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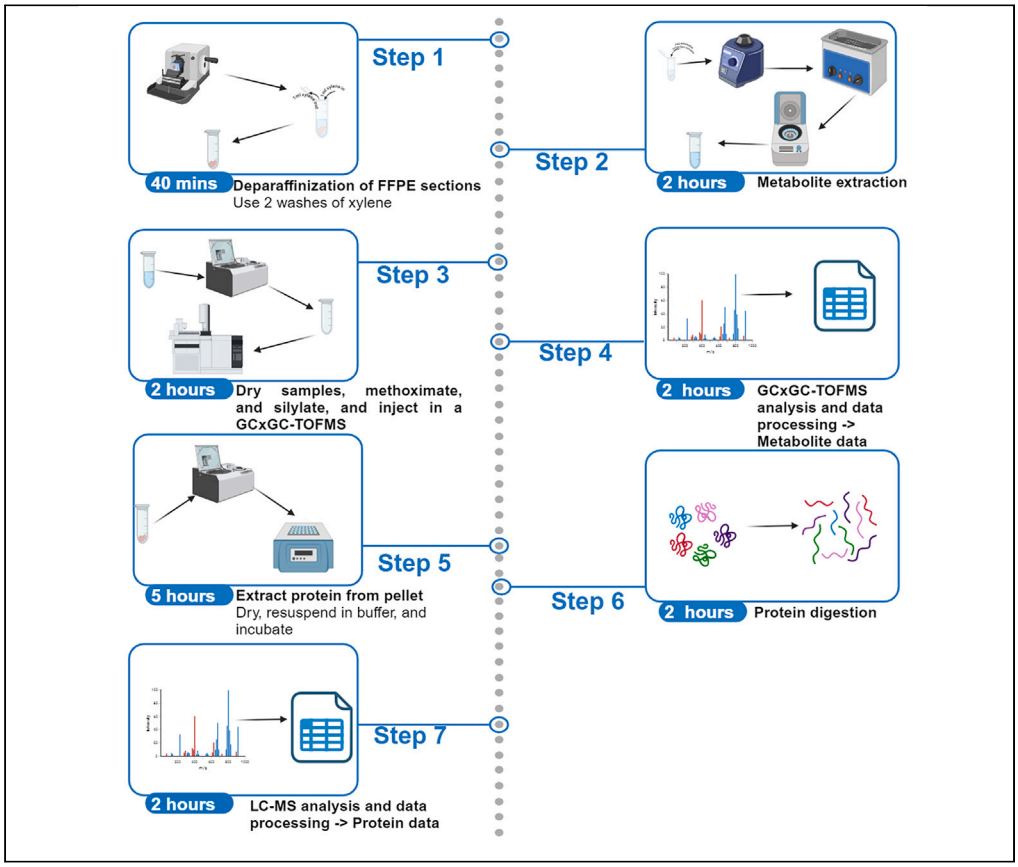
Protocol for unified metabolomics and proteomics analysis of formalin-fixed paraffin-embedded tissue



The use of archival formalin-fixed paraffin-embedded (FFPE) tissue samples for biochemical analyses is problematic because of the formation of a Schiff base, leading to low protein and metabolite yields during analytical extractions. Here, we overcome this issue using a unified protocol on FFPE tissue for metabolomics and proteomics analyses. Using 20 mg of wet mass tissue, this protocol consistently extracted more than 50 metabolites (across 11 classes of metabolites) and over 900 proteins.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Abisola R. Isaiah, Laneke Luies, Du Toit Loots, ..., Marceline Tutu van Furth, Martijn van der Kuip, Shayne Mason

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Highlights

Combined metabolomic and proteomic protocol for FFPE tissue samples

Can identify over 50 metabolites across 11 classes and over 800 proteins

Optimized method that produces reproducible results

Isaiah et al., STAR Protocols 5, 103442
December 20, 2024
https://doi.org/10.1016/j.xpro.2024.103442

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Protocol for unified metabolomics and proteomics analysis of formalin-fixed paraffin-embedded tissue

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https://doi.org/10.1016/j.xpro.2024.103442

SUMMARY

The use of archival formalin-fixed paraffin-embedded (FFPE) tissue samples for biochemical analyses is problematic because of the formation of a Schiff base, leading to low protein and metabolite yields during analytical extractions. Here, we overcome this issue using a unified protocol on FFPE tissue for metabolomics and proteomics analyses. Using 20 mg of wet mass tissue, this protocol consistently extracted more than 50 metabolites (across 11 classes of metabolites) and over 900 proteins.

BEFORE YOU BEGIN

The following protocol describes a step-by-step extraction of metabolites and proteins from FFPE tissue. All microtome sliced sections were pooled (combined) to make one sample after which 20 mg of wet mass was taken in individual DNA LoBind microcentrifuge tubes (triplicate). Using 2 × 10 μm thick sections should be able to produce the same results if the sections weigh 20 mg. The metabolite extraction procedure was adapted with modifications from the study by Wojakowski et al. (2015),[1] and the protein extraction protocol introduces a new buffer for the extraction of proteins from FFPE tissue.

Institutional permissions

Ethics approval was obtained from the North-West University's Ethics committee with approval number NWU-00482-20-A1 and from the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa (ethics approval numbers S12/11/298 and N09/07/185).

Preparation 1: Sample procurement and preparation

1. Obtain FFPE tissue samples from the pathology laboratory.
2. Use a microtome cutter to cut FFPE tissue sections as thinly as desired. For this protocol, we used 20-micron deparaffinized sections with a weight of no less than 20 mg.

a. Microtome slicing.
   i. Set the slicer to the desired thickness (20 microns).
   ii. Mount the FFPE block on the microtome slicer and slice.
   iii. Remove the sliced sections using sterile forceps and place them in an appropriate, sterile biological specimen container.

