Electronic Supplementary Material (ESI) for Analytical Methods.

SUPPORTING INFORMATION

Optimisation of *in vitro* sample preparation for LC-MS metabolomics applications on HepaRG cell cultures

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SI-1 Seeding and cultivation of cell material

Undifferentiated HepaRG cells were cultivated to confluence in cell flasks (75 cm^2). Culture medium was composed of Williams E medium, glutamic acid (2 mM), Foetal Bovine Serum (10 %), penicillin/streptogramin (10 000 IU/mL; 10 000 µg/mL), hydrocortisone (0.05 mM), insulin (5 µg/mL). Cells were cultivated at 37 °C and 5 % CO_2, the medium was renewed every two days until the cells reached confluence.

Splitting procedure was as followed: the cells were detached by rinsing them three times with PBS before using 3 mL Tripl-E for 3 min. The activity of Tripl-E was quenched using 10 mL of fresh medium. Cells were centrifuged at 257 g for 5 min and the cell pellet was reconstituted in fresh medium to a) 0.2 x 10^6 cells/ mL when they were transferred to new flasks for further cultivation, or b) to 0.5 x 10^6 cells/cm^2, when they were seeded in wells for extraction. Extraction wells were further cultivated for one week until they reached confluence.

For the confirmation of our optimisation experiments, the differentiated cells were seeded with General Hepatic Medium and Thaw and Seed supplement. The cells were seeded in chamber slides with 1.5 ml medium at a concentration of 0.6 x 10^6 cells/mL. Medium was renewed the next day, after 3 and after 5 days of seeding with General hepatic medium and maintenance and metabolism supplement. After seven days, the cells were ready for extraction.

SI-2 Sample preparation

SI-2.1 Extraction

SI-2.1.1.1 Original protocol

Prior to extraction, cells were washed twice with PBS. The PBS was aspirated and cell metabolism was quenched using liquid nitrogen. Cells were detached using 300 µL of a cooled (-80 °C) 80 % (*v/v*) MeOH/water solution. A scraper was used to physically detach the cells from the well bottom, the liquid was recovered in the extraction vial. Afterwards, the well was rinsed with another 300 µL of the 80 % MeOH solution to recover potential remnants.

SI-2.1.1.2 Sonication

Cells were washed twice with PBS. The PBS was aspirated and cell metabolism was quenched using liquid nitrogen. Metabolism was fully quenched using 300 µL of a cooled (-80 °C) 80 % (*v/v*) MeOH/water solution. The cells were sonicated in this solution for 90 s using a Branson 550 sonicator (Branson, Danbury, USA). The extract was recovered and the cells were rinsed another time with 300 µL of the 80 % MeOH solution.

SI-2.1.1.3 Chamber slides

A pincer was put on the edge of the carrier glass. The wall and silicon of the carrier glass was manually removed and the cells were quickly submerged consecutively in two glasses of PBS (37 °C). Afterwards the carrier glass was flash frozen in liquid nitrogen. Metabolism was quenched with 200 µL of a cooled (- 80 °C) 80 % (*v/v*) MeOH/milliQ water solution. The liquid was recovered and another 200 µL was used to scrape all cells from the carrier glass. Finally, another 200 µL of the quenching solution was used to rinse the carrier and the scraper. All liquids were recovered into the LLE-vial

SI-2.1.2 Liquid-liquid extraction

SI-2.1.2.1 Original method

The extract was transferred to an extraction vial containing 420 µL $CHCl_3$ and 500 µL water. The well was rinsed with another 300 µL of the cooled MeOH/water mixture, which was also transferred to the extraction vial, resulting in a total of 420 µL chloroform, 620 µL water and 480 µL MeOH (2/3/2 $CHCl_3/H_2O$/MeOH). Liquid-liquid extraction was performed by vortexing, followed by and centrifugation at 2200 g for 7 min resulting in a polar upper phase and a non-polar lower phase.

