**Supplemental Information**

## EXPERIMENTAL PROCEDURE

### Synthesis of Reference BA 3-Sulfates

7,12-Diformylcholic acid, 7-formylchenodeoxycholic acid, and 12-formyldeoxycholic acid were synthesized according to a previous method (1) with small modifications. Briefly, 2.5 g of CA, CDCA, or DCA was dissolved in 10 ml of 90% formic acid containing 2 drops of 70% perchloric acid. After stirring at 55$^{o}$C for 1.5 hr, the mixture was allowed to cool to about 40$^{o}$C. Approximately 8 ml of acetic anhydride was added dropwise at a rate that maintained the temperature between 55-60$^{o}$C until a large quantity of bubbles appeared. The mixture was then cooled to room temperature and poured into 100 ml of water. The precipitate was collected, washed with water, and dried under vacuum. The products were then dissolved in boiling ethanol (25 ml), diluted with 25 ml of boiling water, and recrystallized as white needles (formylated BA). A suspension of formylated BA (CA: 2.46 g; CDCA and DCA: 2.24 g) in 25 ml of acetone to which 50 ml of 0.2 N NaOH was added dropwise for 30 min, and then maintained at room temperature for 1 hr. The solution was acidified with 2.5 ml of dilute acetic acid (1.3 g glacial acetic acid in 10 ml water), and then extracted with 50 ml of ethyl acetate (AcOEt). The organic layer was washed with water 3 times, dried over anhydrous $Na_2SO_4$, and evaporated.

The 3-monohydroxy formylated compounds (7,12-diformylcholic acid, 7-formylchenodeoxycholic acid, and 12-formyldeoxycholic acid) were thus obtained and used for preparation of 3-sulfates according to a previous method (2) with small modifications. To a solution of 3-monohydroxy formylated compound (2 mmol) in 4 ml of dimethylformamide (DMF), sulfur trioxide-triethylamine complex (2.02 mmol), synthesized according to a previous method (3), was added. After 30 min at room temperature, the reaction mixture was extracted with AcOEt, evaporated, and dried under vacuum. The product was dissolved with

20 ml of 0.5 N NaOH in a 30-ml centrifuge tube and heated in a steam bath for 10 min. After cooling to room temperature, the solution was acidified to pH 5-6 with dilute HCl. Four ml of 1 M *p*-toluidine hydrocholoride was added and stirred vigorously. The oily suspension that formed was centrifuged and washed with ice-cold water (1 ml). The final syrup-like product was dissolved in 20 ml of 0.2 N methanolic NaOH, and poured into 50 ml of ether. The precipitate (BA 3-sulfate) was collected, washed with ether, and dried under vacuum.

The taurine-conjugated BA 3-sulfates were synthesized by the following method. In a 30-ml centrifuge tube, BA 3-sulfate (0.2 mmol), EEDQ (CA and CDCA 3-sulfate: 0.28 mmol; DCA 3-sulfate: 0.54 mmol), taurine (0.22 mmol), and triethylamine (40 µl) were dissolved in 1 ml of DMF, stirred in a 90$^{o}$C bath for 1 hr, and maintained at room temperature for another 1 hr. The solution was added to 10 ml of ice-cold ether and centrifuged to collect the oily product. After washing with another 10 ml of ether, the oily product was dissolved in 2 ml of 0.5 N NaOH, heated on a steam bath for 5 min, and cooled to room temperature. After adjusting the pH to 7 with dilute HCl, 2 ml of 1 M *p*-toluidine hydrochloride was added. The suspension was centrifuged and the lower syrupy layer was dissolved in 2 ml of 0.2 N methanolic NaOH. The solution was diluted with 10 ml of ether and centrifuged again. The lower precipitate (taurine-conjugated BA 3-sulfate) was collected, washed with ether, and dried under vacuum.

The glyine-conjugated BA 3-sulfates were synthesized by the following method. A suspension of 3-monohydroxy formylated BA (2 mmol), ethyl glycinate HCl (2.8 mmol), EEDQ (2.8 mmol), and triethylamine (400 µl) in 30 ml of AcOEt was refluxed for 4 hr. After cooling to room temperature, the mixture was washed successively with 0.5 N NaOH (10 ml), 0.5 N HCl (10 ml X3), and water (20 ml X2). The organic layer was dried over anhydrous $MgSO_4$, evaporated, and dried under vacuum. The solid product so obtained was transferred into a 30-ml centrifuge tube, dissolved in 4 ml of DMF, and sulfur trioxide-triethylamine (3.2 mmol) was added and maintained at room temperature for 30 min. After adding 30 ml of ether, the suspension was centrifuged. The lower oily product was washed with ether, dried under

vacuum, and hydrolyzed, precipitated as a *p*-toluidinium salt, and converted to the disodium salt (glycine-conjugated BA sulfate) as described for taurine-conjugated BA 3-sulfates. The BA 3-sulfates synthesized using this method include cholic acid 3-sulfate (CA3S), chenodeoxycholic acid 3-sulfate (CDCA3S), deoxycholic acid 3-sulfate (DCA3S), taurocholic acid 3-sulfate (TCA3S), tauro- or glyco-chenodeoxycholic acid 3-sulfate (TCDCA3S, GCDCA3S), tauro- or glyco-deoxycholic acid 3-sulfate (TDCA3S, GDCA3S), as well as glyco-lithocholic acid 3-sulfate (GLCAS). The final products were characterized and verified by their mass and NMR spectra (data not shown).

**Synthesis of Other Reference BAs**

Cholic acid 7-sulfate (CA7S) and cholic acid 12-sulfate (CA12S) were synthesized according to a previous method (4) and verified by their mass and NMR spectra. 3α,12α-Diol-7-oxo-5β–cholan-24-oic acid (7-dehydroCA) was a hydrolysis product of methyl 7-oxo-3α,12α-diol-5β-cholan-24-oate, which was an intermediate during CA7S and CA12S synthesis. TωMCA was synthesized by conjugation of taurine with ωMCA according to the previous method (5).