Note: Ensure that new microtome blades are used to cut sections.

⚠ CRITICAL: Always wear cut-resistant gloves when handling the blades. Before adjusting a mounted block, ensure that the rotary is locked.

Preparation 2: Laboratory setup

3. Ensure that the heating block is set to 70°C and label all DNA LoBind tubes appropriately.
4. Set the microcentrifuge to 4°C.
5. Run blanks (i.e., using an empty vial without any solvents) on the GC-MS and LC-MS instruments to clean the respective columns.
   a. Check that all freshly prepared washing solutions and mobile phases are filled to the top.
   b. For GC analysis, perform tune and leak checks, and replace the liner and septum, and when using a 2D GC system, a defrosting cycle should also be performed.
6. Wash the carbide tungsten beads by following the steps below:
   a. Rinse with Milli-Q water.
   b. Wash with phosphate-free soap.
   c. Rinse with methanol.
   d. Rinse with acetonitrile.
   e. Rinse again with Milli-Q water.
   f. Dry in the oven (until dry) at 90°C for at least 30 min.

⚠ CRITICAL: Acetonitrile can be irritating to the eyes and skin. Always work in the fume hood, with the appropriate PPE — gloves and laboratory coats — when handling this chemical.

Preparation 3: Prepare solvents

Prepare the following solvents before the sample extraction.

7. Metabolomics internal standard (IS) solution (50 ppm):
   a. Weigh 25 mg of 3-phenylbutyric acid using a disposable weighing boat.
   b. Transfer to a glass volumetric flask and add Milli-Q water up to the 50 mL mark to make up a 500 ppm stock concentration of the IS.
   c. Using a glass bulb pipette 5 mL of the 500 ppm 3-phenylbutyric acid solution into a 50 mL volumetric flask and add Milli-Q water up to the 50 mL mark. This will create a 50 ppm IS working solution.

Note: The excess 500 ppm IS stock solution can be stored in a glass Schott bottle in a chemical cabinet and used to prepare additional 50 ppm IS solutions if needed.

⚠ CRITICAL: The volume of the solvent used in making up the metabolomics IS solution should be subtracted from the total volume of the extraction solvent prepared later (if used in the extraction solvents). For example, in this case, if the IS dissolved in 50 μL Milli-Q water, then add 100 μL of water in the extraction solvent instead of 150 μL Milli-Q water, as described later. The IS can be reused and is stable for as long as needed.

8. Polar phase metabolite extraction solution:
   a. Prepare a 1:1 mixture of methanol with Milli-Q water.

⚠ CRITICAL: Take note of the number of samples to be analyzed and prepare sufficient volumes of extraction solutions as needed. For example, for 50 samples, prepare 10 mL of the polar extraction solution (5 mL methanol and 5 mL Milli-Q water), assuming that each sample requires 200 μL. Properly cap the remaining extraction solvents to prevent evaporation and store them in a refrigerator for later use. The polar phase metabolite extraction solution can be used for as long as needed.

⚠ CRITICAL: Methanol is a toxic and flammable solvent and should be kept away from any open flame. In addition, gloves and laboratory coats must be worn when handling this chemical.

9. Apolar phase metabolite extraction solution:
   a. Prepare a 3:1 mixture of dichloromethane to methanol.

⚠ CRITICAL: Again, take note of the number of samples to be analyzed and prepare sufficient volumes of extraction solutions as needed. For example, for 50 samples, prepare 13 mL of the apolar extraction solution (9 mL dichloromethane and 4 mL methanol), assuming that each sample requires 250 μL. Properly cap the remaining extraction solvents to prevent evaporation and store them in a refrigerator for later use.

   b. Glassware, not plasticware, should always be used when using organic solvents, such as chloroform, during apolar phase extraction to avoid plastics leaching into the solvent.
   c. Only DNA LoBind tubes are recommended for metabolite and protein extractions.

⚠ CRITICAL: Dichloromethane can irritate the skin and eyes and should be handled in the fume hood while wearing gloves and protective laboratory coats.

10. Methoxyamine hydrochloride (MOX-HCl):
   a. Prepare a 20 mg/mL solution by dissolving 20 mg of MOX-HCl in 1 mL of pyridine in a glass GC-MS vial.

Note: The MOX-HCl solution must be prepared fresh – daily or as needed. To avoid wastage, it is advisable to calculate the amount of MOX-HCl required per day (assuming samples are divided into smaller batches). For example, if 25 μL is required per sample and you have 9 samples (i.e., 225 μL MOX-HCl is required for all the samples), then 5 mg of MOX-HCl in 250 μL pyridine can be prepared for the day. However, it may be difficult to work with too small amounts, which might require the preparation of larger volumes.

Note: Keep the prepared solution away from light.

Note: MOX-HCl should be used within one year of purchase, since expired MOX-HCl produces less repeatable results.

⚠ CRITICAL: MOX-HCl causes burns. Wear suitable protective clothing, gloves, and eye/face protection. Pyridine can be irritating to the eyes and skin. Wear appropriate PPE — gloves and laboratory coats — while handling this chemical. The analysis using this solvent must be done in the fume hood.

