Insights into Phosphatidylethanol (PEth) 16:0/18:1 Formation and Degradation Analysis by Supercritical Fluid Chromatography-Tandem Mass Spectrometry

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21st February 2025

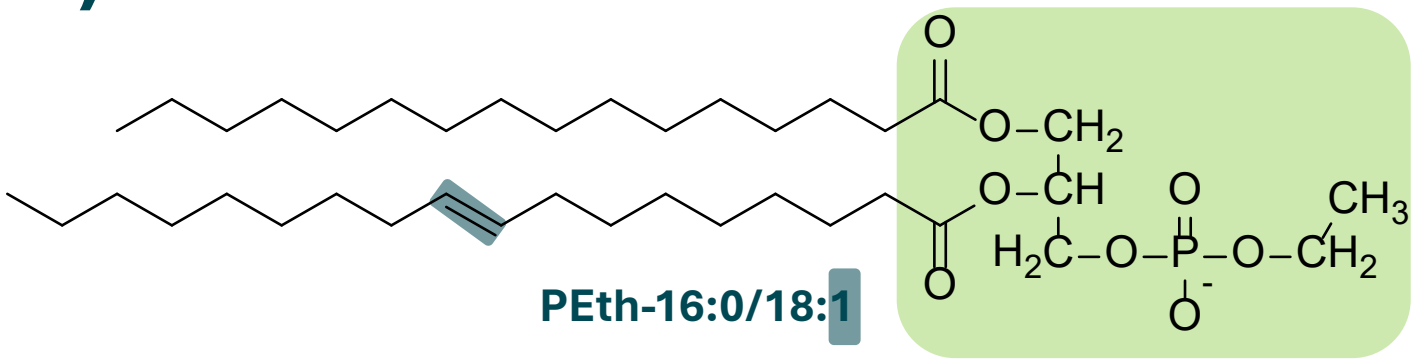
EMERGING FORENSIC SCIENTIST AWARD

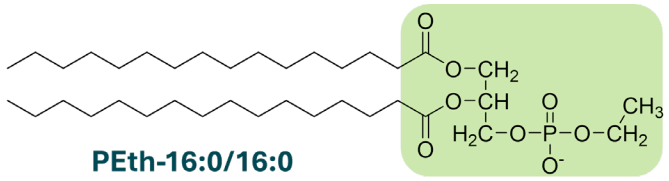
Disclaimer

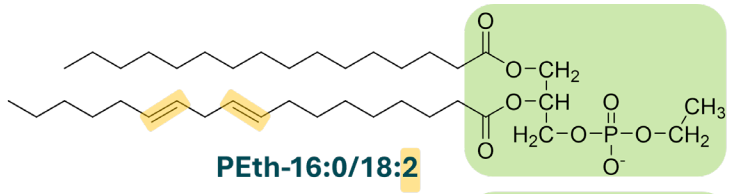
- The views and opinions expressed in this presentation are solely mine, and do not represent any official views or opinions of the American Academy of Forensic Sciences.
- I have no financial or conflicts of interest to disclose.

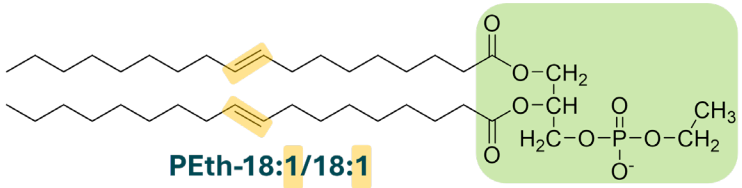
Phosphatidylethanol (PEth)

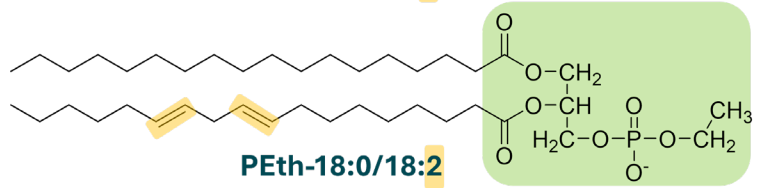
- Abnormal phospholipids formed in erythrocyte membranes in the presence of ethanol
- PEth 16:0/18:1 predominant homologue
- Highly correlated to alcohol intake, even at low doses
- Relatively insensitive to unintentional alcohol exposure
- Long detection window (~12 days) after single drinking event

