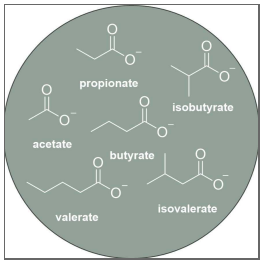


DEC 15, 2023

OPEN ACCESS

**DOI:**
dx.doi.org/10.17504/protocols.io.e6nvwddj7lmk/v1

**Protocol Citation:** Margaux Billen, Scott G Denham, Joanna P Simpson, Natalie ZM Homer 2023. Targeted analysis of Short Chain Fatty Acids (SCFAs) in human serum using derivatization and LC-HRMS analysis. **protocols.io** https://dx.doi.org/10.17504/protocols.io.e6nvwddj7lmk/v1



**Created:** Aug 16, 2023

**Last Modified:** Dec 15, 2023

**PROTOCOL integer ID:** 86538

**Keywords:** Short Chain Fatty Acids, High Resolution Mass Spectrometry, SCFA profile, LC-MS method, SCFA derivatisation, ThermoFisher Exploris 240 Orbitrap, Human serum

**Funders Acknowledgement:**
Fonds Wetenschappelijk Onderzoek (FWO) Vlaanderen
Grant ID: V424223N
Biotechnology and Biological Sciences Research Council
Grant ID: BB/V019066/1

# Targeted analysis of Short Chain Fatty Acids (SCFAs) in human serum using derivatization and LC-HRMS analysis

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Margaux Billen

ABSTRACT

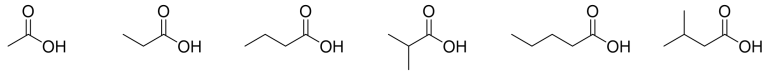
This protocol describes the sample preparation and analysis of short chain fatty acids in human serum samples. Short chain fatty acids (SCFAs) - acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid and 2-isobutoxyacetic acid (internal standard) were derivitised using 3-nitrophenylhydrazine (3-NPH). The derivatives were analysed by liquid chromatography high-resolution mass spectrometry (LC-HRMS).

A targeted LC-MS method was developed to measure six different SCFAs, by utilising approaches adapted from different published methods. These included Dei Cas et al, 2020 who profiled short and medium chain fatty acids following derivatisation (Dei Cas et al, 2020), Liao et al, 2021 who profiled kynurenine metabolites, short chain fatty acids and bile acids in samples following NPH derivatisation, and Nagatomo et al, 2022 who developed a method for application to plasma and tissue from a mouse model to profile SCFAs.

Short chain fatty acids were extracted from human serum using protein precipitation. Sample extracts were then derivatised alongside a calibration curve. Analysis of the derivatised samples was carried out by liquid chromatography high resolution mass spectrometry (LC-HRMS) in full scan negative mode on a ThermoScientific Exploris 240 Orbitrap. The amount of each analyte in each sample was calculated using linear regression of the peak area ratio of the analytes to the internal standard.

Our developed method used only 50 µL of serum with limits of detection of acetic acid at 1 ug/mL, 200 ng/mL for propionic acid and butyric, 20 ng/mL for isobutyric acid, isovaleric acid and valeric acid. Due to the low mass of the analytes in this method we used high resolution mass spectrometry in full scan mode as an alternative to triple quadrupole mass spectrometry which utilises fragmentation of analytes and multiple reaction monitoring. Sensitivity obtained using this HRMS method was found to be comparable to published literature.

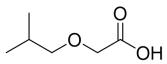
## Analytes



**Acetic acid** **Propionic acid** **Butyric acid** **Isobutyric acid** **Valeric acid** **Isovaleric acid**

Short chain fatty acids (SCFAs) analysed using this protocol.

## Internal standard



**2-isobutoxyacetic acid**

The internal standard used in this project is the SCFA-analogue 2-isobutoxyacetic acid.