**Liquid Chromatographic and Mass Spectrometric Conditions**

A Waters ACQUITY ultra performance LC system (Waters, Milford, MA) was used for BA quantification. Chromatographic separations were performed on an ACQUITY UPLC BEH C18 column (1.7 μm, 100 x 2.1 mm I.D.) heated to 45 $^{o}$C in the column compartment, using a gradient elution of mobile phase A (10 mM ammonium acetate in 20% acetonitrile) and mobile phase B (10 mM ammonium acetate in 80% acetonitrile) as follows: 5% B (0-5 min), 5-14% B (5-14 min), 14-25% B (14-14.5 min), 25% B (14.5-17.5 min), 25-50% B (17.5-18 min), 50% B (18-22 min), 50-80% B (22-22.5 min), 80% B (22.5-24.5 min), 80-5% B (24.5-25 min), and 5% B (25-28 min). All BAs were separated well (Supplemental figure 1). The injection

volume was 7.5 µl, and the flow rate set at 0.4 ml/min. The sample manager system temperature was maintained at 4 $^{o}$C. The injection volume of all samples was 7.5 µl.

The mass spectrometer was a Waters Quattro Premier XE triple quadrupole instrument with an ESI source (Waters, Milford, MA). The entire LC-MS system was controlled by MassLynx 4.1 software. All BAs were detected in the negative mode. The capillary, extractor, and RF voltages were set at 3 kV, 4 V, and 0 V, respectively. The source, desolvation, and collision gases were set at 650 L/h, 75 L/h, and 0.15 ml/min, respectively. The multiple reaction monitoring (MRM) transitions for all BAs are shown in supplemental table 1. The inter-channel delay, inter-scan delay, and dwell time were 5, 5, and 20 ms, respectively.

## RESULTS

### Synthesis of BAs

TCA3S, CA3S, TCDCA3S, GCDCA3S, CDCA3S, TDCA3S, GDCA3S, DCA3S, GLCAS, CA7S, and CA12S were synthesized according to previous methods (2, 6) with modifications. However, synthesis of GCA3S and UDCA monosulfates was unsuccessful using the same methods. CDCA7S and DCA12S were synthesized according to a previous method by Tserng and Klein (4), but the products had the same retention time and mass spectra as CDCA3S and DCA3S (data not shown). This might indicate that our products were CDCA3S and DCA3S, instead of CDCA7S and DCA12S.

### Identification of BA Sulfation Position in Mouse Livers

In the present study, sulfates of GCDCA, GDCA, and GLCA were not detected in any group of mouse livers (data not shown). CA can theoretically be sulfated at the 3-, 7-, or 12-OH positions to form CA3S, CA7S, or CA12S, respectively. In control livers of male and female mice, one peak was found in the chromatograph window of CA3S but with a different

retention time than CA3S standard. This peak was also found at high concentrations in intestinal contents (data not shown). By comparing the retention time and mass spectra with CA7S and CA12S standards, this peak was identified as CA7S (Supplemental figure 2a). To further confirm the position of CA sulfation, liver samples were mixed with one of the CA sulfate standards and separated on UPLC. Only one peak was found when liver samples were mixed with CA7S, but not when mixed with CA3S or CA12S (data not shown). One peak was found in the chromatograph window of TCA3S, with a similar retention time as TCA3S (Supplemental figure 2b). Because CA was sulfated at the 7-position, it was likely that sulfation of TCA was also at the 7-position (TCA7S), which might not separate from TCA3S under the current chromatographic conditions. CDCA can theoretically be sulfated at its 3-OH and 7-OH positions, whereas DCA can theoretically be sulfated at its 3-OH and 12-OH positions. One peak was found in the chromatograph window of TCDCA3S and TDCA3S (Supplemental figure 3a). This peak increased markedly in livers of mice fed CDCA, whereas it was almost non-detectable in livers of mice fed DCA or UDCA. This suggests the peak is a sulfate of TCDCA. One peak in the chromatograph window of CDCA3S and DCA3S was found in livers of mice fed CDCA, but not other BAs (Supplemental figure 3b). This suggests the peak is a sulfate of CDCA. In addition, these two peaks have different retention times than TCDCA3S and CDCA3S. Because CDCA can only be sulfated at either the 3-OH or 7-OH position, these two peaks are TCDCA7S and CDCA7S, respectively. Feeding DCA and UDCA did not generate any peaks in the chromatograph window of dihydroxy BA sulfates (data not shown), suggesting that sulfation is not a major detoxification pathway of DCA or UDCA. In regard to LCA sulfates, LCAS was only detected in livers of female mice fed UDCA (Supplemental figure 4a), whereas TLCAS was detected in both livers of female mice fed LCA and UDCA (Supplemental figure 4b). This suggests that LCA sulfation is a female predominant pathway in mouse livers.

**Reference List**


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2. Tserng, K. Y., and P. D. Klein. 1978. Bile-Acid Sulfates .2. Synthesis of 3-Monosulfates of Bile-Acids and Their Conjugates. Lipids 13: 479-486.
3. Tserng, K. Y., and P. D. Klein. 1977b. Synthesis of sulfate esters of lithocholic acid, glycolithocholic acid, and taurolithocholic acid with sulfur trioxide-triethylamine. J Lipid Res 18: 491-495.
4. Tserng, K. Y., and P. D. Klein. 1979. Bile acid sulfates. III. Synthesis of 7- and 12-monosulfates of bile acids and their conjugates using a sulfur trioxide-triethylamine complex. Steroids 33: 167-182.
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6. Goto, J., H. Kato, F. Hasegawa, and T. Nambara. 1979. Synthesis of monosulfates of unconjugated and conjugated bile acids. Chem Pharm Bull (Tokyo) 27: 1402-1411.

**Supplemental figure legends**

**Supplemental figure 1.** Representative UPLC-MS/MS chromatograms of various BAs under the final chromatography and detection conditions. 1, TωMCA; 2, TαMCA; 3, TβMCA; 4, THCA; 5, TCA; 6, GLCAS; 7, TMDCA; 8, TUDCA; 9, THDCA; 10, TCDCA; 11, TDCA; 12, CA3S; 13, TLCA; 14, CDCA3S; 15, DCA3S; 16, GCA; 17, LCAS; 18, $^{2}H_{4}$-GCDCA; 19, GMDCA; 20, GUDCA; 21, GHDCA; 22, GCDCA; 23, GDCA; 24, GLCA; 25, ωMCA; 26, αMCA; 27, βMCA; 28, HCA; 29, CA; 30, 7-oxoDCA; 31, 12-oxoCDCA; 32, 3-dehydroCA; 33, $^{2}H_{4}$-CDCA; 34, MDCA; 35, UDCA; 36, HDCA; 37, isoDCA; 38, CDCA; 39, DCA; 40, 3-DCA; 41, 6-oxoDCA; 42, 6-oxo-alloLCA; 43, 7-oxoLCA; 44, 12-oxoLCA; 45, alloLCA; 46, isoLCA; 47, LCA; 48, dehydroLCA; 49, TCA3S; 50, TCDCA3S; 51, TDCA3S; 52, TLCAS.