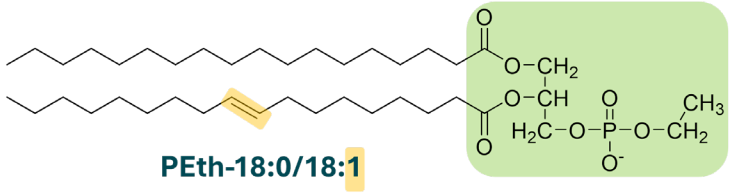


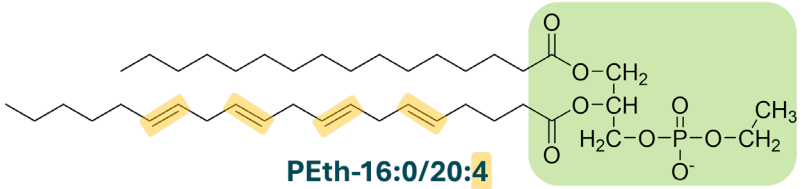












PEth Testing

- Detect changes in drinking behavior earlier and more accurately compared to other biomarkers (e.g. EtG, EtS etc)
  - Useful for abstinence monitoring, diagnosing alcohol use disorder
- Postmortem (PM) PEth provides information on alcohol consumption in the weeks preceding death
  - Useful in determining chronic drinking in cases where alcohol is absent
- 2022 Consensus of Basel
  - *Harmonize PEth analysis and interpretation on a global scale*

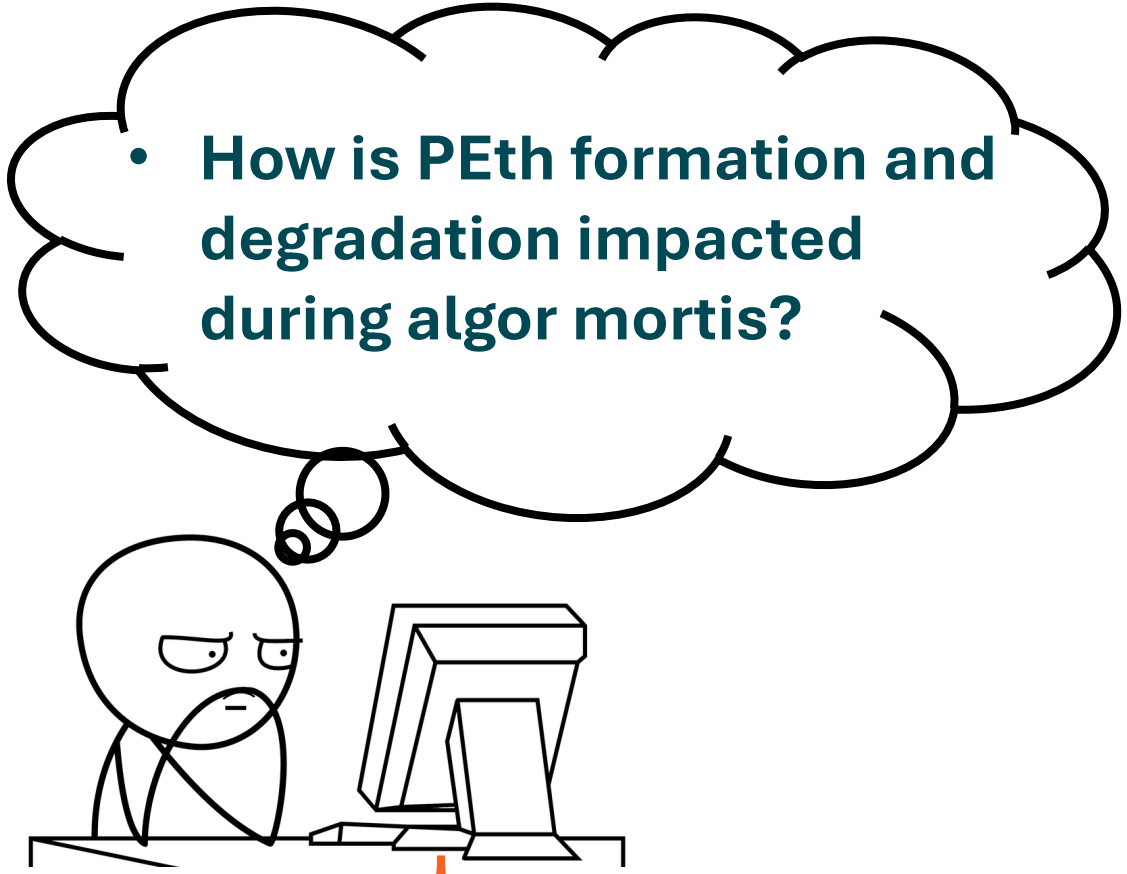
Received: 24 June 2022 | Accepted: 27 June 2022
DOI: 10.1002/dta.3340