## Analyte information

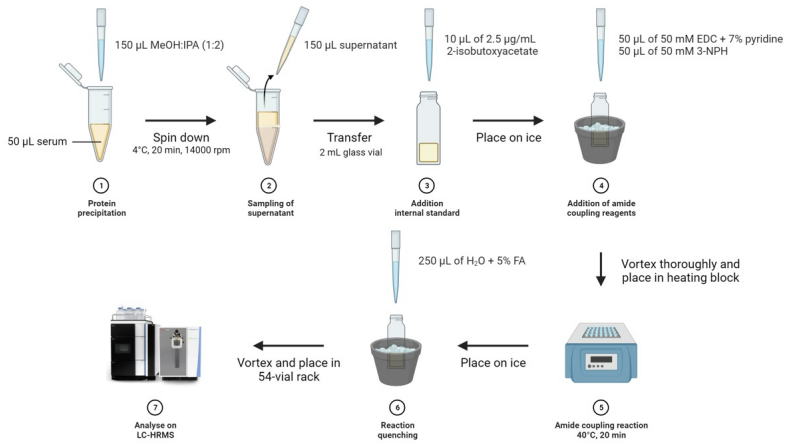
| | Name | Abbreviation | Chemical Formula | Monoisotopic mass (g/mol) | NPH derivatised chemical formula | Monoisotopic Mass - NPH (g/mol) |
|---|---|---|---|---|---|---|
| | Acetic acid | AA | C2H4O2 | 60.05 | C8H9N3O3 | 194.06 |

| | Name | Abbreviation | Chemical Formula | Monoisotopic mass (g/mol) | NPH derivatised chemical formula | Monoisotopic Mass - NPH (g/mol) |
|---|---|---|---|---|---|---|
| | Propionic acid | PA | C3H6O2 | 74.08 | C9H11N3O3 | 208.07 |
| | Butyric acid | BA | C4H8O2 | 88.11 | C10H13N3O3 | 222.09 |
| | Isobutyric acid | isoBA | C4H8O2 | 88.11 | C10H13N3O3 | 222.09 |
| | Valeric acid | VA | C5H10O2 | 102.13 | C11H15N3O3 | 236.10 |
| | Isovaleric acid | isoVA | C5H10O2 | 102.13 | C11H15N3O3 | 236.10 |
| | 2-isobutoxyacetic acid | / | C6H12O3 | 132.08 | C12H17N3O4 | 267.12 |

**Derivatisation reaction**


Hydrazide coupling used for SCFA derivatisation. 50mM of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) + 7% pyridine and 50mM of 3-nitrophenylhydrazine (3-NPH) in acetonitrile:H2O (1:1) are added to 50 µL of human serum.

**Derivatisation protocol overview**




Workflow for sample preparation (50 µL serum), derivatisation and analysis of short chain fatty acids in human serum samples.

## GUIDELINES

Ensure all training is up-to-date for operating the necessary lab equipment.

## MATERIALS

**Consumables**

| | Item | Supplier | Part no. | Quantity |
|---|---|---|---|---|

| | Item | Supplier | Part no. | Quantity |
|---|---|---|---|---|
| | 1.75 mL glass vials with lids | Scientific Laboratory Supplies Ltd | TUB1200 | 10 |
| | 7 mL glass vials with lids | Scientific Laboratory Supplies Ltd | TUB1220 | 5 |
| | 28 mL tall form glass vials with lids | VWR | T008/04 | 2 |
| | TruView Total Recovery 2mL glass vials with screw cap | Waters | 186005663 CV | 54 |
| | 1.5 mL Microcentrifuge SafeLock Tubes | Eppendorf | STK-TUBE-035 | 54 |
| | Kinetex 2.6 um C18 50 x 2.1mm | Phenomenex | 00B-4462-AN | 1 |

**Chemicals**

| | Item | Supplier | Article no. |
|---|---|---|---|
| | Water (HPLC grade) | Fisher Scientific | C-10449380-X |
| | Acetonitrile (LC-MS grade) | VWR | 83640.320 |
| | Methanol (LC-MS grade) | VWR | 83638.320 |
| | Water (LC-MS grade) | VWR | 83645.320 |
| | Formic acid (LC-MS grade) | Fisher Scientific | 10596814 |
| | 2-Propanol (LC-MS grade) | VWR | 84881.320 |
| | Acetic acid (5 mL) | Sigma Aldrich | 71251-5ML-F |
| | Propionic acid (1 mL) | Sigma Aldrich | 94425-1ML-F |
| | Butyric acid (5 mL) | Sigma Aldrich | 19215-5ML |
| | Isobutyric acid (500 mg) | Sigma Aldrich | 46935-U |
| | Valeric acid (1 mL) | Sigma Aldrich | 75054-1ML |
| | Isovaleric acid (1 mL) | Sigma Aldrich | 78654-1ML |
| | 2-isobutoxyacetic acid | Sigma Aldrich | CDS014100-250MG |
| | Pyridine (LC-MS grade) | Fisher Scientific | 3951366 |
| | 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide | Sigma Aldrich | 341006 |
| | 3-Nitrophenylhydrazine hydrochloride (5 g) | Sigma Aldrich | N21804-5G |