**Supplemental figure 2.** UPLC-MS/MS chromatograms of liver samples from control male mice. (a) The peaks at m/z 486.8>96.8 from liver samples of control mice and CAS standards. Standard CA7S eluted at the same time as the sample peak. (b) The peaks at m/z 296.7>496.3 eluted at the similar time between liver samples and standard TCA3S.

**Supplemental figure 3.** UPLC-MS/MS chromatograms of liver samples from control and CDCA-fed male mice. (a) The peaks at m/z 288.7>480.3. The peak from livers of mice fed CDCA was larger than that from control mice, and eluted at a different time than TCDCA3S and TDCA3S standards. (b) The peaks at m/z 470.8>96.8. The peak from livers of mice fed CDCA eluted at a different time than CDCA3S and DCA3S standards.

**Supplemental figure 4.** UPLC-MS/MS chromatograms of liver samples from control, LCA-fed, and UDCA-fed female mice. (a) The peaks at m/z 454.8>96.8. The peak from livers of mice fed UDCA eluted at the same time as LCAS. (b) The peaks at m/z 280.3>464.2. Peaks from livers of mice fed LCA and UDCA eluted at the same time as TLCAS.

**Supplemental figure 5.** UPLC-MS/MS chromatograms of liver samples from control, CDCA-fed, and UDCA-fed male mice. (a) The peaks at m/z 389.4>389.4. Three major peaks were increased markedly in livers of mice fed CDCA. (b) The peaks at m/z 567.1>391.5. No peaks were detected in livers of control male mice, whereas five peaks appeared in livers of mice fed UDCA.

**Supplemental figure 6**. Conjugated (a) and unconjugated (b) BA concentrations in livers of mice fed 1% CA for 7 days. All BA data are expressed as mean ± S.E. for five mice in each group. *, statistically significant difference between the same gender of control and CA-fed groups ($p<0.05$). #, statistically significant difference between male and female mouse livers in the same group ($p<0.05$).

**Supplemental figure 7**. Conjugated (a) and unconjugated (b) BA concentrations in livers of mice fed 0.3% DCA for 7 days. All BA data are expressed as mean ± S.E. for five mice in each group. *, statistically significant difference between the same gender of control and DCA-fed groups ($p<0.05$). #, statistically significant difference between male and female mouse livers in the same group ($p<0.05$).

**Supplemental figure 8**. Conjugated (a) and unconjugated (b) BA concentrations in livers of mice fed 0.3% CDCA for 7 days. All BA data are expressed as mean ± S.E. for five mice in each group. *, statistically significant difference between the same gender of control and CDCA-fed groups ($p<0.05$). #, statistically significant difference between male and female mouse livers in the same group ($p<0.05$).

**Supplemental figure 9.** Conjugated (a) and unconjugated (b) BA concentrations in livers of mice fed 0.3% LCA for 7 days. All BA data are expressed as mean ± S.E. for five mice in each group. *, statistically significant difference between the same gender of control and LCA-fed groups ($p<0.05$). #, statistically significant difference between male and female mouse livers in the same group ($p<0.05$).

**Supplemental figure 10**. Conjugated (a) and unconjugated (b) BA concentrations in livers of mice fed 3% UDCA for 7 days. All BA data are expressed as mean ± S.E. for five mice in each group. *, statistically significant difference between the same gender of control and

UDCA-fed groups ($p<0.05$). #, statistically significant difference between male and female mouse livers in the same group ($p<0.05$).

**Supplemental figure 11.** Conjugated BAs, unconjugated BAs, and total BAs in livers of mice fed BAs and resin. All BA data are expressed as mean ± S.E. for five mice in each group. *, statistically significant difference between the same gender of control and BA-fed groups ($p<0.05$). #, statistically significant difference between male and female mouse livers in the same group ($p<0.05$).