LETTER TO THE EDITOR

WILEY

Consensus for the use of the alcohol biomarker phosphatidylethanol (PEth) for the assessment of abstinence and alcohol consumption in clinical and forensic practice (2022 Consensus of Basel)

PEth 16:0/18:1 concentration cutoff	Interpretation
<20 ng/ml	Compatible with abstinence or low alcohol consumption
≥20 ng/ml but <200 ng/ml	Alcohol consumption
≥200 ng/ml	Strongly suggestive of chronic excessive alcohol consumption



Drinking event

+

Time of death

Blood collection and storage at -80°C

- Risk of post-sampling formation of PEth in blood containing ethanol
  - Dried blood spots
  - PLD inhibitors (FIPI, $NaVO_3$, Na_2WO_4)

Research Objectives

1. Develop and validate the first SFC-MS/MS method to quantify PEth 16:0/18:1 in PM blood
   - *per ANSI/ASB 036 recommendations and in-house validation protocols*

Liquid-liquid extraction

- Weigh *blood (0.25 g)*
- Add *250 µL of pH 9 borate buffer (0.6M)*
- Add *2 mL of 80:20 (v/v) heptane:2-propanol*
- Centrifuge (4000 rpm, 10 minutes)
- Freeze at *−80°C* for 15 min
- Decant organic layer
- Evaporate to dryness at 40°C under nitrogen (1.5 mL/min, 15 min)
- Reconstitute in *200 µL methanol*