11. Proteomics ethanolamine buffer solution: Prepare all buffer solutions in a glass Schott bottle.

Reagent	Final concentration	Amount
Ethanolamine (100 mM)	100 mM	59 μL
SDS	4%	0.4 g
TCEP (100 mM)	100 mM	400 μL
Tris (100 mM), pH 8	100 mM	0.1211 g
DTT (100 mM)	100 mM	0.1543 g
Milli-Q water	N/A	up to 10 mL
Total	**N/A**	**10 mL**

⚠ CRITICAL: Ethanolamine is extremely corrosive and highly flammable and should be stored in a safety cabinet. Wear gloves and work in a fume hood when handling this chemical. Store the ethanolamine buffer solution (for proteomics extraction) at 4°C since DTT is unstable at room temperature.

Note: This buffer should be used within 6 h of preparation. The waste should be discarded in a designated chemical waste container.

12. Proteomics NaCl exchange buffer solution:

Reagent	Final concentration	Amount
NaCl	100 mM	0.1 g
SDS	1%	0.1 g
Tris (100 mM), pH 8	100 mM	0.1211 g
Milli-Q water	N/A	up to 10 mL
Total	**N/A**	**10 mL**

⚠ CRITICAL: The exchange buffer is needed as a blank during the proteomics nanodrop concentration check, so it is important to prepare enough of this buffer for the samples and the blank.

Note: This buffer should be used within 24–48 of preparation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Human brain formalin-fixed paraffin-embedded (FFPE) tissue samples	Tygerberg Hospital (Age of block 34 years)	N/A
Chemicals, peptides, and recombinant proteins		
Xylene	Sigma-Aldrich	Cas# 1330-20-7 Cat# 214736-1L PCode 1003437921
3-Phenylutyric acid	Aldrich	Cas# 4593-90-2 Cat# 116807-5G PCode: 102077614 Lot# STBH0614
Methoxyamine hydrochloride (MOX-HCl)	Sigma-Aldrich	Cas# 593-56-6 Cat# 89803-5G Lot# BCCG1261 PC code 102478205
Pyridine	Sigma-Aldrich	Cas# 110-86-1 Cat# 270970-25ML Source SHBP0527 PC code 1003369500

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA)	Sigma-Aldrich	Cas# 25561-30-2 Cat# 646547–10X1ML PC code 102458180
Chloroform	Honeywell	Cat# AH049-4 Cas# 67-66-3 Lot# DZ647-ZA
Methanol	Honeywell	Cat# AH230-4 Cas# 67-56-1 Lot# DW767-ZA
Milli-Q water	N/A	N/A
Acetonitrile	Honeywell	Cat# 017-4 Cas# 75-05-8 Lot# DW848-ZA
Sodium dodecyl sulfate (SDS)	Merck	PC code 102531578 Cat# L3771-25G Cas# 151-21-3
Tris(hydroxymethyl)aminomethane hydrochloride	Melford	Cas# 77-86-1 Cat#B2005-5G
Ethanol amine	Sigma-Aldrich	PC code 1003588518 Source MKCP6930 Cas# 141-43-5 Cat# E9508-100ML
Dithiothreitol (DTT)	Merck	PC code 1003556714 Cat#D9760-1G Cas# 16096-97-2
Tris(2-carboxyethylphosphine) (TCEP)	Sigma-Aldrich	PC code 1003539994 Cat# 646547–10X1ML Cas# 51805-45-9
Sodium chloride (NaCl)	Sigma	Cas# 7647-14-5 Lot# BCBG9115V Cat# 53014-5KG
Liquinox phosphate-free liquid detergent	Sigma	Cas: Z742916-3.8L Lot# MKCN6265 PCode: 1003183101
DNA LoBind tube	Eppendorf	Cat. No.: 022431021 Lot: M209842H
Other		
Microtome cutter	Thermo Scientific	HM 340E semi-automated microtome
Microcentrifuge	Eppendorf	Centrifuge 5430R
Vortex	Lasec	Vortex mixer
Ultrasonic cleaner	Scientech	Ultrasonic mixer
Vibration mill	Retsch	MM 400
SpeedVac	Eppendorf	Concentrator plus
GCxGC-TOFMS	LECO	Pegasus 4D-C
Ultimate 3000 RSLC-MS nano system	Thermo Scientific Tribrid mass spectrometer	Orbitrap Fusion
Nanodrop	Thermo Scientific	NanoDrop One

STEP-BY-STEP METHOD DETAILS

A broad overview of the entire protocol, including preparation of the FFPE sample, metabolomics analysis, and proteomics analysis, is shown in Figure 1.

Deparaffinization of FFPE sections

Timing: 40 min

This section describes how the FFPE tissue blocks are sliced and deparaffinized. The wet mass is then collected after deparaffinization.

