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Protocol status: Working
We use this protocol and it's working

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Extraction, derivatisation and GC-MS/MS analysis of d2-glucose and d5-glycerol from human plasma

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ABSTRACT

Tracer glucose and tracer glycerol is used to assess glucose kinetics and turnover.

Here we have improved upon existing gas chromatography mass spectrometry (GC-MS) methods for the quantitation of glucose, D2-glucose, glycerol and D5-glycerol in plasma.

Plasma samples (200 µL) were extracted by automated 96-well protein precipitation (PPT+) using **acetonitrile** and as an organic solvent, carried out on an Extrahera automated sample handler.

Extracts were derivatised using acetic anhydride to form glucose pentacetate and glycerol triacetate, with internal standard butanetriol and ¹³C₆-glucose pentacetate.

Derivatives were separated on a **GC 9000** on an TG-WAX MS column (30 m x 0.25 mm; 0.25 µm) and a carrier gas mobile phase of helium. The run time was 30 minutes, followed by mass spectral analysis on a Quantum tandem quadrupole mass spectrometer (ThermoFisher) operated in chemical ionisation mode.

This method has been used to analyse glucose, D2-glucose, glycerol, D5-glycerol. Validation demonstrates that this method is sensitive, specific, and suitable for simultaneous measurement of glucose and glycerol in plasma (200 µL) and glucose turnover

ATTACHMENTS

[EXAMPLE_PLATE
MAP_NZMH.xlsx](#)

PROTOCOL integer ID: 95547

Keywords: tandem mass spectrometry, GC-MS/MS, tracer glucose, tracer glycerol, stable isotope tracers

Funders Acknowledgement:

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Grant ID: EDCRF

GUIDELINES

Ensure all training is up-to-date for operating the necessary laboratory instrumentation and equipment.

MATERIALS

Consumables Table

A	B	C	D
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	10
28 mL tall form glass vials with lids	VWR	T008/04	2
2 mL deep well 96 well collection plate	Biotage	121-5203	1
Biotage PPT+ 96 well plate	Biotage	120-2040-P01	1
96 Extrahera 1000 μ L pipette tips	Biotage	414141	2
2 mL deep well 96 well collection plate	Waters	186002482	1
96 well plate sealing film	Merck	Z369659-100EA	1
300 μ L fixed insert glass autosampler vials (100 pk)	Agilent	9301-1388	1
Crimp caps aluminium 11 mm (100 pk)	Agilent	5181-1211	1
Thermo TGWAX-MS 30 m x 0.25 mm, 0.25 μ m	Thermo	26088-1420	1

Consumables for extraction and derivatisation of glucose and glycerol**Chemicals and Analytical Standards Table**

A	B	C
Item	Supplier	Article no.
Water (HPLC grade)	Fisher Scientific UK Ltd (QMRI Stores)	STK-SOLV-175
Acetonitrile (HPLC grade)	Fisher Scientific UK Ltd (QMRI Stores)	STK-SOLV-025
Pyridine, anhydrous 99.8%	Merck	270970-100ML
Acetic Anhydride (Reagent Plus)	Merck	320102-100ML
n-Heptane	Fisher Scientific UK	10598800
Glucose	Sigma-Aldrich	1.08337.1000
d2-Glucose (6,6-d2)	Cambridge Isotope Laboratories	DLM-349-SP-PK

A	B	C
Glycerol (liquid)	Sigma-Aldrich	G9012
d5-Glycerol (1,1,2,3,3-d5)	Cambridge Isotope Laboratories	DLM-1229-1
13C6-glucose	Sigma-Aldrich	389374-1G
Butanetriol (liquid)	Sigma-Aldrich	309710

Chemicals and analytical standards

Solutions Required

- 1:1 Acetic anhydride:Pyridine (20 mL)
 - o Add 10 mL acetic anhydride to 10 mL pyridine. Mix thoroughly. This will give enough solution for derivatisation of 100 samples. Prepare freshly for each batch.