**Equipment**

| | Item | Model | Supplier |
|---|---|---|---|
| | Dri-block | DB.3A | Techne |
| | Microtube centrifuge | 1-15 | Sigma |
| | Liquid Chromatography Pump | Vanquish uHPLC | Thermo |
| | Autosampler | Vanquish uHPLC | Thermo |
| | Column oven | Vanquish uHPLC | Thermo |
| | Mass spectrometer | Exploris Orbitrap 240 | Sciex |
| | Balance | PS-100 | Fisher Scientific |

SAFETY WARNINGS

Adhere to local lab rules.
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) is a highly toxic reagent, handle with care and follow all necessary safety rules.

ETHICS STATEMENT

When handling human clinical samples, ensure you are following local guidelines including adherence to Good Clinical Practice. In particular, ensure that samples are analysed without identifiable patient data.

## Solvent preparation

15m

1 **Prepare Mobile Phase A:** water + 0.1% Formic Acid
- Add 1 L of LC-MS grade water to a 1L glass bottle.
- Add 1 mL of LC-MS grade Formic Acid to the water.
- Mix thoroughly.

2 **Prepare Mobile Phase B:** Acetonitrile
- Add 1 L of LC-MS grade Acetonitrile to a 1L glass bottle.

3 **Prepare Autosampler Seal Wash:** 10% Acetonitrile
- Add 100 mL LC-MS grade Acetonitrile to 900 mL LC-MS grade water in a 1L glass bottle.
- Mix thoroughly.

## Derivatisation solution preparation

20m

4 Prepare all solution fresh each time the protocol is carried out.
All solutions (for derivatisation, internal standards and calibration standards) are stored at -20°C after preparation until use.

5 **Prepare derivatisation solution A: Acetonitrile:Water (50:50)**
- Add 10 mL of LC-MS grade water to a 28 mL glass vial labelled Acetonitrile:Water (50:50).
- Add 10 mL of LC-MS grade Acetonitrile.
- Mix thoroughly.

6 **Prepare derivatisation solution B: Acetonitrile:Water (50:50) + 7% pyridine**
- Add 8.6 mL of LC-MS grade water to a 28 mL glass vial labelled Acetonitrile:Water (50:50) + 7% pyidine.
- Add 1.4 mL of LC-MS grade pyridine to give a 14% pyridine in water solution.
- Dilute with 10 mL of LC-MS grade Acetonitrile to give a Acetonitrile:Water (50:50) + 7% pyridine solution.
- Mix thoroughly.

7 **Prepare derivatisation solution C: 50 mM 1-Ethyl-3(3dimethyl-aminopropyl)carbodiimide (EDC) + 7% pyridine.**
- Using the PS-100 balance, an appropriate amount of EDC was weighed into a 7 mL glass vial. **EDC is toxic and highly corrosive and so this was added to the 7 mL vial in the fume hood and transferred to the balance with the lid on the vial.**
- To this the correct amount of acetonitrile:water (50:50) + 7% pyridine was added to achieve a 50 mM EDC solution in 50:50 acetonitrile:water + 7% pyridine. **EDC has a molecular weight (MW) of 155.24 g/mol and so 7.72 mg is used per mL.**

8 **Prepare derivatisation solution D:** 50 mM 3-NPH (3-nitrophenylhydrazine)
- Using the PS-100 balance, an appropriate amount of 3-NPH was weighed into a 7 mL glass vial.
- To this the correct amount of acetonitrile:water (50:50) was added to achieve a 50 mM 3-NPH solution in 50:50 acetonitrile:water. **3-NPH has a MW of 189.60 g/mol and so 9.45 mg is used per mL.**

9 **Prepare quenching solution:** water + 5% Formic Acid

- Add 475 L of LC-MS grade water to a 500 mL glass bottle.
- Add 25 mL of LC-MS grade Formic Acid to the water.
- Mix thoroughly.