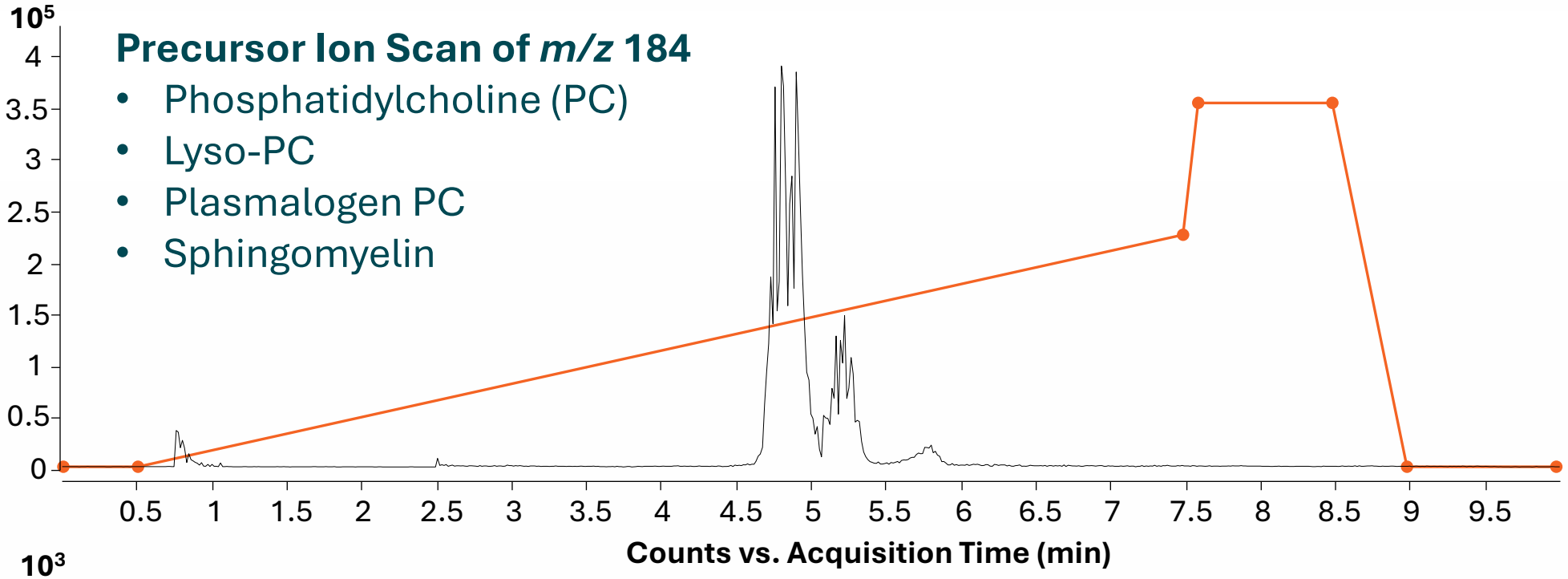
Method Parameters

Agilent 1260 Infinity II SFC control module and 1260 Infinity II Liquid Chromatograph	
Column and Temperature (°C)	Waters Corp Torus DEA column (1.7 µm, 3 mm X 100 mm) at 50°C
Mobile Phase	**A**: Supercritical CO_2 **B**: 0.1% ammonium acetate in 95:5 methanol: water
Flow Rate	1.0 mL/min
Injection volume	2 µL

Agilent Ultivo Triple Quadrupole Mass Spectrometer	
Ionization type	Electrospray Ionization (ESI) in negative mode
Acquisition Type	Multiple Reaction Monitoring
PEth 16:0/18:1	**Quantifier**: *m/z* 701.5 → *m/z* 281.2; **Qualifier**: *m/z* 701.5 → *m/z* 255.2
PEth 16:0/18:1-d_5	**Quantifier**: *m/z* 706.5 → *m/z* 281.2

