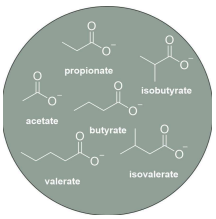
Dec 15, 2023

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



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We use this protocol and it's working

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Abstract

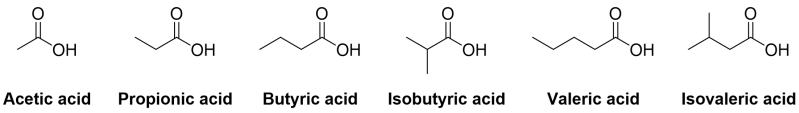
This protocol describes the sample preparation and analysis of short chain fatty acids in human serum samples. Short chain fatty acids (SCFAs) - acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid and 2-isobutoxyacetic acid (internal standard) were derivitised using 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.

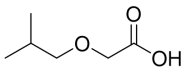
Our developed method used only 50 µL of serum with limits of detection of acetic acid at 1 ug/mL, 200 ng/mL for propionic acid and butyric, 20 ng/mL for isobutyric acid, isovaleric acid and valeric acid. Due to the low mass of the analytes in this method we used high resolution mass spectrometry in full 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



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

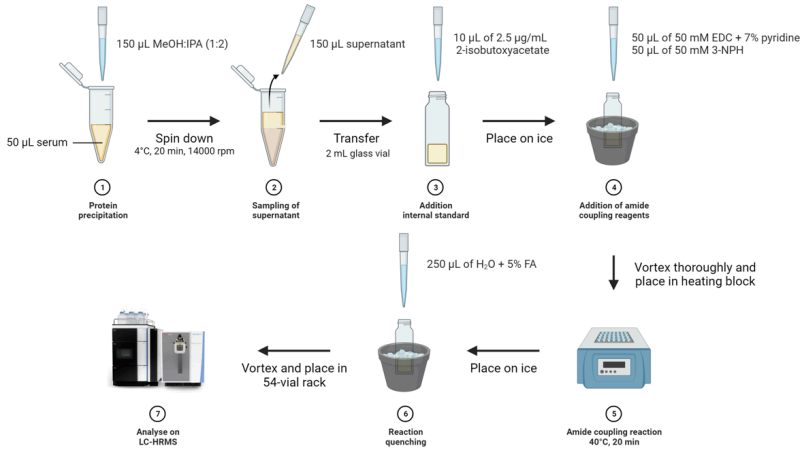
	Name	Abbreviation	Chemical Formula	Monoisotopic mass (g/mol)	NPH derivatised chemical formula	Monoisotopic Mass - NPH (g/mol)
	Acetic acid	AA	C2H4O2	60.05	C8H9N3O3	194.06
	Propionic acid	PA	C3H6O2	74.08	C9H11N3O3	208.07
	Butyric acid	BA	C4H8O2	88.11	C10H13N3O3	222.09
	Isobutyric	isoBA	C4H8O2	88.11	C10H13N3O3	222.09

	Name	Abbreviation	Chemical Formula	Monoisotopic mass (g/mol)	NPH derivatised chemical formula	Monoisotopic Mass - NPH (g/mol)
	acid					
	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
	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	186005663CV	54
	1.5 mL Microcentrifuge SafeLock Tubes	Eppendorf	STK-TUBE-035	54
	Kinetex 2.6 um C18 50 × 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

	Item	Supplier	Article no.
	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

Troubleshooting

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 × 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 × 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 × 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 + 100 µL of 1 mg/mL PA + 100 µL of 1 mg/mL BA + 100 µL of 1 mg/mL isoBA + 100 µL of 1 mg/mL VA + 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.

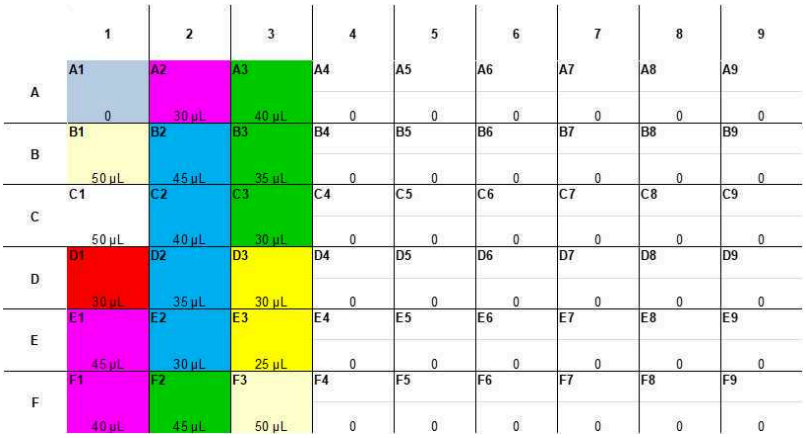
14 Complete a 54-vial (Thermo Vial Rack) map for standards and samples (**make sure to place them column-wise**) using the design as shown. The number of samples that can be analysed per batch is 36, alongside a 15-point calibration curve.

	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:



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 × 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

	Time (min)	Flow (mL/min)	%A	%B	Curve
	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.

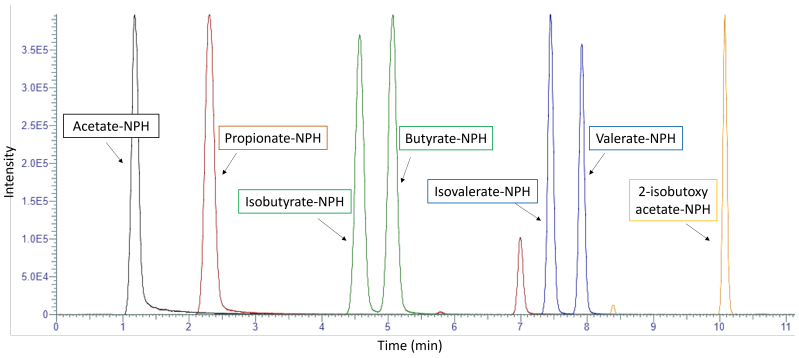
38 Create a batch in Xcalibur using the electronic plate map - use the correct position for the Thermovial 54 rack, the correct position of the column, the correct lines for the mobile phases and the correct LC-MS/MS method. Name and save the **Batch acquisition file.** Use the same naming convention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

Protocol references

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