SI-2.1.2.2 Adaptations

Two experiments were performed changing the composition of the extractions solvents.
In the first experiment, the use of a buffer was investigated by adding NH_4Ac to the water fraction in a concentration of 10 mM or 50 mM (pH 6.7). In a second experiment, the use of antioxidants (vitamin C and BHT) at 1 mM and chelator ($(NH_4)_2EDTA$) at 0.5 mM was evaluated. One group was treated with the 10 mM acetate buffer solution, the second and third group were treated with one additive only (antioxidants or chelator, respectively). The fourth group was treated with a combination of antioxidants and a chelator, evaluating potential synergistic effects.

SI-2.1.3 recovery, evaporation and reconstitution

From the non-polar phase, 250 µL was collected, evaporated to dryness under nitrogen, and stored at -80 °C prior to analysis. In later experiments, 240 µL was collected, two times 20 µL was pooled in QC-pools and the remaining 200 µL was divided in two separate vials to prevent an extra freeze thaw cycle. Samples were reconstituted in 50 µL of 35/65 (*v/v*) IPA/MeOH.
For the polar fraction, two times 400 µL was recovered. From each vial, 40 µL aliquots were pooled together in a QC pool. For the HILIC analysis, the first polar extracts were reconstituted in 40 µL 60 % (*v/*v) ACN/milliQ water. The second polar cell extracts were reconstituted in 20 % (*v/v*) MeOH/milliQ water. All samples were filtered over a 0.22 µm cartridge (VWR, USA) at 14 000 g for 2 min using a Microfuge® Centrifuge (Beckman Coulter, Brea, USA).

SI-2.2 MS-maintenance

Calibration and tuning were performed using a reference solution which contained six calibrant masses with *m/z* over a range of 100 to 1700. During the analysis, reference *m/z* 112.9856 and 980.0153 were used in negative ionisation mode, and *m/z* and 121.0509 and 922.0098 in positive ionisation mode to recalibrate the Q-TOF MS during analysis. The source was not cleaned within an analytical batch; cleaning was performed between the analytical runs (e.g. when changing column or ionisation mode)

SI-3 Analytical methods

SI-3.1 Non-polar fraction, positive ionisation mode

Separation was performed on a Kinetex XB-C18 (150 x 2.1 mm; 1.7 µm particle size, Phenomenex, Utrecht, the Netherlands) RP-column. A buffer of 5 mM NH_4AC and 0.1 % (*v/v*) HAc in milliQ water (pH 4.2) was prepared to stabilise the pH of the mobile phases. Mobile phase A was composed of 1/1 ACN/buffer, mobile phase B was made of 2/10/88 buffer/ACN/IPA. A gradient elution with following program was performed: 55 % B for 1 min, increasing to 70 % at 5 min. The percentage of B was further increased to 98 % at 25 min and kept for a 4 min rinse at 100 % B to complete the analytical separation. The column was re-equilibrated for 9 min, the flow rate was kept constant at 0.25 mL/min. Column temperature was 55 °C.

MS conditions were constant during the entire run: drying gas temperature and flow were 325 °C and 8 L/min respectively. Capillary, nozzle and fragmentor voltage were 3500 V, 500 V and 175 V, respectively.

SI-3.2 Non-polar fraction, negative ionisation mode

Separation was performed on a Kinetex XB-C18 (150 x 2.1 mm; 1.7 µm particle size) RP-column. Mobile phase A was composed of 1/1 MeOH and 10 mM NH_4AC in milliQ water (pH 6.7), mobile phase B was made of 2/10/88 10mM NH_4Ac in milliQ water/MeOH/IPA. A gradient elution with following program was performed: 55 % B for 1 min, increasing to 70 % B at 5 min. Afterwards, percentage of B was increased to 91 % at 20 min and 6 min rinse at 100 % B to complete the analytical separation, the column was re-equilibrated for 9 min. The flow rate was 0.25 mL/min. The column was heated to 55 °C.

MS source conditions were constant during the entire run: drying gas temperature and flow were 350 °C and 8 L/min respectively. Capillary, nozzle and fragmentor voltage were 3 750 V, 0 V and 175 V, respectively. After 21 min, the capillary and nozzle voltage were slightly altered to 3 500 V and 500 V, respectively.