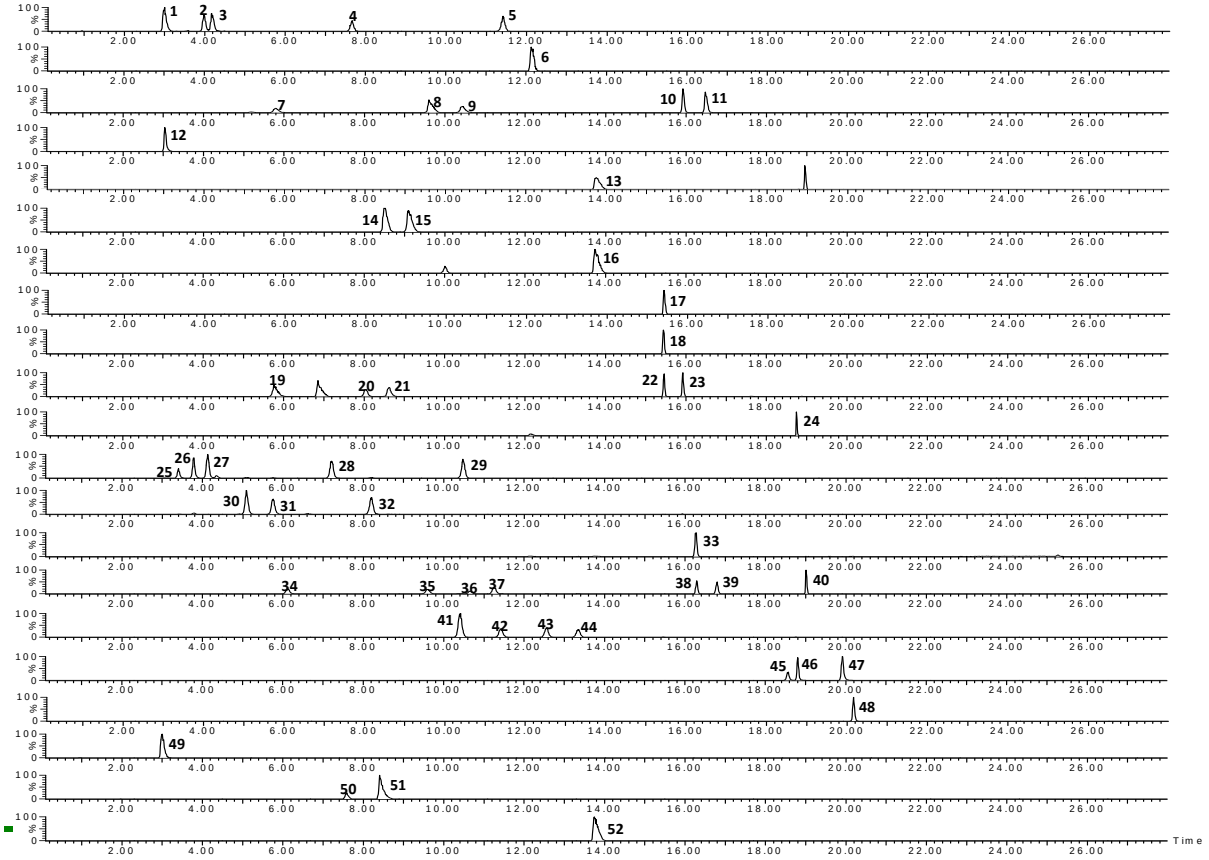
**Supplemental figure 12**. Conjugated (a) and unconjugated (b) BA concentrations in livers of mice fed 2% resin for 7 days. All BA data are expressed as mean ± S.E. for five mice in each group. *, statistically significant difference between the same gender of control and resin-fed groups ($p<0.05$). #, statistically significant difference between male and female mouse livers in the same group ($p<0.05$).

**Supplemental table 1. Mass spectrometer conditions for quantification of various BAs.**

| Name | Precursor (m/z) | Product (m/z) | Cone (V) | Collision (eV) |
|---|---|---|---|---|
| TLCAS | 280.3 | 464.2 | 35 | 25 |
| Tauro-dihydroxy BAS | 288.7 | 480.3 | 40 | 25 |
| TCA3S | 296.7 | 496.3 | 40 | 25 |
| DehydroLCA | 373.4 | 373.4 | 65 | 15 |
| Monohydroxy BA | 375.4 | 375.4 | 65 | 20 |
| oxoLCA | 389.4 | 389.4 | 70 | 15 |
| Dihydroxy BA | 391.4 | 391.4 | 65 | 15 |
| 2H4-CDCA | 395.3 | 395.3 | 60 | 18 |
| DehydroCA | 405.4 | 405.4 | 70 | 15 |
| Trihydroxy BA | 407.4 | 407.4 | 65 | 15 |
| GLCA | 432 | 432 | 60 | 15 |
| Glyco-dihydroxy BA | 448 | 448 | 60 | 15 |
| 2H4-GCDCA | 452.1 | 452.1 | 60 | 18 |
| LCAS | 454.8 | 96.8 | 60 | 42 |
| GCA | 464 | 464 | 60 | 15 |
| Dihydroxy BAS | 470.8 | 96.8 | 60 | 45 |
| TLCA | 482.1 | 80 | 90 | 70 |
| Trihydroxy BAS | 486.8 | 96.8 | 60 | 45 |
| Tauro-dihydroxy BA | 498.1 | 79.9 | 90 | 70 |
| GLCAS | 511.7 | 432 | 50 | 32 |
| Tauro-trihydroxy BA | 514.1 | 79.9 | 90 | 70 |
| Glyco-dihydroxy BAS | 528 | 448.3 | 50 | 30 |
| Glyco-trihydroxy BAS | 544.3 | 464.3 | 50 | 30 |
| Monohydroxy BAG | 551.5 | 375.5 | 35 | 40 |
| Dihydroxy BAG | 567.1 | 391.5 | 45 | 40 |
| Trihydroxy BAG | 583.1 | 407.2 | 46 | 36 |

BAS: BA sulfate; BAG: BA glucuronide

# Supplemental figure 1



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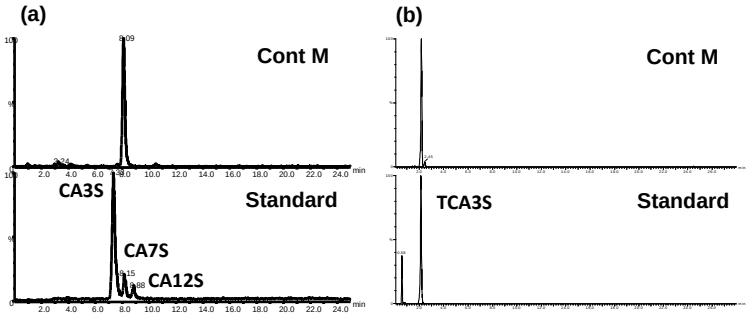

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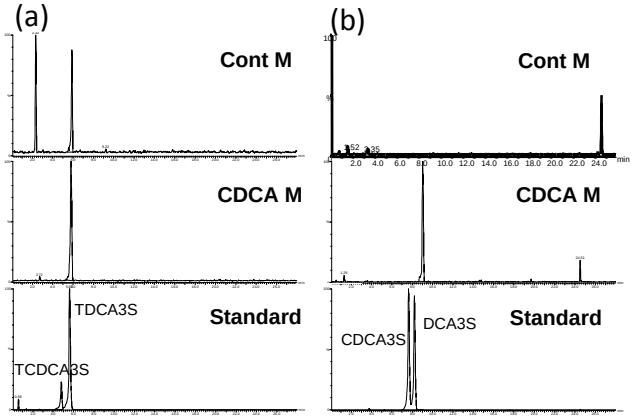