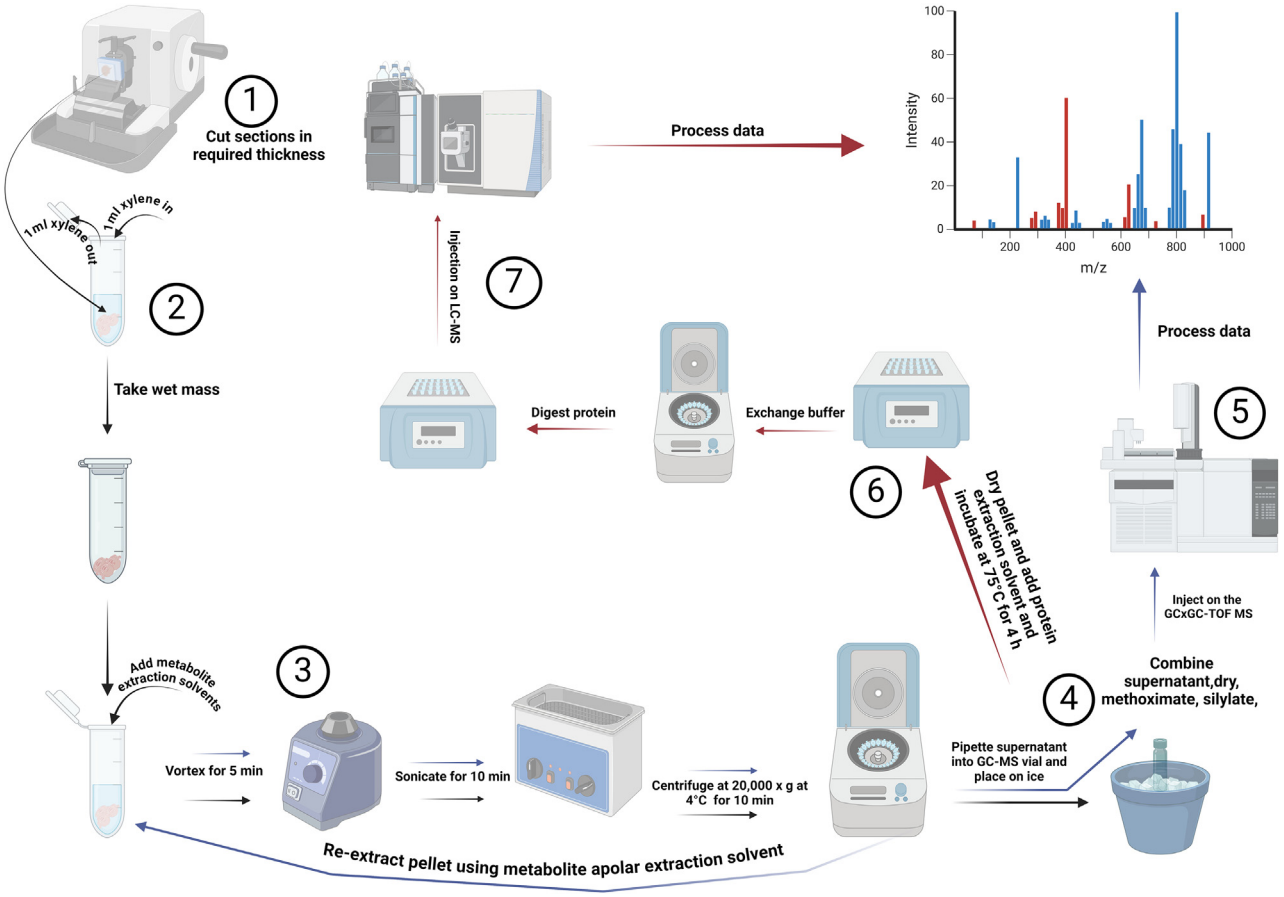

Figure 1. A comprehensive overview of the entire protocol, encompassing FFPE sample preparation, metabolomics analysis, and proteomics analysis

This protocol includes the following steps: (1) slicing of the FFPE sections, (2) deparaffinization of FFPE sections, (3) metabolite extraction procedure [MeOH:H_2O] (1:1) and CH_2Cl_2:MeOH (3:1)], dry and derivatization via (4) methoximation and silylation, (5) GCxGC-TOFMS analyses, (6) protein extraction, (7) protein digestion and LC-MS analyses. The black arrows (→) denote the 1st metabolite extraction, the blue arrows (➔) denote the 2nd metabolite extraction, and the red arrows (➔) denote the protein extraction from the pellet.

1. First deparaffinization:
   a. Heat the FFPE sections in microcentrifuge tubes at 70°C for 1 min before adding 1 mL xylene.

   Note: This melts the wax, facilitating complete wax removal by xylene. Reduced contaminants were observed in the results from FFPE samples after extraction.[2]

   b. Homogenize FFPE tissue slices using a 3 mm carbide tungsten bead in a 1.5 mL microcentrifuge tube in a vibration mill at 30 Hz in 1 mL xylene for 5 min.
   c. Centrifuge for 5 min at 20,000 × *g* at 4°C.
   d. Discard the supernatant and proceed to the next deparaffinization using the remaining pellet.
2. Second deparaffinization:
   a. Add 1 mL of xylene to the pellet and incubate at room temperature for 5 min.
   b. Centrifuge at 20,000 × *g* at 4°C for 5 min.
   c. Discard the supernatant.
   d. Weigh the samples.

⚠ CRITICAL: This protocol requires a minimum deparaffinized sample weight of 20 mg for repeatable results.

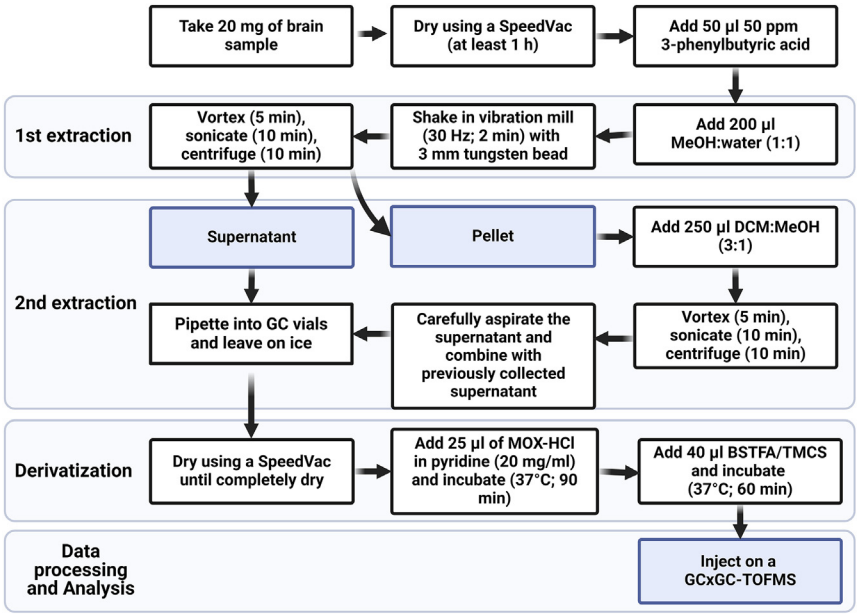

Figure 2. Workflow of FFPE brain tissue metabolomics extraction, incorporating adapted protocol modifications