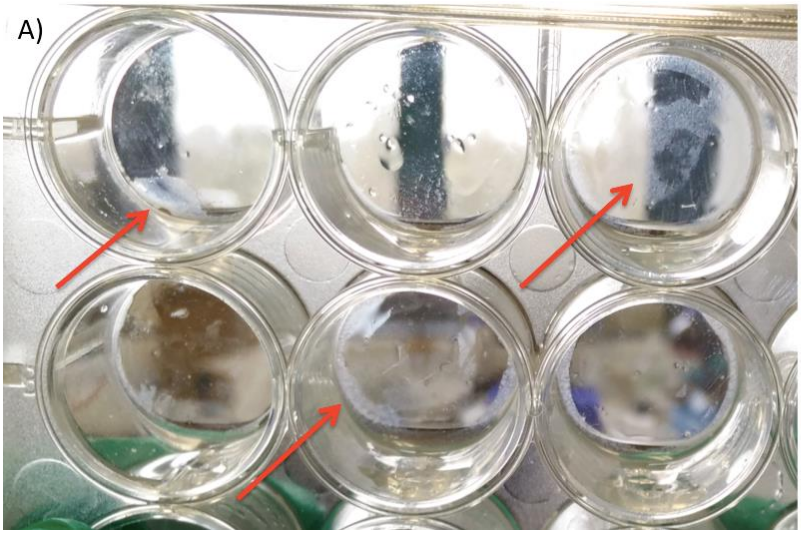
SI-3.3 Polar fraction, positive ionisation mode

An iHILIC Fusion (100 x 2.1 mm; 1.8 µm particle size, HILICON, Umea, Sweden) column was used in the final method. Mobile phase A was made of 10 mM NH4F and 0.1 % FA (*v/v*) (pH 3.15) and mobile phase B was ACN/MeOH (98/2 *v/v*), flow rate was 0.3 mL/min. A gradient elution started with 95 % B. After 2 min, the percentage of B decreased linearly to 65 % at 8 min, and decreased further to 25 % at 13 min. A rinse was performed with 25 % of B for 2 min and the column was re-equilibrated for 6 min. Column was heated to 30 °C to prevent temperature related changes in chromatography. MS conditions were constant throughout the run. Drying gas temperature and flow rate were 250 °C and 8 L/min. Sheet gas was 350 °C at 11 L/min. Capillary and fragmentor voltages were 2 000 V and 150 V, respectively. The nebulizer was set at 45 psig.

SI-3.4 Polar fraction, negative ionisation mode

A Gemini® Phenyl-hexyl (150 x 2 mm, 3 µm particle size, Phenomenex) was used in the final method. An isocratic pump with isolated tubing prevented contamination of pairing agents on the conventional hardware. The mobile phase consisted of 25 % MeOH with 10 mM TBA and 0.02 % FA (*v/v*) (pH = 9) and was pumped through the column at a flow rate of 0.2 mL/min. Runtime was set at 16 min. Drying gas temperature and flow rate were 250 °C and 10 L/min. Sheet gas was 250 °C at 10 L/min. Capillary and fragmentor voltages were 2 000 V and 100 V, respectively. The nebulizer was set at 45 psig.

A)



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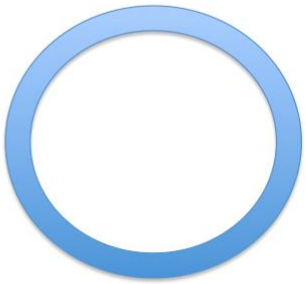
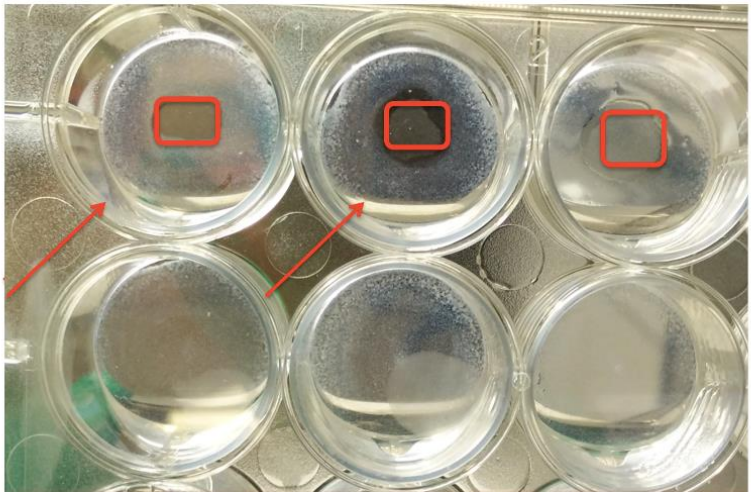