- Heptane + 5% Acetic anhydride (10 mL):
 - o Add 10 mL acetic anhydride to 10 mL pyridine. Mix thoroughly. This will give enough solution for resuspension of 100 samples. Prepare freshly for each batch.

Equipment

A	B	C
Item	Model	Supplier
Liquid Handling Robot	Extrahera	Biotage
Evaporator	TurboVap 96 Dual	Biotage
Manual adjustable volume pipettes, various	ErgoOne	Starlab
Repeater pipette	Repetman	Gilson
Microtube centrifuge	1-15	Thermo Scientific
Deepwell Plate shaker	-	Starlab
Gas Chromatography System	Trace GC Ultra	Thermo Scientific
Autosampler	TriPlus (liquid injection)	Thermo Scientific
Mass spectrometer	TSQ Quantum	Thermo Scientific

Equipment required for homogenisation, extraction and steroid analysis

SAFETY WARNINGS

- ! Ensure risk assessments are up to date and that all local laboratory guidelines are followed for handling chemicals and biological samples

ETHICS STATEMENT

All human studies were approved by the University of Edinburgh NHS Lothian ACCORD Ethical Review Board and samples analysed in a Good Clinical Practice laboratory.

BEFORE START INSTRUCTIONS

Ensure all consumables are in stock and all compounds and reagents are freshly prepared

Preparation of solutions, calibration standards and enrichment curves

1 Preparation of Internal Standard Solution

Label vials as follows:

- 1 x 28 mL glass vials labelled INT STD mix
 - Internal standard mix ($^{13}\text{C}_6$ Glucose 5 mg/mL + Butanetriol 0.25 mg/mL)
 - Weight out 50 mg of $^{13}\text{C}_6$ Glucose in a 28 mL glass vial. Add pL 2.5 μL butanetriol and make up to mL 10 mL in water - Make fresh every 3-4 weeks
- (This should be sufficient for 5 plates of samples)

2 Preparation of Calibration Standard Solutions

Pipette volume of each solution into glass vials as defined below.

Note: Amount of glucose is 50 x that of d2 glucose and 200x that of Glycerol and d5-glycerol

- **GLUCOSE A** - 50 mg/mL Glucose + 1 mg/mL d2-glucose (need 100 μL max) so – Prepare 2 mg/mL d2-glucose in water. Weigh out 50 mg glucose in a 7 mL glass vial. Add 500 μL x 2 mg/mL d2-glucose + 500 μL water to the 50 mg glucose. This dilutes the 2 mg/mL d2-glucose 1:1 and results in 1 mL of a 50 mg/mL Glucose/ 1 mg/mL d2 glucose solution. Vortex thoroughly.
- **GLUCOSE B** - 5 mg/mL Glucose + 0.1 mg/mL d2-glucose: Add 100 μL of Glucose A solution to 900 μL water in a 1.75 mL glass vial. Vortex thoroughly.
- **GLUCOSE C** - 0.5 mg/mL Glucose + 0.01 mg/mL d2-glucose: Add 100 μL of Glucose B solution to 900 μL water in a 1.75 mL glass vial. Vortex thoroughly.
- **GLYCEROL A** – 250 $\mu\text{g}/\text{mL}$ (Glycerol + d5-glycerol): Prepare by weighing out 2 mg glycerol and 2 mg d5-glycerol in separate 7 mL glass vials. Add 4 mL water to each to produce 500 $\mu\text{g}/\text{mL}$ solutions of each.

Vortex thoroughly. Combine 500 µL of the 500 µg/mL glycerol solution and 500 µL of the 500 µg/mL d5-glycerol solution in a 1.75 mL glass vial giving a combined 250 µg/mL solution. Vortex thoroughly.

- **GLYCEROL B** – 25 µg/mL (Glycerol + d5-glycerol): Add 100 µL of Glycerol A solution to 900 µL water in a 1.75 mL glass vial. Vortex thoroughly.
- **GLYCEROL C** – 2.5 µg/mL (Glycerol + d5-glycerol): Add 100 µL of Glycerol B solution to 900 µL water in a 1.75 mL glass vial. Vortex thoroughly.