Precursor Ion Scan of *m/z* 184

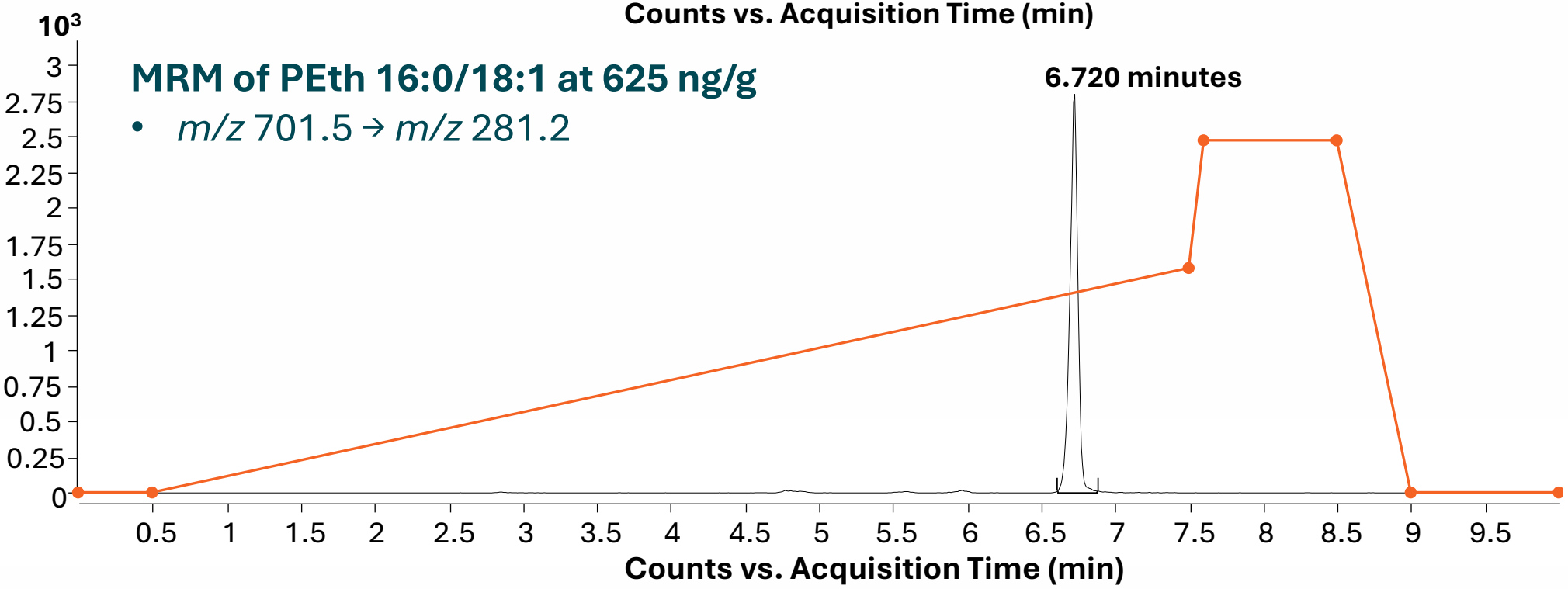
- Phosphatidylcholine (PC)
- Lyso-PC
- Plasmalogen PC
- Sphingomyelin



Gradient	
Minutes	% B
0	1
0.5	1
7.5	45
7.6	70
8.5	70
9	1
10	1

MRM of PEth 16:0/18:1 at 625 ng/g

- *m/z* 701.5 → *m/z* 281.2



Parameters	Validation Procedure **LQC: 20 ng/g, MQC: 200 ng/g, HQC: 2000 ng/g*	Results
Recovery (%)	Evaluated at LQC and HQC concentrations in 10 sources	59% (LQC), 48% (HQC)
Linearity	Least square regression and residual plot with 6 non-zero calibrators over 8 days	Linear 1/x between 10 – 2500 ng/g
LOD/LLOQ	Lowest calibrator extracted in triplicate with 3 autopsy sources over 3 runs (n=9)	LOD administratively set at LLOQ (10 ng/g)
Bias	Extracted in triplicate over 8 days	-16.4 to 17.7% (LQC), -8.8 to 12.5 % (MQC), -15.2 to 10.4% (HQC)
Within-run %CV		2.9 to 17.1% (LQC), 0.7 to 7.6% (MQC), 1.3 to 10.5% (HQC)
Between-run %CV	Combined replicate pools of triplicate extracted QCs over 8 days	8.3% (LQC), 5.6% (MQC), 5.7% (HQC)
Matrix Effects	Post-extraction addition with 10 sources (n=20)	20% (LQC), 1% (HQC)
Interference studies	Monitor (1) Negatives, (2) neat homologues (PEth 18:0/18:1, 16:0/16:0, 18:1/18:1, 18:0/18:2, 16:0/18:2, and 16:0/20:4), (3) 10 'blank' autopsy sources, (4) ULOQ with no internal standard	No interferences
Dilution integrity	1:5 dilution of HQC (n=5)	-17.6 % (Bias), 2.3% (Precision)
Carryover	Re-injecting extracted blank after highest calibrator (2500 ng/g) over 8 runs	No carryover
Processed sample stability	Peak area of PEth/PEth-d_5 after 72 hrs at 8°C for 20 ng/g (LQC) and 2000 ng/g (HQC) concentration	≤ 15% signal decrease from the t_0 response