Figure SI-1: A) extraction efficiency with the original protocol. Arrows show zones where low scraping efficiency was noticed. B) Schematic overview of cell extraction efficiency.

A)



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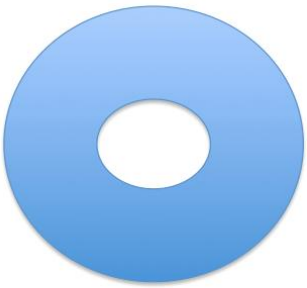

Figure SI-2: A) Extraction efficiency during sonication. Red circled zones are fully extracted; blurred surfaces in the well are cells still present in the well. B) Schematic overview of cell extraction efficiency.

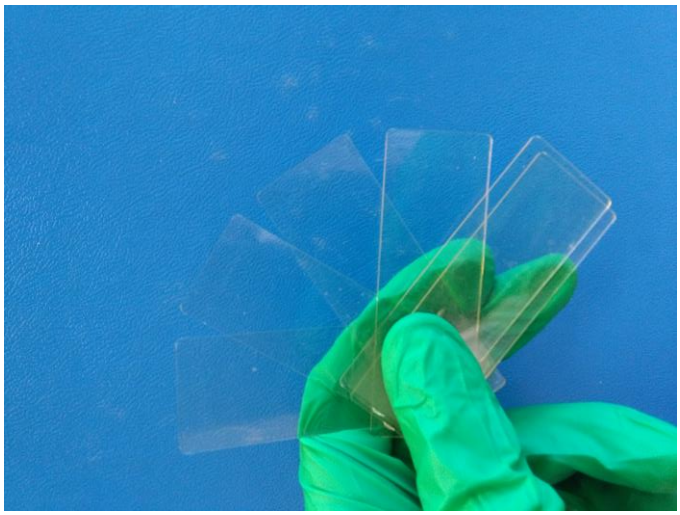

Figure SI-3: Cell extraction on chamber slides: no cell remnants were present after extraction.