3 Calibration standards

A Standard name	B Glucose (µg)	C d2-Glucose (µg)	D Glycerol (µg)	E d5-Glycerol (µg)	F Vol Glucose and d2-Glucose (µL)	G Vol Glycerol and d5-Glycerol (µL)	H Vol Water (µL)	I Vol Int Std (µL)
Blank	0	0	0	0	0	0	200	0
Std 0	0	0	0	0	0	0	200	20
Std 1 (10 µg Glucose)	10	0.2	0.05	0.05	20 x Glucose C	20 x Glycerol C	160	20
Std 2 (20 ug)	20	0.4	0.1	0.1	40 x Glucose C	40 x Glycerol C	120	20
Std 3 (50 ug)	50	1	0.25	0.25	10 x Glucose B	10 x Glycerol B	180	20
Std 4 (100 ug)	100	2	0.5	0.5	20 x Glucose B	20 x Glycerol B	160	20
Std 5 (150 ug)	150	3	0.7	0.75	3 x Glucose A	3 x Glycerol A	194	20

A	B	C	D	E	F	G	H	I
			5					
Std 6 (200 ug)	200	4	1	1	4 x Glucose A	4 x Glycerol A	192	20
Std 7 (300 ug)	300	6	1.5	1.5	6 x Glucose A	6 x Glycerol A	188	20
Std 8 (400 ug)	400	8	2	2	8 x Glucose A	8 x Glycerol A	184	20
Std 9 (500 ug)	500	10	2.5	2.5	10 x Glucose A	10 x Glycerol A	180	20

Table Y - Preparation of Calibration standards

Note that **Std 1** (10 ug) is 10 ug glucose, 0.2 ug D2-glucose, 0.05 ug glycerol and 0.05 ug D5-glycerol. **Std 9** (500 ug) is 500 ug glucose, 10 ug D2-glucose, 2.5 ug glycerol and 2.5 ug D5-glycerol

Extraction of glucose, glycerol, D2-glucose and D5-glycerol from human p...

- 4 Perform protein precipitation of samples and standards in a 96-well plate with  200 µL e and 600 µL acetonitrile
-
-
-
- 4.1 Prepare a map of calibration standards and samples in a 96-well format as below, using Column-wise plate layout for automated extraction on an Extrahera liquid handling robot (Biotage, Sweden):

