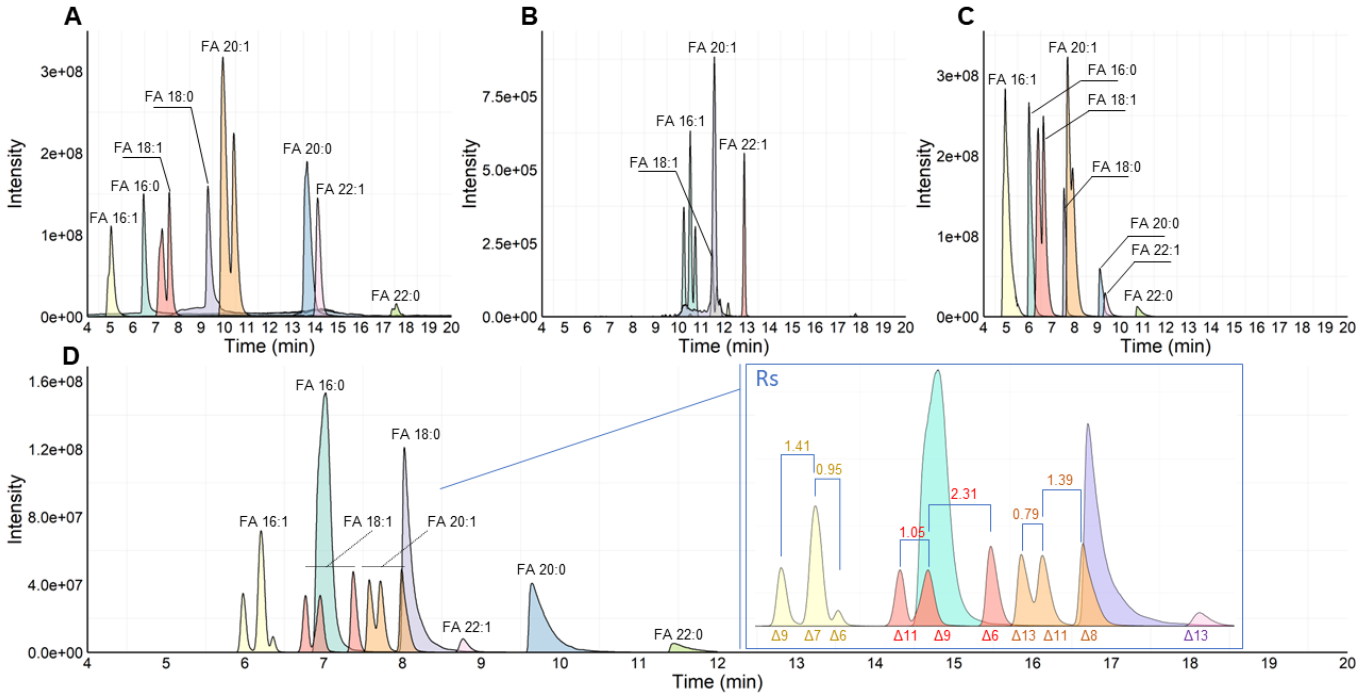
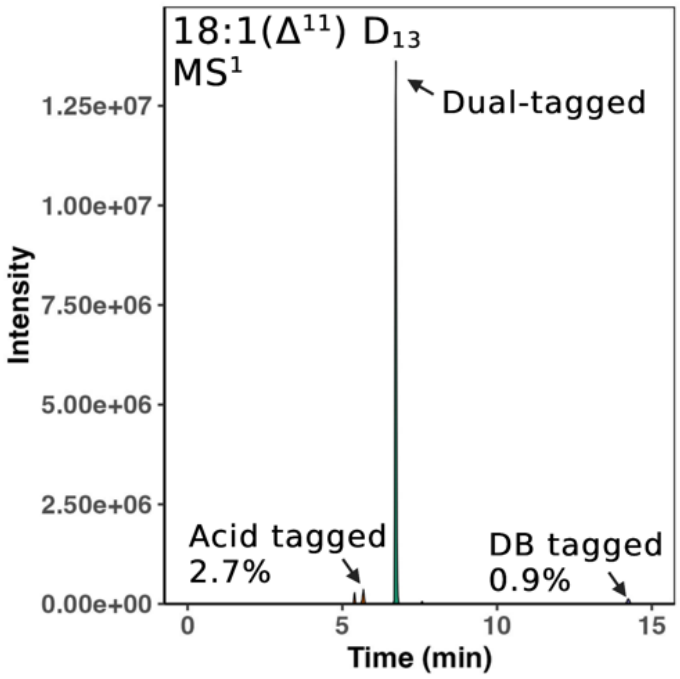


Figure S4: Chromatographic separation of free and derivatized acids **using the capillary LC method**.