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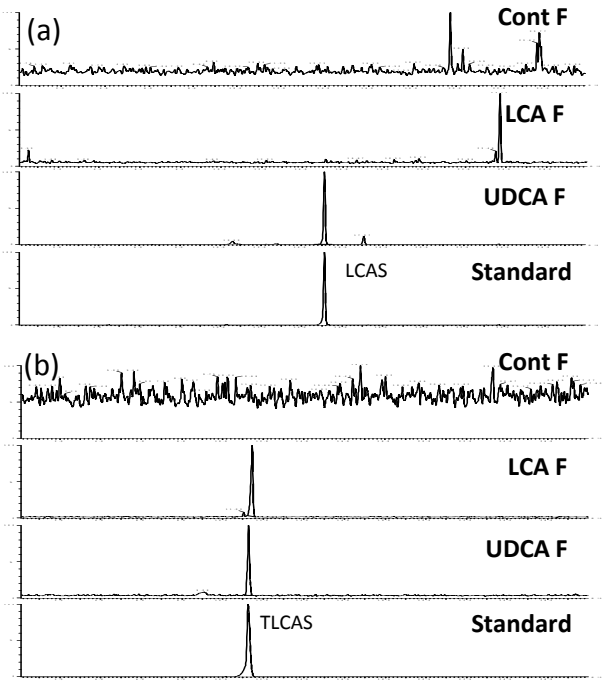

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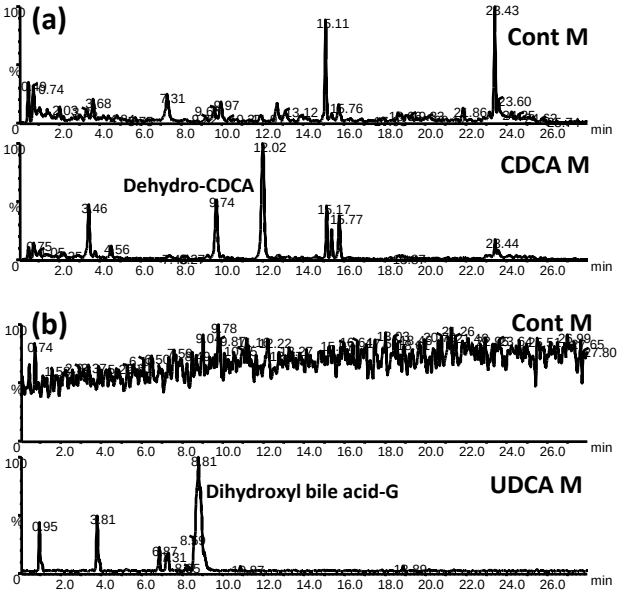

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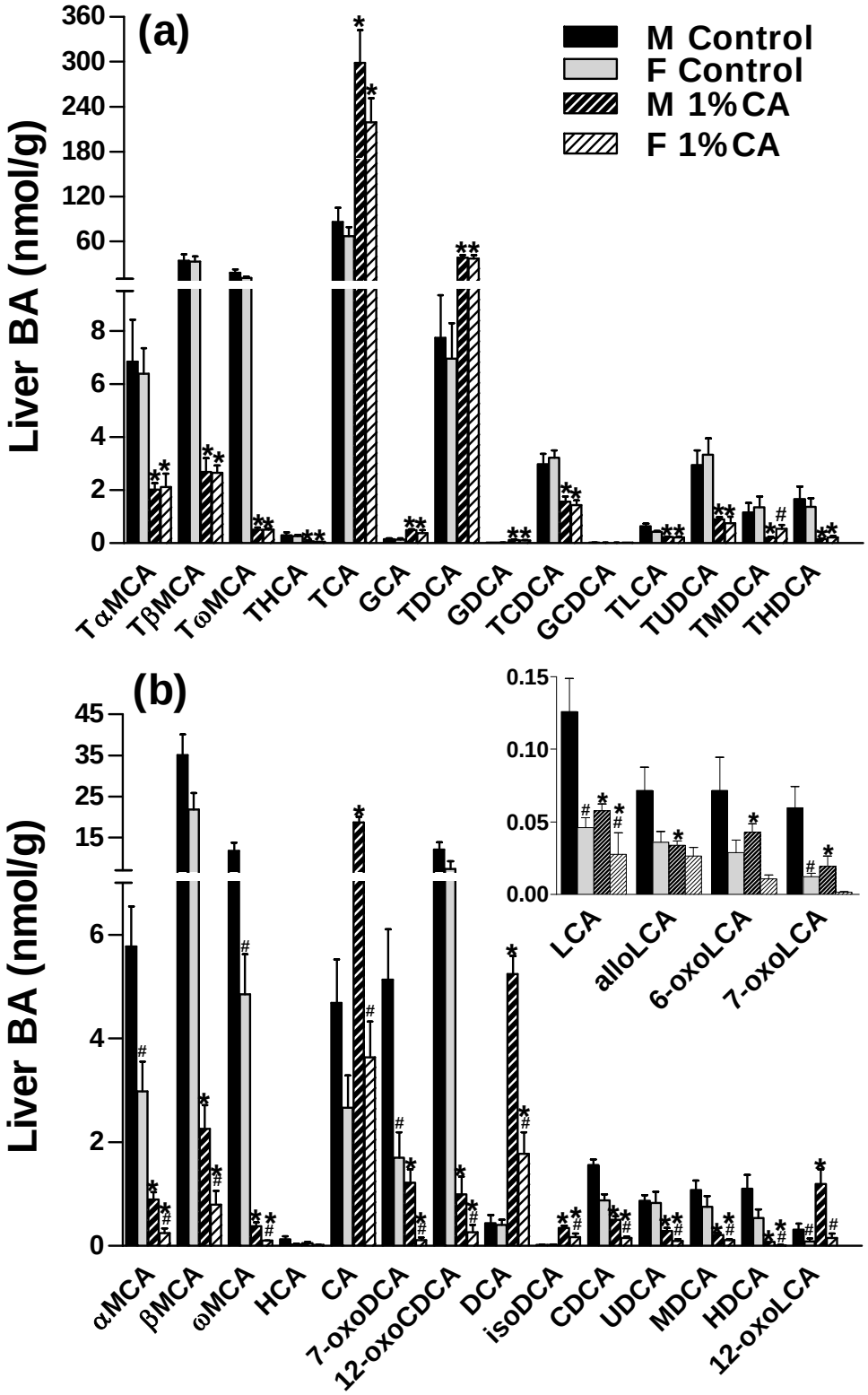