	1	2	3	4	5	6	7	8	9	10	11	12	
A	A1 (1) Double blank	A2 (9) Std 7 - Glu	A3 (17) Sample 4	A4 (25) Sample 12	A5 (33) Sample 20	A6 (41) Sample 30	A7 (49) Sample 36	A8 (57) Sample 44	A9 (65) Sample 52	A10 (73) Sample 60	A11 (81) Sample 68	A12 (89) Sample 76	
B	B1 (2) Std 0 - Glu	B2 (10) Double blank	B3 (18) Sample 5	B4 (26) Sample 13	B5 (34) Sample 21	B6 (42) Sample 29	B7 (50) Sample 37	B8 (58) Sample 45	B9 (66) Sample 53	B10 (74) Sample 61	B11 (82) Sample 69	B12 (90) Sample 77	
C	C1 (3) Std 1 - Glu	C2 (11) Double blank	C3 (19) Sample 6	C4 (27) Sample 14	C5 (35) Sample 22	C6 (43) Sample 30	C7 (51) Sample 38	C8 (59) Sample 46	C9 (67) Sample 54	C10 (75) Sample 62	C11 (83) Sample 70	C12 (91) Sample 78	
D	D1 (4) Std 2 - Glu	D2 (12) Double blank	D3 (20) Sample 7	D4 (28) Sample 15	D5 (36) Sample 23	D6 (44) Sample 31	D7 (52) Sample 39	D8 (60) Sample 47	D9 (68) Sample 55	D10 (76) Sample 63	D11 (84) Sample 71	D12 (92) Sample 79	
E	E1 (5) Std 3 - Glu	E2 (13) Double blank	E3 (21) Sample 8	E4 (29) Sample 16	E5 (37) Sample 24	E6 (45) Sample 32	E7 (53) Sample 40	E8 (61) Sample 48	E9 (69) Sample 56	E10 (77) Sample 64	E11 (85) Sample 72	E12 (93) Sample 80	
F	F1 (6) Std 4 - Glu	F2 (14) Double blank	F3 (22) Sample 9	F4 (30) Sample 17	F5 (38) Sample 25	F6 (46) Sample 33	F7 (54) Sample 41	F8 (62) Sample 49	F9 (70) Sample 57	F10 (78) Sample 65	F11 (86) Sample 73	F12 (94) Sample 81	
G	G1 (7) Std 5 - Glu	G2 (15) Double blank	G3 (23) Sample 2	G4 (31) Sample 10	G5 (39) Sample 18	G6 (47) Sample 26	G7 (55) Sample 34	G8 (63) Sample 42	G9 (71) Sample 50	G10 (79) Sample 58	G11 (87) Sample 66	G12 (95) Sample 74	Sample 82
H	H1 (8) Std 6 - Glu	H2 (16) Double blank	H3 (24) Sample 11	H4 (32) Sample 19	H5 (40) Sample 27	H6 (48) Sample 35	H7 (56) Sample 43	H8 (64) Sample 51	H9 (72) Sample 59	H10 (80) Sample 67	H11 (88) Sample 75	H12 (96) Solvent	

Plate Map design for automated 96-well extraction by protein precipitation (PPT+)

4.2

Defrost plasma samples, vortex, and centrifuge ( 100 x g, 00:05:00)

5m

4.3

Set up Extrahera robot for PPT+ extraction.

Turn on Air Compressor. Make sure a pressure of ~9 bar is achieved and that the compressor goes into Standby (indicated by green flashing light). Switch on fumehood. Turn on Extrahera. Purge line S3 for acetonitrile ensure sufficient number of standard bore solvent tips (Deck position 1) and standard bore sample tips (Deck position 2) are on the deck.

4.4

Place a labelled PPT+ plate in deck position 3. Check plate orientation is correct.

4.5

Place a labelled Waters 2 mL 96-well collection plate is in carousel position A

4.6

Take a Biotage 2 mL deep well collection plate and label with batch details. Add 200 uL water to blank wells according to plate map designed.

- 4.7 Add required amount of water and Glucose and Glycerol standards to the calibration standard wells according to the table in the PREPARATION OF CALIBRATION STANDARDS section. Due to the small volumes of standard being pipetted, ensure that the standard is pipetted INTO the water.
- 4.8 Add 200 uL of each plasma sample from the sample tubes to the appropriate well plate positions
- 4.9 Using the Gilson Repetman and a 1 mL tip, add 20 uL of the internal standard solution to wells except for Double Blank and Solvent Blank wells.
- 4.10 Seal the plate using a Merck well plate sealing film and shake the plate on a plate shaker for 2 mins to ensure that the standards and internal standards are sufficiently mixed.
- 4.11 Remove the plate seal and place the sample plate on the deck of the Extrahera in position 4.
- 4.12 Select and run the PPT+ method. It will load 600 uL acetonitrile onto the PPT+ plate. Then it will transfer the contents of the sample plate column by column onto the acetonitrile loaded PPT+ plate.
- 4.13 The robot will leave the sample to interact with the acetonitrile for 20 minutes, before applying positive pressure onto the PPT+ plate and collecting the eluent of the sample in the Waters 2 mL deep well 96-well plate. Check the full volume has been eluted into the plate.