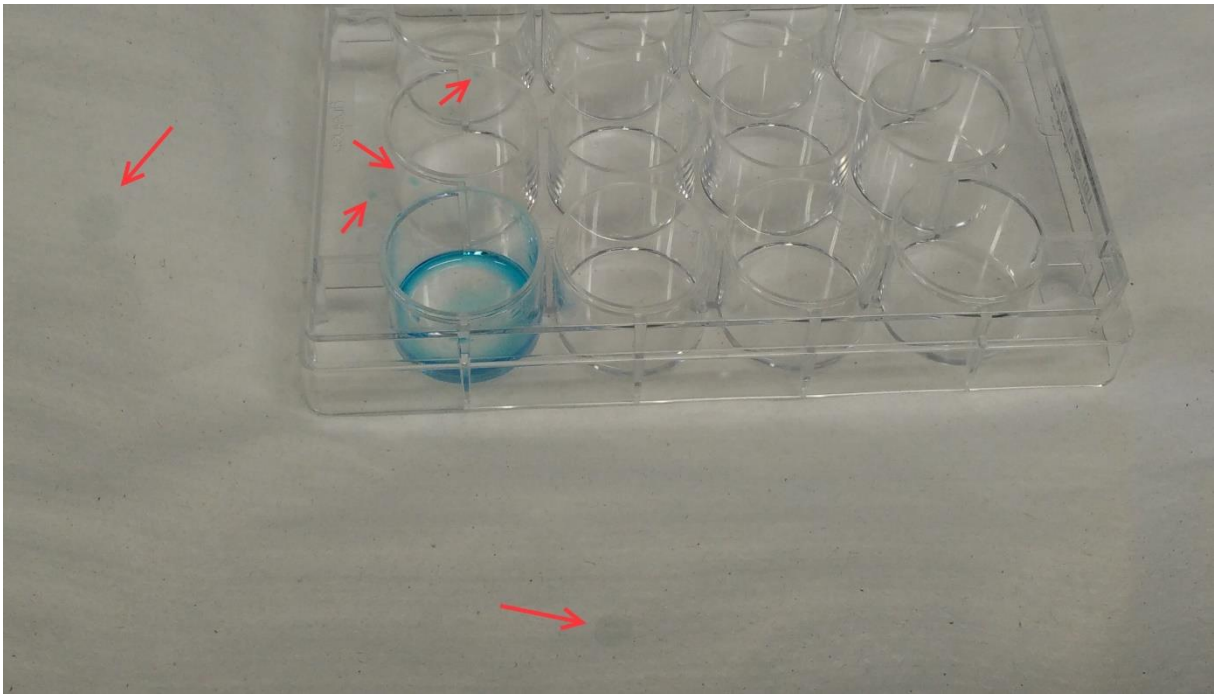

Figure SI-4: Spills during processing of a well. The red arrows show zones were spills had been detected after scraping of the well.

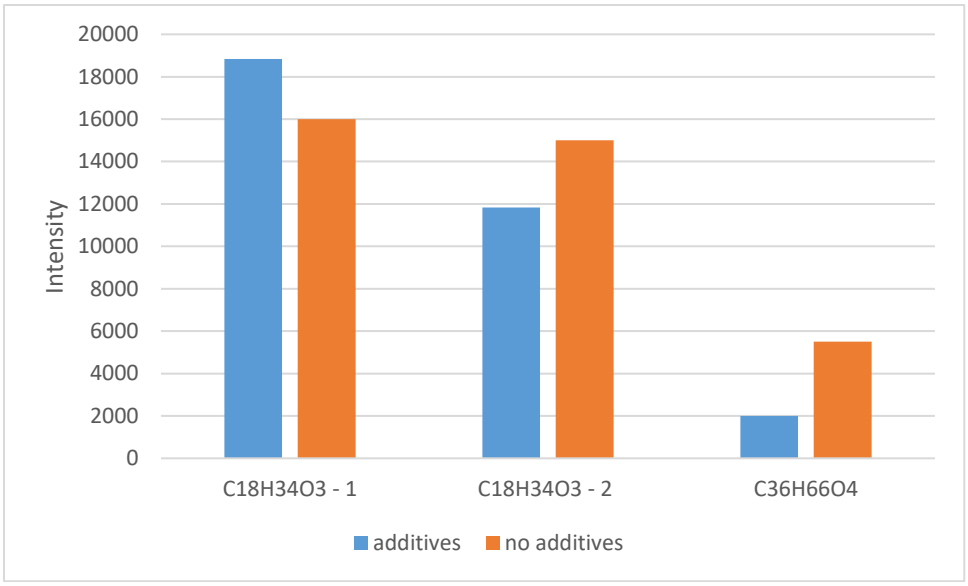

Figure SI-5: Degradation products of Oleic acid when the samples were processed with and without additives.