## Internal standard preparation

20m

10 Prepare Internal Standard stock solution:

**2-isobutoxyacetic acid stock solution (1 mg/mL)**
Add 500 µL of water (HPLC grade) to a manufacturer's vial of 0.5 mg 2-isobutoxyacetic acid and vortex thoroughly to give a 1 mg/mL 2-isobutoxyacetic acid stock solution.

11 Prepare Internal Standard mix dilution according to the table below:

- 1 x 28 mL glass vials labelled "2.5 µg/mL 2-isobutoxyacetic acid (IS) in water".

| Stock concentration | Amount of stock | Volume of water (µL) | Final volume (µL) |
|---|---|---|---|
| 2.5 µg/mL | 25 µL x 1 mg/mL of 2-isobutoxyacetic acid stock solution | 9975 | 10000 |

## Calibration standards mix preparation

30m

12 Prepare calibration standard stock solutions.

12.1 Label vials as follows:

- 6 x 7 mL glass vials labelled 1 mg/mL acetic acid (AA), propionic acid (PA), butyric acid (BA), isobutyric acid (isoBA), valeric acid (VA), isovaleric acid (isoVA)
- 5 x 1.75mL glass vials labelled: 100 µg/mL, 50 µg/mL, 5 µg/mL, 500 ng/mL and 50 ng/mL.

12.2 **SCFA stock solutions (1 mg/mL)**

The following standards were prepared as 1 mg/mL solutions in methanol. 5 µL of each standard was added to a glass 7 mL vial. As all of the standards are liquids, the density was used to account for the volume of methanol added to each standard and is shown below.

| Standard name | MW | Chemical Formula | Chemical formula-NPH | Density (g/ml) | Mass of std (mg) in 5 µL | Vol MeOH (µL) |
|---|---|---|---|---|---|---|
| Acetic acid | 60.05 | C2H4O2 | C8H9N3O3 | 1.05 | 5.25 | 5250 |
| Propionic acid | 74.08 | C3H6O2 | C9H11N3O3 | 0.99 | 4.95 | 4950 |
| Butyric acid | 88.11 | C4H8O2 | C10H13N3O3 | 0.96 | 4.80 | 4800 |
| Iso-butyric acid | 88.11 | C4H8O2 | C10H13N3O3 | 0.95 | 4.75 | 4750 |
| Valeric acid | 102.13 | C5H10O2 | C11H15N3O3 | 0.94 | 4.70 | 4700 |
| Iso-valeric acid | 102.13 | C5H10O2 | C11H15N3O3 | 0.93 | 4.65 | 4650 |

12.3 Prepare **Calibration Standard mix dilutions** according to the table:

**Standard mix dilutions:**

| Solution name / Concentration | Amount of stock | Vol water (µL) | Final vol (µL) |
|---|---|---|---|
| 100 µg/mL | 100 µL of 1 mg/mL AA<br>+ 100 µL of 1 mg/mL PA<br>+ 100 µL of 1 mg/mL BA<br>+ 100 µL of 1 mg/mL isoBA<br>+ 100 µL of 1 mg/mL VA<br>+ 100 µL of 1 mg/mL isoVA | 400 | 1000 |
| 50 µg/mL | 500 µL x 100 µg/mL **SCFAs mix** above | 500 | 1000 |
| 5 µg/mL | 100 µL x 50 µg/mL of **SCFAs mix** above | 900 | 1000 |
| 500 ng/mL | 100 µL x 5 µg/mL of **SCFAs mix** above | 900 | 1000 |
| 50 ng/mL | 100 µL x 5 µg/mL of **SCFAs mix** above | 900 | 1000 |

## Derivatisation procedure with NPH

2h

13 Prepare an electronic list of the samples to be analysed using this method. The sample list needs to include a unique ID, as well as recording all relevant experimental details.