5 Preparation of Enrichment curve for glucose and D2-Glucose

1. Label 7 mL glass vials for Glucose enrichment curve (i.e Blank, 0%.....8%) and a 'tracer only' sample (n=9).
2. Pipette volume of each stock solution into 7 mL glass vials as defined in table below.

3. All solutions made fresh every 3-4 weeks:
4. 2 mg/mL d2-glucose prepared by 10 mg d2-glucose in 5 mL water
5. 2 mg/mL Glucose prepared by 100 mg in 50 mL water

These are made up to 5000 µL or 500 µL and then a 200 µL aliquot is taken for extraction. No internal standard is required for the enrichment curve.

A	B	C	D
Standard	d2-glucose (mL)	Glucose (mL)	Water (mL)
Blank	0	0	500
0%	0	500	0
0.26%	12.5	4987	0
0.5%	25	4975	0
1%	50	4950	0
2%	100	4900	0
4%	200	4800	0
8%	400	4600	0
d2 TRACER ONLY	40	0	160

Table X - Enrichment curve for D2-glucose / Glucose

Transfer of extracts to glass vials for derivatisation

- 6 Transfer eluent to individually labelled glass vials and dry down on a Dry Block under nitrogen gas at 40C, gas flow at 25 L/min. Typical dry down is 35-40 mins. Do not overdry. Store at -20C at this stage if needed.
- 7 Preparation of Glycerol Enrichment curve for glycerol and d5-glycerol

1. Label 7 mL glass vials for Glycerol enrichment curve (i.e Blank, 0%.....8%) and a 'tracer only' sample (n=9)
2. Pipette volume of each stock solution into 7 mL glass vials as defined in table below.

3. All solutions made fresh every 3-4 weeks:

- 2 mg/mL d5-glycerol prepared by adding 10 mg d2-glucose in 5 mL water
- 2 mg/mL Glycerol prepared by adding 100 mg into 50 mL water

These are made up to  500 µL and then a 200 µL aliquot is taken for extraction. No internal standard is required for the enrichment curve.

A	B	C	D
Standard	d5-glycerol (µL)	Glycerol (µL)	Water (µL)
Blank	0	0	500
0%	0	500	0
0.26%	12.5	4987	0
0.5%	25	4975	0
1%	50	4950	0
2%	100	4900	0
4%	200	4800	0
8%	400	4600	0
d5 TRACER ONLY	40	0	160

Table X - Enrichment curve for D5-glycerol

Derivatisation of glucose and glycerol and tracers with acetic anhydride

8

- 1) Using the Gilson Repetman and a 5 mL tip, add  200 µL of Pyridine:Acetic Anhydride (1:1) to the dried extracts. Cap vial and vortex thoroughly.
- 2) Leave on the bench at room temperature for 15 minutes.
- 3) Dry down the vial contents using the TurboVap 96 Dual system with Gas Flow set to 25L/min, Gas Temperature set to 40°C and the Plate Temperature set to 40°C. Typical dry down time is 30-45 mins. Do not over dry.

- 4) Using the Gilson Repetman and a 5 mL tip, resuspend the dry sample in 100 µL of Heptane + 5% Acetic Anhydride. Cap and vortex thoroughly.
- 5) Transfer the contents of each 1.75 mL sample vial to individual 300 µL fixed insert glass autosampler vials. Crimp caps and store at -20°C until analysis.

Set up of GC-MS/MS instrumentation into Chemical Ionisation mode

- 9 Ensure the GC-MS/MS INSTRUMENT is in CI mode** using Methane as reagent gas and Argon as collision gas

Note

Ensure argon and methane cylinders are regularly checked for supply level

- 10 Set up the Gas Chromatography system with autosampler, injector and oven temperature settings as below**

A	B
Injection	Hydrogen (from Trace hydrogen generator)
Carrier gas	Hydrogen (from Trace hydrogen generator)
Carrier gas flow (mL/min)	2.0
Liner	PTV Siltek Metal Liner 120 mm x 2.75 mm OD x 2mm ID Part no: 45322044
Septum	BTO 50 mm septum Part no: 31303233-BP
Temperature	75 °C
Operating mode	Splitless
Split flow control	On
Split flow	15.0
Splitless time	1.00 min
Purge flow control	On
Purge flow	5.00 mL/min
Constant septum purge	On