Key modifications include pre-deparaffinization heating of FFPE samples for 1 min, utilization of 20 mg of deparaffinized tissue for analysis, drying of samples using a SpeedVac for 1 h (reduced from 2 h in a desiccator), incorporation of 3-phenylbutyric acid as an internal standard, omission of centrifugation on a 0.22 nm filter, and implementation of BSTFA silylation without agitation. Based on Wojakowska et al.[1]

e. Dry samples in SpeedVac for 1 h or until the samples are completely dry.

Extraction of metabolites from the deparaffinized sample

Timing: 2 h

This section describes the metabolite extraction using 20 mg wet mass of deparaffinized tissue.

Figure 2 provides an overview of the metabolomics protocol.

3. Add 50 µL of the 50 ppm metabolomics IS solution to the weighed (20 mg) samples.

⚠ CRITICAL: The IS should be added first to all samples before the extraction solvents.

Note: Testing concentrations of the IS at 25 ppm, 10 ppm, 5 ppm, and 1 ppm did not yield improved chromatograms.

4. Add 200 µL polar metabolite extraction solution.
   a. Homogenize at 30 Hz in a vibration mill for 5 min using a 3 mm tungsten bead.
   b. Vortex for 5 min.
   c. Sonicate at high frequency for 10 min without heat.
   d. Centrifuge at 20,000 × *g* at 4°C for 10 min.
   e. Transfer the supernatant to a GC vial using a glass Pasteur pipette and place on ice.
5. Add 250 µL apolar metabolite extraction solution to the pellet.
   a. Repeat points 4a–e.
6. Combine both supernatants for metabolomics analysis. Keep the pellet for later proteomics extraction and analysis (see point 17).
7. Dry the supernatant using a SpeedVac.

⚠ CRITICAL: Ensure that the samples are completely dry before proceeding with methoximation and silylation.

Derivatization via methoximation and silylation

Timing: 2 h 30 min

This section describes the steps involved in two derivatization methods, namely methoximation and silylation.

8. Add 25 μL of 20 mg/mL MOX-HCl (methoximation agent) and incubate at 37°C for 90 min.

Note: Drying after methoximation (point 8), before silylation (point 9),[3] was tested, but it did not improve the results in any way.

9. Add 40 μL of BSTFA with 1% TMCS, and incubate at 37°C for 60 min.

⚠ CRITICAL: Flammable liquid. Handle and store under inert gas.

10. Transfer all contents to an insert in a GC vial, and cap securely.
11. Inject into the GCxGC-TOFMS for metabolomics output (see Figure 1, step 5).

GCxGC-TOFMS analysis

Timing: 2 h 45 min

This section describes how to configure the GCxGC-TOFMS for metabolomics analysis.

12. Inject 1 μL of the sample into a LECO Pegasus 4D GCxGC-TOFMS fitted with an Agilent 7890 GC system and TOFMS (LECO Africa), equipped with a Restek Rxi-5Sil primary capillary column (28.2 m; 250 μm diameter; 0.25 μm film thickness), and a Restek Rxi-17 secondary capillary column (1.3 m; 250 μm diameter; 0.25 μm film thickness).
13. Use a split ratio of 1:5, or splitless, on all samples.
14. Set the following GC settings:
    a. Use purified helium as the carrier gas, set at a constant flow of 1.4 mL/min.
    b. Set a 3 mL/min septum purge flow.
    c. Set the gas saver flow to 15 mL/min.
    d. Maintain the front inlet temperature at 250°C throughout the entire run.
    e. Program the primary oven as follows:

Rate (°C/min)	Target temperature (°C)	Duration (min)
Initial	70	1
5	100	0
8	180	0
10	220	0
13	325	3
Off	0	0

    f. Set the secondary oven to a temperature offset of +5°C.
    g. Enable the modulator, with a temperature of 15°C. Also, set the modulation period to 3 s, pulsing every 0.7 s.
    h. Enable the chiller, set at −80°C.
    i. Set the transfer line temperature to 225°C.