Free fatty acids (A), chloramine-T tagged FFAs (B), acid tagged FFAs (C), and dual-tagged FFA standards (D) were separated **using the capillary LC method**. Resolution between double bond isomers is shown in blue for dual tagged analytes.

Figure S5: Reaction completion in a HepG2 tagged lysate.



Dual-tagged HepG2 cells were analyzed as described in the main text and separated on the capillary LC system. MS^1 extracted ion chromatograms are shown for the possible reaction products of the internal standard. Dual tagged internal standard is shown in green. The two possible incomplete reaction products, acid only or double bond only tagged, are shown in orange and purple. The acid only tagged product is observed at 2.7% and the double bond tagged product is observed at 0.93% of the intensity of the dual-tagged product.

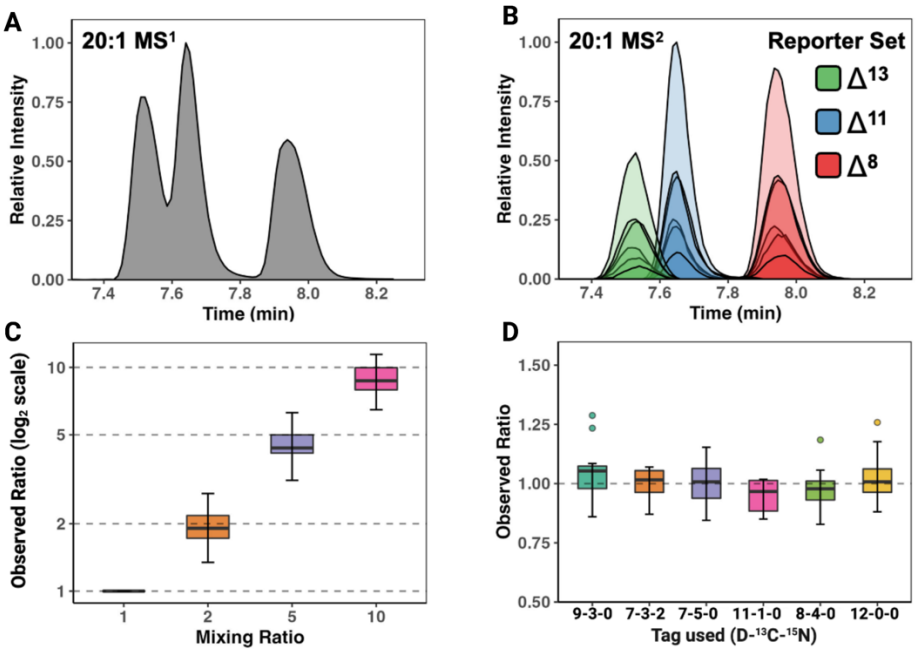


Figure S6: Analytical performance of the **capillary LC method**.

Six-plex dual tagged 20:1 isomers were separated using the **capillary LC** method (A). The shown 20:1 double bond isomers were mixed as a 6-plex at a ratio of 1:2:2:5:5:10 and each reporter set corresponds to one isomer (B). Peak ratios were taken for all standards mixed across an order of magnitude (C). Tags produce similar ratios when mixed 1:1 (D) suggesting minimal tag influence on signal response.

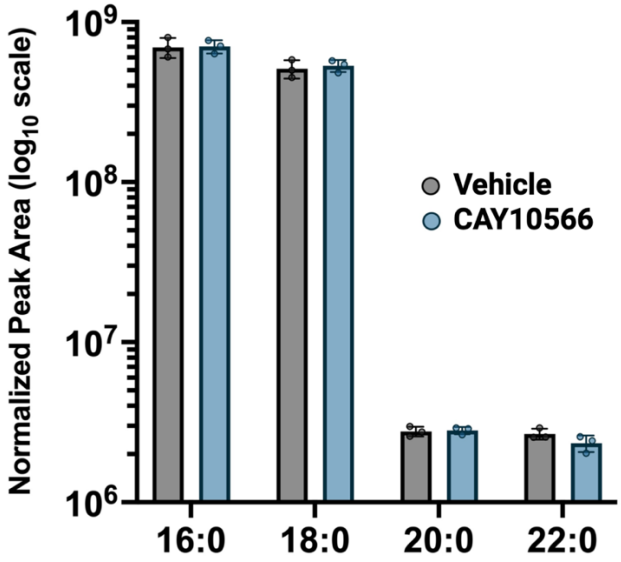


Figure S7: Saturated acid comparison upon SCD1 inhibition.