A	B
Vacuum compensation	On
Enable gas saver mode	Off
Enable evaporation phase	On
Enable clean phase	On
Enable pressure ramps	Off
Post cycle temperature	CoolDown

Gas Chromatography Injector Settings

A	B
Carrier gas	Hydrogen (from Trace hydrogen generator)
Carrier gas flow (mL/min)	2.0
GC column	Thermo TGWAX-MS 30 m x 0.25 mm, 0.25 µm Part no: 26088-1420
Injection	Programmable Temperature Vaporiser (PTV) (back inlet)

Gas Chromatography Oven Settings

A	B	C	D
Time (min)	Rate (°C/min)	Target value (Rate (°C))	Hold time (min)
0.00	0.0	60.0	0.65
0.65	36.80	150	0.00
17.187	12.10	260	5.00

A	B	C	D
20.00	Stop run		

Gas Chromatography temperature gradient settings, fitted with TGWAX-MS (30 m x 0.25 mm; 0.25 µm)

Set up of mass spectrometry settings following GC separation

11 TSQ GC-MS/MS mass spectrometry settings

A	B
Instrument	Thermo TSQ9000
Ion volume, Ionisation Mode	Chemical Ionisation
Reagent gas	Methane
Reagent gas flow	1 mL/min
Scan Mode, Polarity	SIM & SRM, Positive & Negative
Resolution (Q1/Q3)	unit/unit
MS transfer line tempature	240 °C
Ion source temperature	175 °C

TSQ GC-MS/MS mass spectrometry settings for glucose and glycerol analysis

12 Mass transitions for each acetate derived compound monitored:

A	B	C	D	E	F
Compound name	Mode	Ionisation	Q1 (m/z)	Q3 (m/z)	Retention time (mins)
Glucose	SIM	CI negative	287.1	/	12.6
D2-glucose	SIM	CI negative	289.1	/	12.6
Glycerol	MRM	CI positive	159.1	43.1	6.0
D5-glycerol	MRM	CI negative	164.1	43.1	6.0
13C6-glucose	SIM	CI negative	293.1	/	12.6

A	B	C	D	E	F
butanetriol	MRM	Cl negative	231.1	83.2	6.8

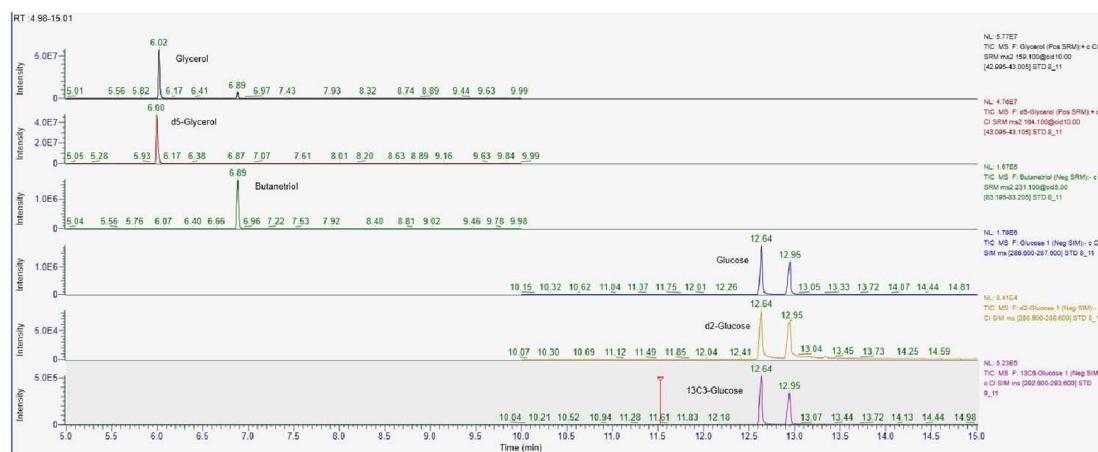
Compound specific mass spectrometry parameters for acetate derivatives on TSQ Quantum, Cl - chemical ionisation, MRM - multiple reaction monitoring, SIM - single ion monitoring