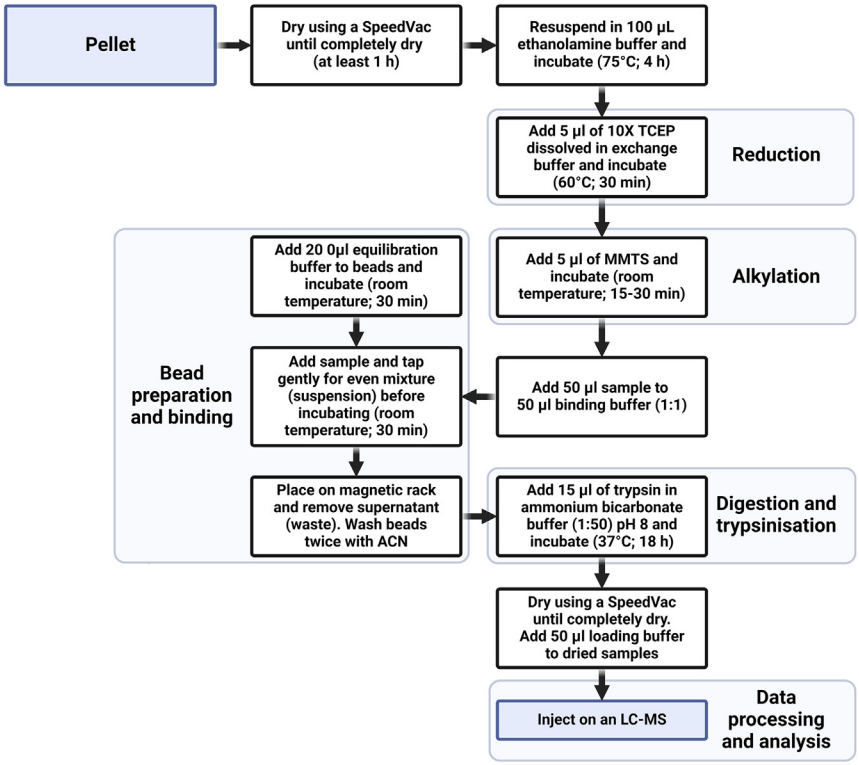

Figure 3. Workflow of FFPE brain tissue proteomics extraction

Protein undergoes reduction using TCEP so the functional groups can be in their charged state, alkylation using MMTS after which it is digested using HILIC magnetic beads in Trypsin.

15. Ensure the following MS settings:
    a. Use a 360 s solvent delay during which no mass spectra are recorded. Notably, this interval should be appended to the time axis on the GC column to reflect accurate retention times.
    b. Mass spectra should be collected over a range of 50–800 *m/z* at an acquisition rate of 20 spectra per second.
    c. Set the filament bias to −70 V and optimize the detector voltage at an offset of 100 V.
    d. Set the ion source temperature to 200°C.
16. For data processing, using ChromaTOF, set the following:
    a. Set the baseline offset to 1.
    b. Smoothing parameters should be automatically selected.
    c. Set the expected peak width to 7 s.
    d. Adjust the signal-to-noise ratio to 300, with a minimum of five apexing peaks.
    e. Set the match required to combine to 800. This means that a metabolite name (similarity match) will only be assigned if it achieves 80% match with the library.

Protein extraction from pellet

Timing: 5 h

This section describes how to extract proteins from the pellet after extracting the metabolites.

Figure 3 provides an overview of the proteomics protocol.

17. Dry sample pellets in a SpeedVac for at least 1 h or until the samples are completely dry.
18. Resuspend the pellets in 100 µL ethanolamine buffer.

⚠ CRITICAL: Ensure that all samples mix properly in the ethanolamine buffer by gently vortexing until the solvent is completely mixed with the pellet.

a. Heat at 75°C for 4 h.
b. Perform a buffer exchange by adding NaCl buffer to the protein in a 30 kDa filter and makeup to the mark (400 μL). Hereafter, centrifuge at 13,000 × *g* at 12°C for 10 min (repeat this process 3 times).
c. Check protein concentrations of the supernatant at 280 nm using a NanoDrop One instrument.

19. Proceed to protein digestion (see next step).

Protein digestion

Timing: 23 h

This section describes the protein digestion after incubation in the extraction buffer and exchanging the buffer.

This step is on-bead protein digestion of the previously extracted proteins, which is necessary for accurate identification and quantification.

20. Resuspend 50 μg of the extracted protein samples in 5 μL 100 mM Tris-HCl containing 100 mM NaCl and 1% SDS (exchange buffer) and reduce using 5 mM TCEP final concentration.
    a. Add 5 μL S-methyl methane thiosulfate (MMTS) to a final concentration of 20 mM in 100 mM Tris to the samples and incubate for 30 min at room temperature.
    b. Dilute the samples twofold with binding buffer.
21. Add the protein solution to MagResyn HILIC magnetic particles prepared according to the manufacturer's instructions.
    a. Incubate at room temperature for 30 min.

⚠ CRITICAL: Gently tap the bottom of the microcentrifuge tube to mix the magnetic particles with the sample and to avoid the magnetic particles adhering to the side of the microcentrifuge tube, as this can lead to sample loss.

    b. Remove the supernatant and wash the magnetic particles twice in 95% acetonitrile (ACN).
    c. Suspend the magnetic particles in 50 mM triethylammonium bicarbonate (TEAB) containing trypsin in a final ratio of 1:50 (for example, 6 μL trypsin to 300 μL TEAB).

Note: Ammonium bicarbonate can be substituted for TEAB.

    d. Incubate for 18 h at 37°C.
    e. Place on a magnet.

⚠ CRITICAL: Do not centrifuge to separate the pellet from the supernatant as this could damage the magnetic beads.

    f. Pipette out supernatant in a different microcentrifuge tube.
    g. Extract the peptides by adding 50 μL 0.5% trifluoroacetic acid (TFA) to the beads.
    h. Combine the supernatants and centrifuge at 12,000 × *g* for 10 min to pellet the trypsin.
    i. Remove the supernatant, dry, and resuspend it in 50 μL 2% acetonitrile in water.

LC-MS analysis

This section describes how to set up the LC-MS for proteomics analysis.