14 Complete a 54-vial (Thermo Vial Rack) map for standards and samples (**make sure to place them column-wise**) using the design as shown. The number of samples that can be analysed per batch is 36, alongside a 15-point calibration curve.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| A | A1 Solvent blank (H2O) | A2 10.0 STD | A3 500.0 STD | A4 INSP002 | A5 INSP009 | A6 INSP017 | A7 INSP026 | A8 INSP034 | A9 INSP040 |
| B | B1 Reagent blank | B2 25.0 STD | B3 750.0 STD | B4 INSP003 | B5 INSP010 | B6 INSP019 | B7 INSP028 | B8 INSP035 | B9 INSP041 |
| C | C1 0 STD | C2 50.0 STD | C3 1000.0 STD | C4 INSP004 | C5 INSP011 | C6 INSP021 | C7 INSP029 | C8 INSP036 | C9 INSP042 |
| D | D1 1.00 STD | D2 75.0 STD | D3 2000.0 STD | D4 INSP006 | D5 INSP014 | D6 INSP023 | D7 INSP030 | D8 INSP037 | D9 INSP043 |
| E | E1 2.50 STD | E2 100.0 STD | E3 2500.0 STD | E4 INSP007 | E5 INSP015 | E6 INSP024 | E7 INSP032 | E8 INSP038 | E9 INSP044 |
| F | F1 5.00 STD | F2 250.0 STD | F3 Reagent blank | F4 INSP008 | F5 INSP016 | F6 INSP025 | F7 INSP033 | F8 INSP039 | F9 INSP045 |

15 Defrost calibration standard solutions, internal standard solutions and human serum samples.

16 Label 54 Eppendorf Microcentrifuge SafeLock Tubes (1.5 mL) according to the plate map.

17 Add water (LC-MS grade) to the Eppendorfs according to this guide:

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| A | A1 0 | A2 30 µL | A3 40 µL | A4 0 | A5 0 | A6 0 | A7 0 | A8 0 | A9 0 |
| B | B1 50 µL | B2 45 µL | B3 35 µL | B4 0 | B5 0 | B6 0 | B7 0 | B8 0 | B9 0 |
| C | C1 50 µL | C2 40 µL | C3 30 µL | C4 0 | C5 0 | C6 0 | C7 0 | C8 0 | C9 0 |
| D | D1 30 µL | D2 35 µL | D3 30 µL | D4 0 | D5 0 | D6 0 | D7 0 | D8 0 | D9 0 |
| E | E1 45 µL | E2 30 µL | E3 25 µL | E4 0 | E5 0 | E6 0 | E7 0 | E8 0 | E9 0 |
| F | F1 40 µL | F2 45 µL | F3 50 µL | F4 0 | F5 0 | F6 0 | F7 0 | F8 0 | F9 0 |

18 Add the required amount of standards to the Eppendorfs according to the table below. Due to small volumes being pipetted ensure that the standard is pipetted **into** the water.

| Standard name | Amount (ng) | Concentration (ng/mL) for 500 µL standard volume | INT STD vol (µL) | STD vol (µL) |
|---|---|---|---|---|
| 0 STD | 0 | 0 | 10 µL (x 2.5 µg/mL IS) | 0 |
| 1.00 STD | 1.00 | 2.00 | 10 µL (x 2.5 µg/mL IS) | 20 µL x 50 ng/mL |
| 2.50 STD | 2.50 | 5.00 | 10 µL (x 2.5 µg/mL IS) | 5 µL x 500 ng/mL |
| 5.00 STD | 5.00 | 10.0 | 10 µL (x 2.5 µg/mL IS) | 10 µL x 500 ng/mL |
| 10.0 STD | 10.0 | 20.0 | 10 µL (x 2.5 µg/mL IS) | 20 µL x 500 ng/mL |
| 25.0 STD | 25.0 | 50.0 | 10 µL (x 2.5 µg/mL IS) | 5 µL x 5 µg/mL |
| 50.0 STD | 50.0 | 100.0 | 10 µL (x 2.5 µg/mL IS) | 10 µL x 5 µg/mL |
| 75.0 STD | 75.0 | 150.0 | 10 µL (x 2.5 µg/mL IS) | 15 µL x 5 µg/mL |
| 100.0 STD | 100.0 | 200.0 | 10 µL (x 2.5 µg/mL IS) | 20 µL x 5 µg/mL |
| 250.0 STD | 250.0 | 500.0 | 10 µL (x 2.5 µg/mL IS) | 5 µL x 50 µg/mL |
| 500.0 STD | 500.0 | 1000.0 | 10 µL (x 2.5 µg/mL IS) | 10 µL x 50 µg/mL |
| 750.0 STD | 750.0 | 1500.0 | 10 µL (x 2.5 µg/mL IS) | 15 µL x 50 µg/mL |
| 1000.0 STD | 1000.0 | 2000.0 | 10 µL (x 2.5 µg/mL IS) | 20 µL x 50 µg/mL |
| 2000.0 STD | 2000.0 | 4000.0 | 10 µL (x 2.5 µg/mL IS) | 20 µL x 100 µg/mL |
| 2500.0 STD | 2500.0 | 5000.0 | 10 µL (x 2.5 µg/mL IS) | 25 µL x 100 µg/mL |