No significant changes were observed for saturated acids upon SCD1 inhibition by CAY10566 (n = 3).

Table S1: PRM schedule for the UHPLC fragmentation of dual tagged fatty acids.

Retention times were set across the entire targeted isomer elution window of each monounsaturated acid for simplicity. Mass isolation was set to the average m/z of the 6-plex tagged acid. This was done to ensure equal isolation despite the small mDa shifts in the MS^1 scans for the 6-plex tags.

Analyte	Isolated m/z	Start time (min)	End time (min)
16:0	381.4484	1.10	1.70
16:1	548.4525	0.40	1.25
18:0	409.4797	1.95	2.35
18:1	576.4838	1.25	2.10
18:1 Δ11 (D_{13})	589.5654	1.25	1.75
20:0	437.5110	2.20	2.50
20:1	604.5151	1.95	2.40
22:0	465.5423	2.35	2.70
22:1	632.5464	2.15	2.40

Table S2: Isotopic purity of 6-plex tags.

Each tag contains a mix of naturally occurring and synthetic isotope impurities. These purity differences were accounted for reduce tag-induced variation. The theoretical naturally occurring ^{13}C impurity is 7.6% for an unlabeled tag.

Tag	Isotope purity (%)
9-3-0	91.8%
7-3-2	85.8%
7-5-0	85.5%
11-1-0	85.3%
8-4-0	87.2%
12-0-0	85.6%

Table S3: Product m/z for dual tagged acids.

Each isotopically encoded acid tag produces a consistent neutral loss upon fragmentation. The tosylated double bond cleaves to produce a unique set of reporters for each position.

	Theoretical reporter m/z per double bond position and tag variant					
DB position	9-3-0	7-3-2	7-5-0	11-1-0	8-4-0	12-0-0
1	267.0799	268.0769	269.0866	271.1049	273.1116	275.1300
2	281.0955	282.0925	283.1022	285.1206	287.1273	289.1457
3	295.1112	296.1082	297.1179	299.1362	301.1429	303.1613
4	309.1268	310.1238	311.1335	313.1519	315.1586	317.1770
5	323.1425	324.1395	325.1492	327.1675	329.1742	331.1926
6	337.1581	338.1551	339.1648	341.1832	343.1899	345.2083
7	351.1738	352.1708	353.1805	355.1988	357.2055	359.2239
8	365.1894	366.1864	367.1961	369.2145	371.2212	373.2396
9	379.2051	380.2021	381.2118	383.2301	385.2368	387.2552
10	393.2207	394.2177	395.2274	397.2458	399.2525	401.2709
11	407.2364	408.2334	409.2431	411.2614	413.2681	415.2865
12	421.2520	422.2490	423.2587	425.2771	427.2838	429.3022
13	435.2677	436.2647	437.2744	439.2927	441.2994	443.3178
14	449.2833	450.2803	451.2900	453.3084	455.3151	457.3335
15	463.2990	464.2960	465.3057	467.3240	469.3307	471.3491
16	477.3146	478.3116	479.3213	481.3397	483.3464	485.3648
17	491.3303	492.3273	493.3370	495.3553	497.3620	499.3804
18	505.3459	506.3429	507.3526	509.3710	511.3777	513.3961

Table S4: Analytical performance of the dual-tagged acids using the **capillary LC method**. RT = retention time, RSD = relative standard deviation.

Analyte	Intra-scan RT % RSD (n = 6)	Intensity % RSD (n = 6)	R^2	w(4σ) (seconds)
16:0	0.45%	3.9%	0.995	21.12
16:1(Δ^6)	0.35%	21.6%	0.922	8.82
16:1(Δ^7)	0.27%	9.6%	0.990	10.62
16:1(Δ^9)	0.14%	12.1%	0.996	8.28
18:0	0.28%	4.7%	0.992	15.18
18:1(Δ^6)	0.18%	7.1%	0.988	8.82
18:1(Δ^9)	0.15%	8.9%	0.994	12.18
18:1(Δ^{11})	0.12%	7.3%	0.979	10.14
20:0	0.89%	6.7%	0.993	29.58
20:1(Δ^8)	0.14%	6.9%	0.995	8.88
20:1(Δ^{11})	0.11%	10.3%	0.994	12.24
20:1(Δ^{13})	0.11%	10.3%	0.983	12.24
22:0	0.97%	6.7%	0.991	32.52
22:1(Δ^{13})	0.10%	8.2%	0.957	15.24
Average	**0.30%**	**8.9%**	**0.984**	**14.70**