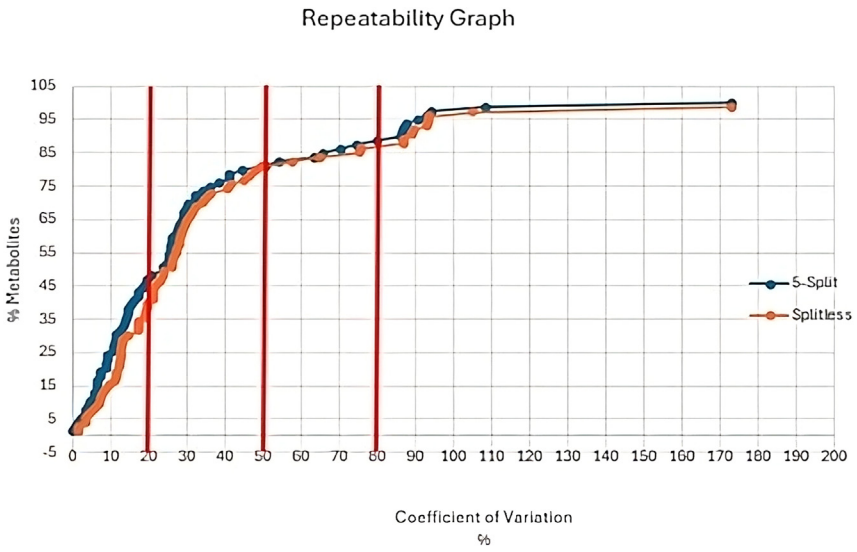

Figure 4. Coefficient of variation plot and the percentage of metabolites for the 1:5 split and splitless injections on the GCxGC-TOFMS

22. Inject 1 μg of peptide into the Thermo-Scientific Ultimate 3000 RS LC nano system equipped with a 5 μm × 20 mm C18 trap column (Thermo-Scientific) and a CSH C18 130A analytical LC column (Waters), with 75 μm × 250 mm dimensions and 1.7 μm particle size.
23. LC-MS information:
    a. Loading solvent: 2% ACN in water; 0.1% formic acid. Mobile phase A: 2% ACN in water; 0.1% formic acid. Mobile phase B: 100% ACN.
    b. Load the samples onto the trap column using the loading solvent.
    c. Set the solvent flow rate at 2 mL/min from a temperature-controlled autosampler at 7°C.
    d. Perform loading 5 min before eluting the sample into the analytical column.
    e. Set the flow rate to 300 nL/min and generate the gradient as follows: 5%–30% mobile phase B over 60 min and 30%–50% mobile phase B from 60–80 min.
    f. Perform chromatography at 45°C and deliver the outflow to the mass spectrometer.
    g. Acquire data in positive ionization mode.
    h. Use Maxquant software for data processing and Skyline for data visualization.

EXPECTED OUTCOMES

When running an untargeted GCxGC-TOFMS metabolomics experiment, an acceptable amount of analytical variation is considered to be a coefficient of variation (CV) value of less than 50%.[4] To test our protocol, we used human-derived FFPE brain tissue. The metabolomics repeatability results obtained from this protocol using a 1:5 split and splitless injection are shown in Figure 4. The percentage of metabolites is presented on the y-axis and the CV is on the x-axis. The red vertical lines indicate CV values at 20%, 50% and 80%. The percentage of metabolites obtained at the 20% CV value using a 1:5 split injection was 46%, which means that 46% of the total metabolites detected in the method had a CV of less than 20%. For 50% CV, the percentage of metabolites was 81%. Considering the sample type (FFPE human brain tissue), we also included a CV of 80%, which covers 87% of the metabolites.

The classes of metabolites obtained (Figure 5) include fatty acids, carboxylic acids, alcohols, amino acids, alkanes, organic acids, lipids, carbohydrates, and inorganic acids, although 35% of these metabolites have not yet been identified with sufficient (80%) confidence.

Previous studies have shown a decline in the total number of metabolites obtained from FFPE compared to fresh-frozen, mainly due to a loss of the polar compounds.[1,2,5] However, fatty acids

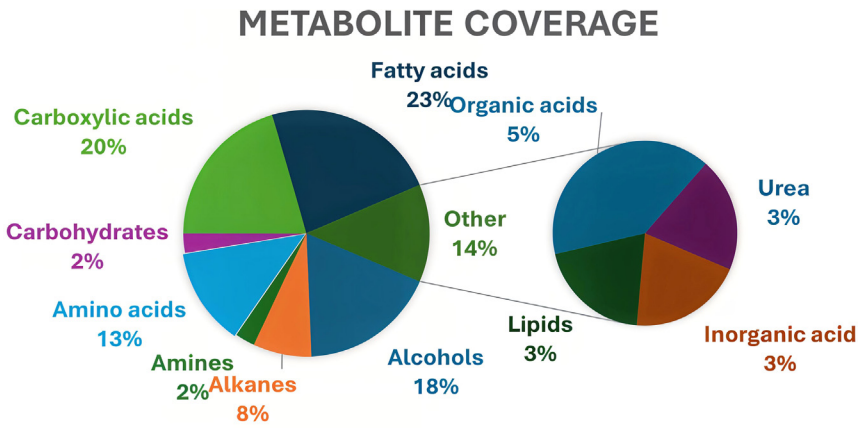

Figure 5. Classes of metabolites identified from the GCxGC-TOFMS metabolomics analysis of FFPE brain tissue, excluding unknown/unannotated compounds

and their esters can still be used in disease diagnosis and biomarker discovery. See 'Data S1: Metabolites' for a list of metabolites obtained from FFPE human brain tissue using this protocol.