19 Add 50 µL of each serum sample into the appropriately labelled Eppendorfs.

20 Add 100 µL of ice-cold isopropanol and 50 µL of ice-cold methanol to all Eppendorfs, except for the solvent blank (A1). Vortex each Eppendorf tube.

21 Centrifuge the samples for 20 minutes at 14 000 RPM and 4°C.

22 **CAREFULLY** transfer 150 µL of the supernatant, without disturbing the pellet, of all Eppendorf tubes to TruView Total Recovery 2mL glass vials.

23 Add 10 µL of 2.5 µg/mL 2-isobutoxyacetic acid (IS) to each vial, except for the solvent blank (A1) and the reagent blanks (B1 and F3).

24 Place all vials on ice.

25 Add 50 µL of derivatisation solution C (50 mM EDC + 7% pyridine in 50:50 ACN:$H_2O$) to each vial, except for the solvent blank (A1).

26 Add 50 µL of derivatisation solution D (50 mM 3-NPH in 50:50 ACN:$H_2O$) to each vial, except for the solvent blank (A1).

27 Screw solid caps on all samples and vortex the solutions.

28 Place all vials in a heating block (Techne dri-block) at 40°C for 00:20:00.

29 After 20 minutes, put all vials back on ice.

30 Add 250 µL of the quenching solution (water + 5% Formic Acid) to each vial, except for the solvent blank (A1).

31 Vortex all samples, exchange the solid caps for caps with a septum.

32 Add 500 µL of LC-MS grade water to the solvent blank. Samples are ready for LC-MS analysis.

## Set up of SCFA LC-HRMS method and analysis

30m

33 Put the freshly prepared mobile phases onto the uHPLC system. Purge lines with mobile phase A and mobile phase B.

34 Install a **Kinetex 2.6 µm C18 (50 x 2.1 mm)** column into the column oven and set the column temperature to **50 °C.** Equilibrate at **90% mobile phase A**, **0.4 mL/min** for at least 15 minutes. Ensure that the pressure is stable and there are no leaks detectable on the system.

35 Create an **acquisition method** in Xcalibur for chromatography and mass spectrometry settings. For chromatography include the following chromatographic gradient conditions in table below.

Add the detail of the column and mobile phases in the method. Make sure the right column position is selected for the valves and the column oven temperature and column pre-heater are set to 50 °C.

| Time (min) | Flow (mL/min) | %A | %B | Curve |
|---|---|---|---|---|
| Initial | 0.400 | 90 | 10 | Initial |
| 2.50 | 0.400 | 90 | 10 | 5 |
| 10.0 | 0.400 | 70 | 30 | 5 |
| 10.5 | 0.400 | 0 | 100 | 5 |
| 12.5 | 0.400 | 0 | 100 | 5 |
| 13.0 | 0.400 | 90 | 10 | 5 |
| 15.0 | 0.400 | 90 | 10 | 5 |

**Chromatographic gradient** for separation of derivatized SCFAs in serum samples on a Kinetex 2.6 µm C18 50 x 2.1mm using a system of water + 0.1% Formic Acid (Mobile Phase A) and Acetonitrile (Mobile Phase B).