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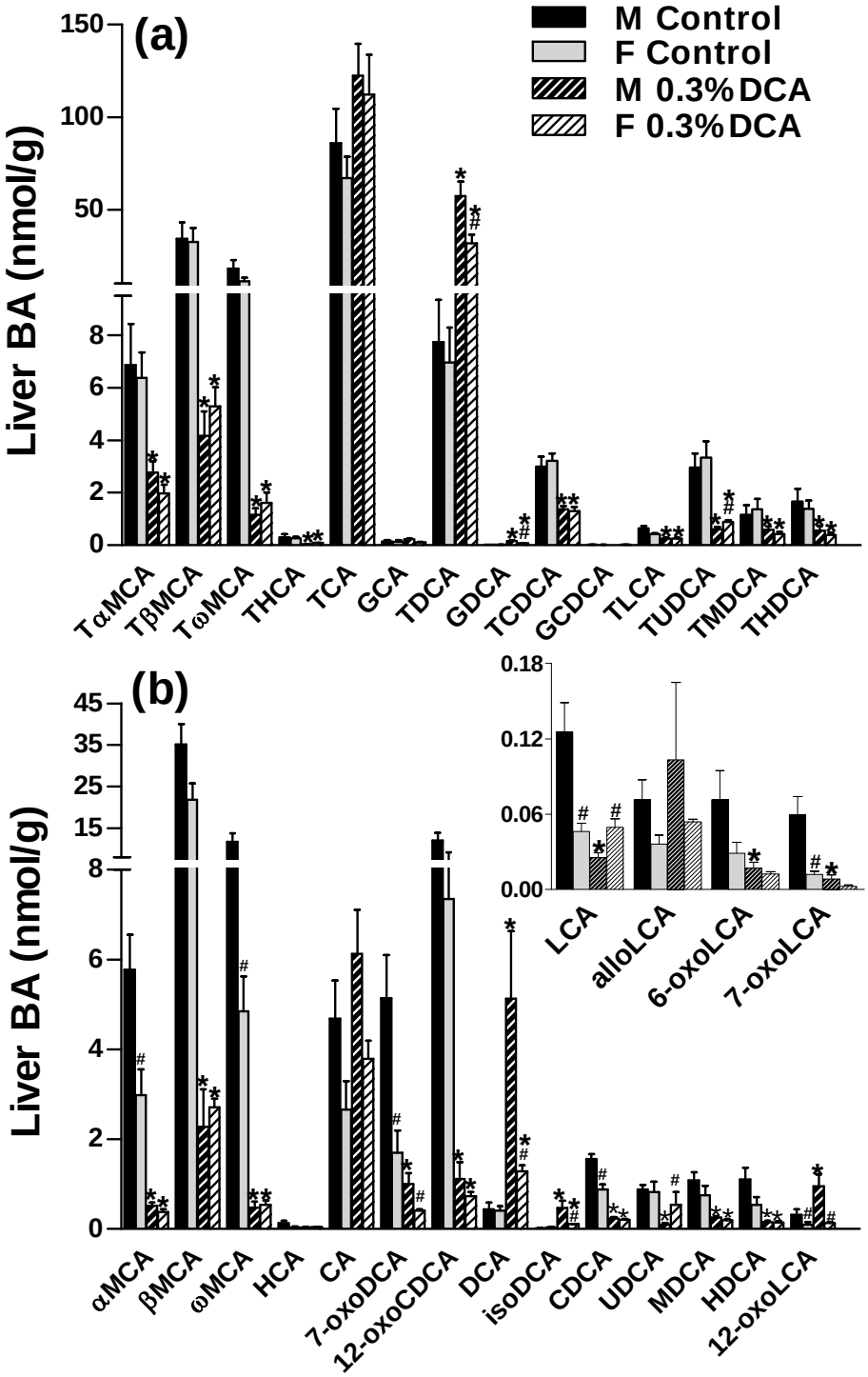

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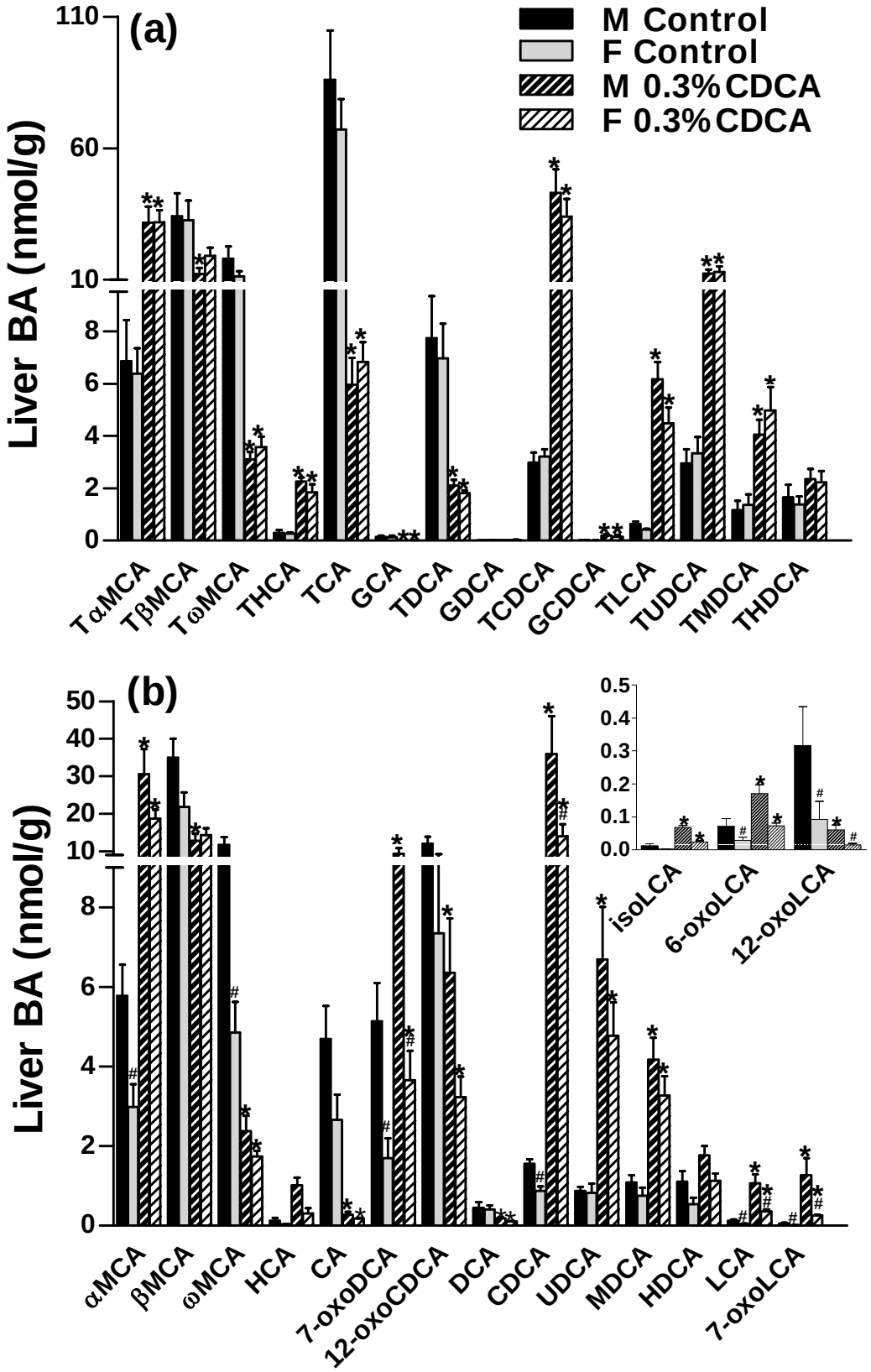