Another problem encountered when extracting compounds, especially proteins, from FFPE tissue is the formation of cross-links during formalin fixation. Formalin-fixing and paraffin-embedding usually lead to a low protein identification from samples fixed and stored over long periods.[5] However, in this protocol, the total number of proteins obtained was 926 (Figure 6) using only 20 mg of tissue sample, with a false discovery rate of 1%. This is a significant improvement from previous studies.[5] See 'Data S2: Proteins' for a list of proteins obtained from FFPE human brain tissue using this protocol.

LIMITATIONS

One limitation of this protocol is the use of analytical equipment other than GCxGC-TOFMS for the metabolomics analysis and LC-MS for proteomics analysis. Using alternative analytical instruments might yield slightly different results because of variations in sensitivity among various analytical tools. However, if more sensitive instrumentation for the metabolomics will be used, it is advisable to proceed after the drying step (Step 3 of the graphical abstract), before derivatization.

Another limitation is the sample size. Working with less than 20 mg of tissue results in insufficient repeatability, making the analysis of limited quantity samples a challenge.

TROUBLESHOOTING

Problem 1

When using the vibration mill to homogenize the samples the micro-centrifuge tubes may crack, leading to loss of samples.

Potential solution

Use a 1.5 mL micro-centrifuge tube instead of a 2 mL tube because the 1.5 mL tube is sturdier. Ensure that the micro-centrifuge tubes are of high quality (see Step 1, point 1b).

Problem 2

Samples take too long to dry.

Potential solution

Use a nitrogen dryer instead of a SpeedVac for drying steps (see Step 1, point 2e; Step 2, point 7; and Step 4, point 17).

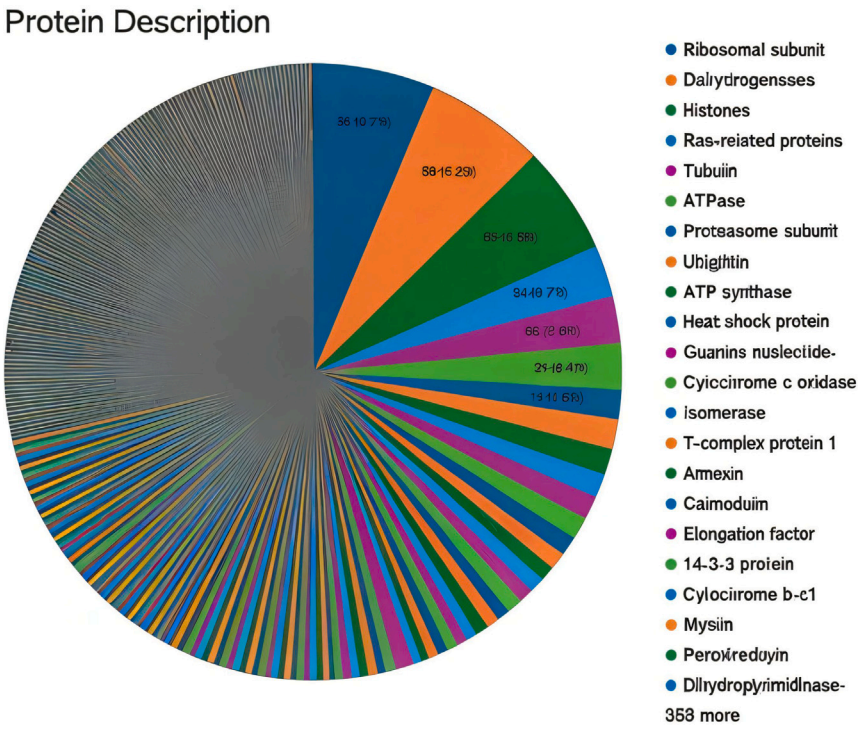

Figure 6. Protein species obtained from 20 mg FFPE brain tissue

Problem 3

An alternative solution is needed for apolar metabolite extraction.

Potential solution

Use chloroform instead of dichloromethane for apolar metabolite extraction. However, dichloromethane has proven to be more efficient in the extraction protocol (see sample preparation 3, point 9).

Problem 4

The volume of solution in the GC vial is too small for the needle to pick up.

Potential solution

Use a spring-bottom vial insert instead of a GC vial insert to accommodate low sample volume obtained after metabolomics extraction (see step 3, point 10).

Problem 5

The high cost of triethylammonium bicarbonate.

Potential solution

Use ammonium bicarbonate instead of triethylammonium bicarbonate (TEAB) (see step 5, point 21c).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shayne Mason (nmr.nwu@gmail.com).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Shayne Mason (nmr.nwu@gmail.com).

Materials availability

No new materials were generated from this protocol.

Data and code availability

See supplementary data (Data S1: Metabolites; Data S2: Proteins) for data availability.

ACKNOWLEDGMENTS

This research was supported by a Work Grant from the Amsterdam Institute for Infection and Immunity and, in part, by the National Research Foundation of South Africa.

AUTHOR CONTRIBUTIONS

A.R.I. wrote the manuscript and generated the images. All coauthors provided feedback, comments, and/or edits on various drafts of the manuscript. All authors approved of the final version of this manuscript. S.M., M.v.d.K., M.T.v.F., and D.T.L. provided supervision for A.R.I. S.M. conceptualized the manuscript and administered the project. M.v.d.K. and M.T.v.F. secured funding for the project. A.R.I., S.M., L.L., and D.T.L. were involved in the formal analysis. L.L. assisted with image corrections. S.M., L.L., D.T.L., and A.A.W. supervised A.R.I. with the metabolomics aspects. M.V. and N.N.C. supervised A.R.I. with the proteomics aspects.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103442.

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