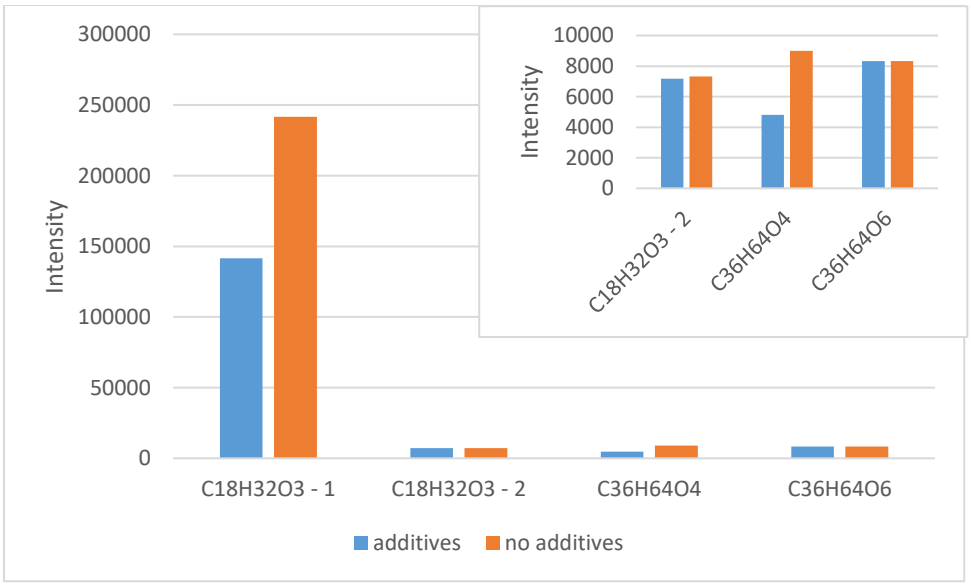

Figure SI-6: Degradation products of Linoleic acid when the samples were processed with and without additives.

Table SI-1: RSD values for the internal standards detected in the non-polar fraction, positive ionisation mode after LLE with different buffer concentrations.

		RSD (%)		
Compound	Formula	10 mM buffer	50 mM buffer	no buffer
Lyso PC (17:0)	C25H52NO7P	33.4	26.5	28.1
PC (17:0/17:0)	C42H84NO8P	49.3	30.2	24.2
Ceramide (17:0)	C35 H69NO3	45.0	29.8	27.5
Cholic acid-d4	C24D4H36O5	37.4	31.8	31.1
TG (3x $^{12}C_{15}{}^{13}C$:0)	[13C]3C48H96O6	31.9	26.7	11.6

Table SI-2: RSD values for the internal standards detected in the non-polar fraction, negative ionisation mode after LLE with different buffer concentrations.

		RSD (%)		
Compound	Formula	10 mM buffer	50 mM buffer	no buffer
Ceramide (17:0)	C35 H69NO3	10.5	9.6	22.0
Misoprostol	C22H38O5	20.9	17.4	32.7
Cholic acid-d4	C24D4H36O5	22.9	28.8	38.6
Lauric acid-d3	C12H21D3O2	11.3	15.8	25.9

Table SI-3: RSD values for the internal standards detected in the non-polar fraction, positive ionisation mode after LLE with different additives. (AOX = antioxidants)

		RSD (%)			
Compound	Formula	10 mM buffer	AOX	EDTA	AOX + EDTA
Lyso PC (17:0)	C25H52NO7P	34.1	139.8	61.7	33.0
PC (17:0/17:0)	C42H84NO8P	67.5	25.3	73.2	28.5
Ceramide (17:0)	C35 H69NO3	36.1	13.6	68.9	28.0
Cholic acid-d4	C24D4H36O5	52.2	160.6	146.3	44.7
TG (3x $^{12}C_{15}$ ^{13}C:0)	[13C]3C48H96O6	44.4	31.7	70.1	31.8

Table SI-4: RSD values for the internal standards detected in the non-polar fraction, negative ionisation mode after LLE with different additives. (AOX = antioxidants)

		RSD (%)			
Compound	Formula	10 mM buffer	AOX	EDTA	AOX + EDTA
Ceramide (17:0)	C35 H69NO3	36.6	17.6	34.7	14.7
Misoprostol	C22H38O5	37.8	61.6	53.8	26.7
Cholic acid-d4	C24D4H36O5	35.8	26.6	31.1	21.7
Lauric acid-d3	C12H21D3O2	42.2	33.1	41.3	21.6

Table SI-5: RSD values for the internal standards detected in the polar fraction, positive ionisation mode after LLE with different additives. (AOX = antioxidants)

		RSD (%)			
Compound	Formula	10 mM buffer	AOX	EDTA	AOX + EDTA
Caffeine	C8H10N4O2	11.2	26.4	4.7	11.8
Lysine- $^{13}C_6$ $^{15}N_2$	[13C]6H14[15N]2O2	7.8	26.8	18.6	20.1
Tryptophane-d5	C11D5H7N2O2	22.4	44.8	16.9	15.2
Leucine-d3	C6D3H10O2N	18.6	38.5	17.4	26.5