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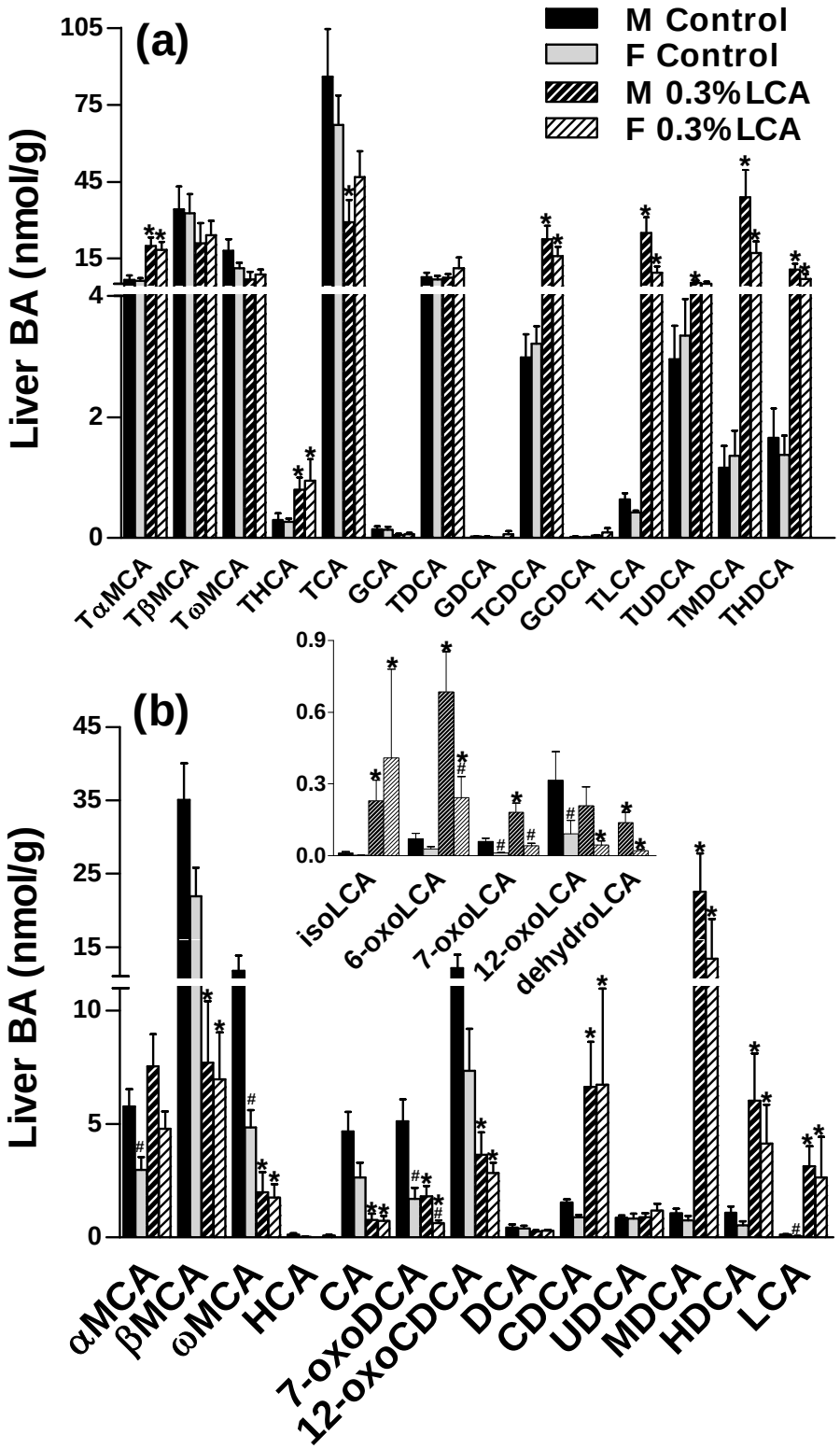