13 Example Chromatogram of Glucose, Glycerol and tracers, of Glucose, Glycerol and tracers with associated retention times

Check the retention times of glucose, glycerol, tracers and internal standards are as expected, as shown in the chromatogram below:

Expected result

Retention times; glycerol at **6 mins**, D5-glycerol at **6 mins**, glucose at **12.6 mins** and D2-glucose at **12.6 mins** and internal standards $^{13}\text{C}_6$ -glucose at **12.6 mins** and butanetriol at **6.8 mins**.



Extracted ion chromatograms of glucose, D2-glucose, glycerol, D5-glycerol, $^{13}\text{C}_6$ -glucose and butan

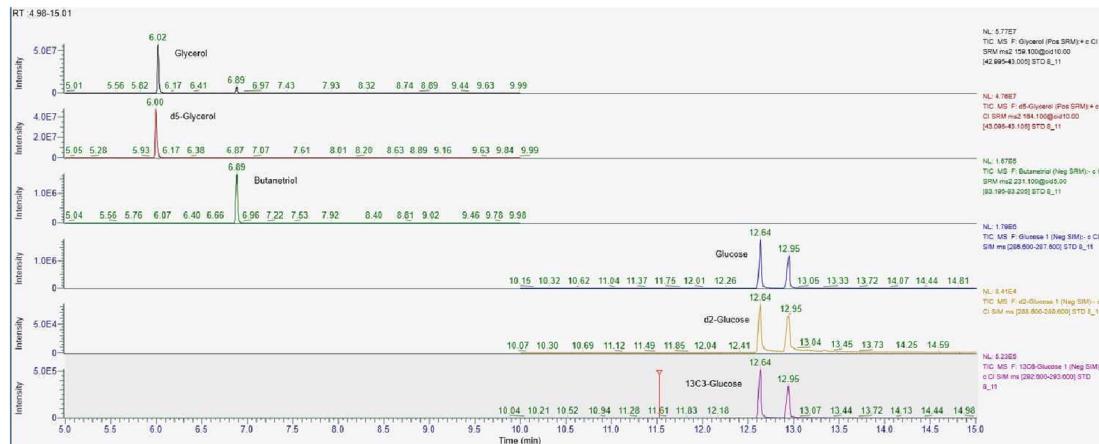
Glucose and Glycerol and tracer acetate derivative analysis by GC-MS/Ms 16m

- 14** Set up an acquisition batch in Xcalibur software using the electronic Microsoft Excel file of the calibration standards and sample list. Set to inject 2 μL per sample and use a method of chromatographic separation as described in **step 10** and mass spectrometer settings as outlined in **step 11**.

- 15** Check the retention times of glucose, glycerol, tracers and internal standards are as expected, as shown in the expected result tab and the chromatogram below:

Expected result

Retention times; glycerol at **x mins**, D5-glycerol at **y mins**, glucose at **y mins** and D5-glycerol at **y mins** and internal standards 13C6-glucose at **x mins** and butanetriol at **y mins**.



Extracted ion chromatograms of Glucose, glycerol and tracers and internal standards

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Once the chromatography of a test solution has been checked and the retention times are consistent, set the batch of samples to analyse. Use Tracefinder software and excel to evaluate the GC-MS/MS data to calculate the concentration of glucose and glycerol and tracers in each sample

Protocol



NAME

Using TraceFinder and Excel software to evaluate and report multi-analyte targeted LC-MS data acquired on an ThermoScientific Exploris 240 Orbitrap

CREATED BY

Margaux Billen

PREVIEW