36 Add the following mass spectrometry method parameters to the acquisition method:

| A | B |
|---|---|
| **Instrument** | Thermo Exploris 240 Orbitrap |
| **Source, Ionisation Mode** | Thermo Scientific™ OptaMax™ NG ion source (H-ESI) |
| **Scan Mode, Polarity** | Full Scan, Negative |
| **Mass range** | 50-500 m/z |
| **Resolution** | 120 000 |
| **Acquisition time** | 15.0 min |
| **Sheath Gas** | 30 |
| **Aux Gas** | 5 |
| **Sweep Gas** | 1 |
| **IonSpray Voltage (IS) (Negative)** | -2500 V |
| **Ion Transfer Tube Temperature** | 300°C |
| **Vaporizer Temperature** | 450°C |
| **Probe position (x – axis)** | 2 |
| **Probe position (y – axis)** | 2 |

37 Place the Thermovial 54 rack with the samples into the autosampler of the chromatography system.

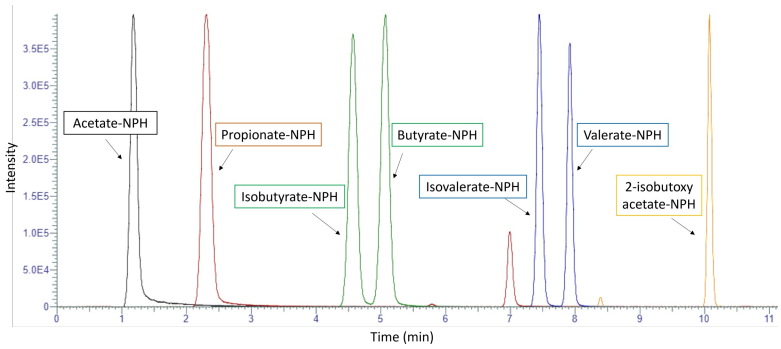
38 Create a batch in Xcalibur using the electronic plate map - use the correct position for the Thermovial 54 rack, the correct position of the column, the correct lines for the mobile phases and the correct LC-MS/MS method. Name and save the **Batch acquisition file.** Use the same naming convention to name the resulting **data file**.

39 Set volume of injection to **5** µL and submit batch to analyse.
Test the system with a mid-standard curve point injection and then complete the batch in order from A1 to F9.

40 Use the deprotonated molecular mass *m/z* $[M-H]^-$ for each compound in the table below to interrogate the data.

| | A | Acetate NPH | Propionate-NPH | Butyrate-NPH | Isobutyrate-NPH | Valerate-NPH | Isovalerate-NPH |
|---|---|---|---|---|---|---|---|
| | m/z [M-H]- | 194.0571 | 208.0728 | 222.0883 | 222.0883 | 236.1039 | 236.1039 |
| | Retention time (min) | 1.18 | 2.30 | 5.07 | 4.57 | 7.92 | 7.45 |

41 Typical chromatography of NPH-derivatised acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid and 2-isobutoxyacetic acid separation is shown below. Separation performed on a Kinetex 2.6 µm C18 50 x 2.1mm using a system of water + 0.1% Formic Acid (Mobile Phase A) and Acetonitrile (Mobile Phase B).




**Chromatographic Separation** of Acetic acid-NPH (1.18 mins), Propionic acid-NPH (2.30 mins), Butyric acid-NPH (5.07 mins), Isobutyric acid-NPH (4.57 mins), Valeric acid-NPH (7.92 mins), Isovaleric acid-NPH (7.45 mins) and 2-isobutoxyacetic acid-NPH (10.07 mins) on a Kinetex 2.6 µm C18 50 x 2.1mm using a system of water + 0.1% Formic Acid (Mobile Phase A) and Acetonitrile (Mobile Phase B). Flow rate 0.4 mL/min, 50 °C and a gradient elution over 15 minutes.

## Data analysis using TraceFinder software

2h

42 Use this protocol to evaluate the data and obtain the SCFA profile of the samples analysed:
Margaux Billen, Scott G Denham, Joanna P Simpson, Natalie ZM Homer 2023. Using TraceFinder and Excel software to evaluate and report multi-analyte targeted LC-MS data acquired on an ThermoScientific Exploris 240 Orbitrap. **protocols.io** https://dx.doi.org/10.17504/protocols.io.n92ldm8z7l5b/v1