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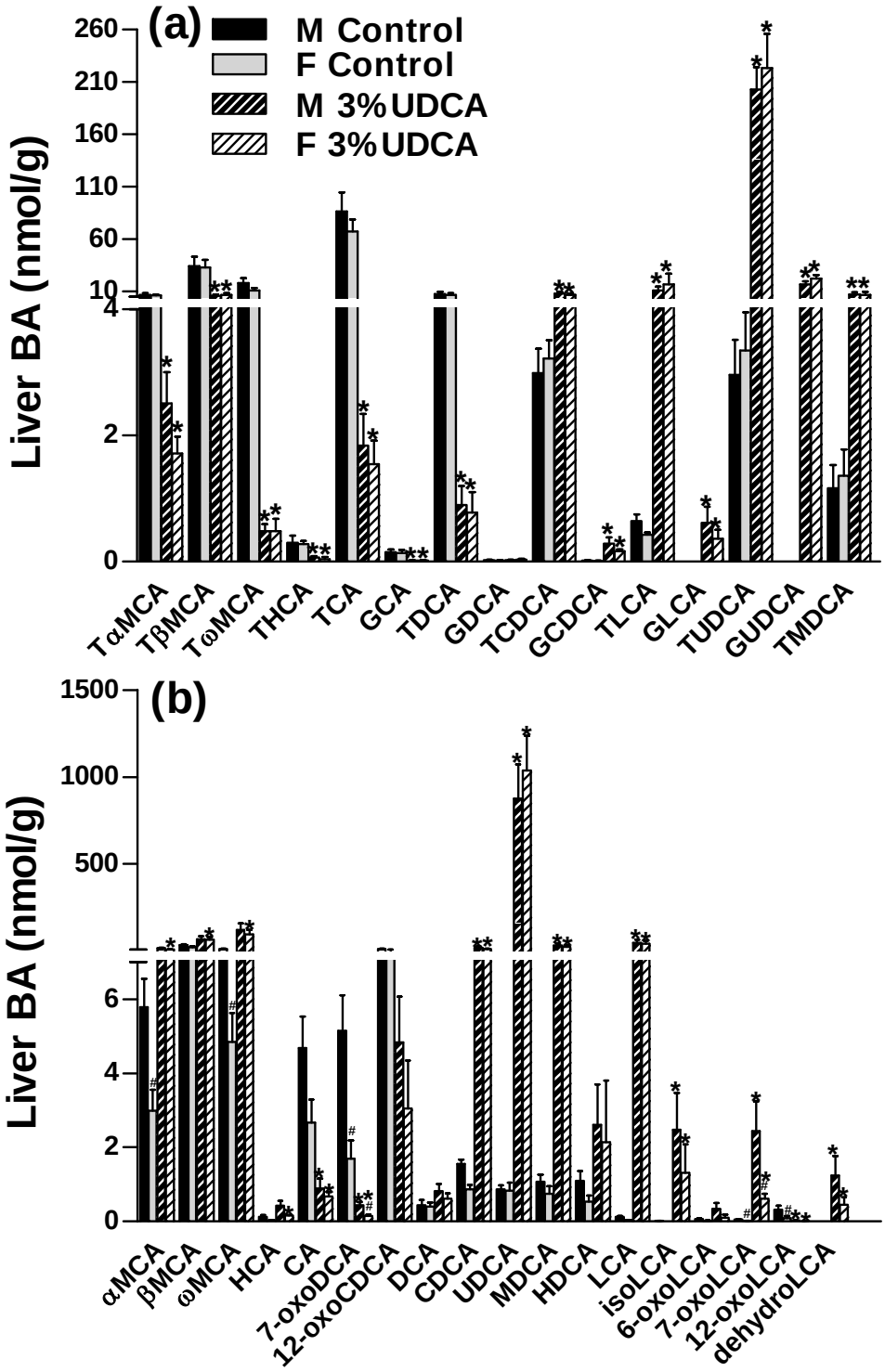

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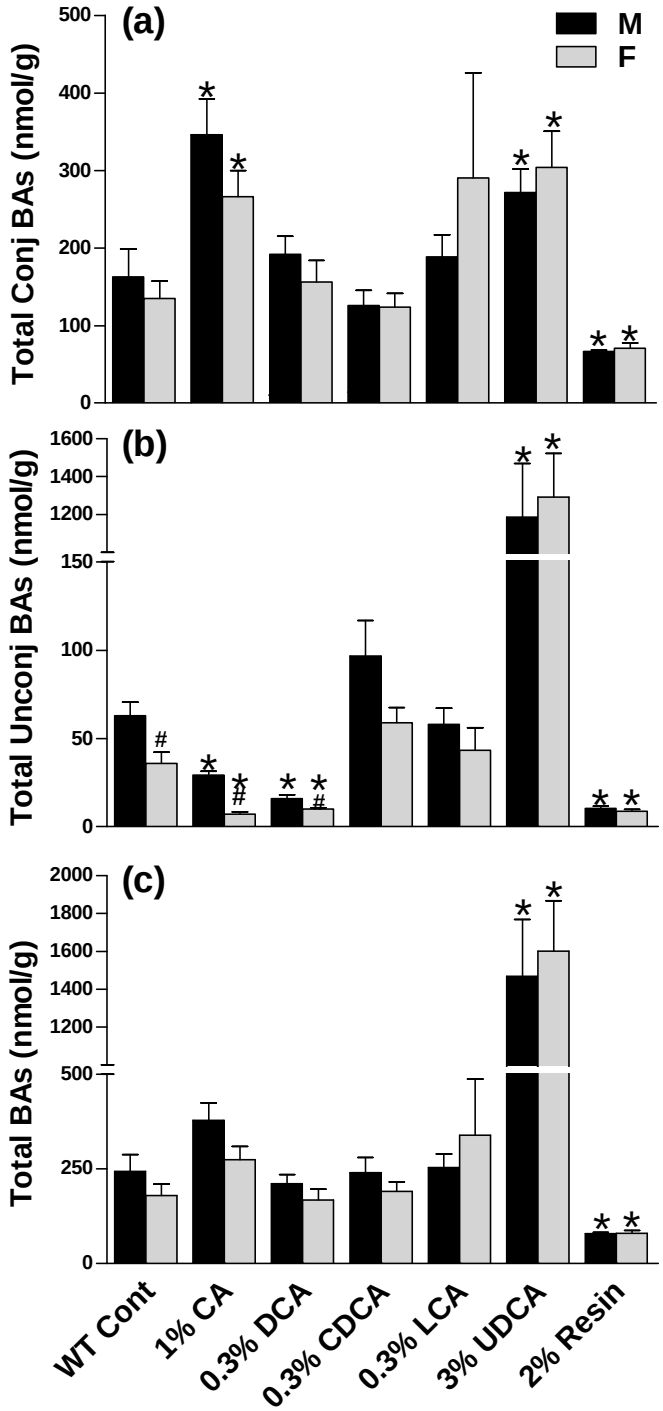

Supplemental figure 12