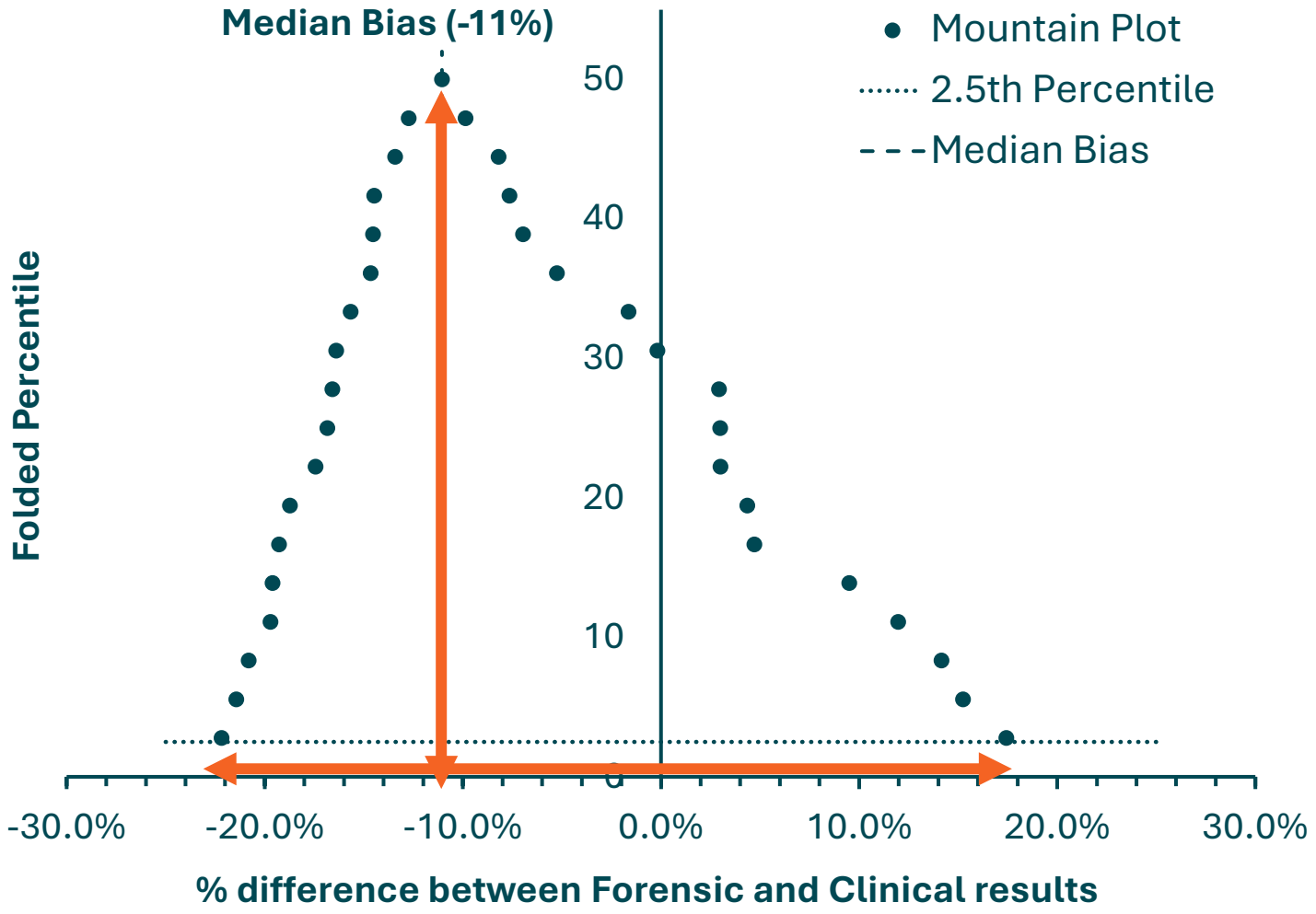
Proof-of-concept:

- 35 autopsy samples ranging between 32.6 to 2476 ng/g
- Mountain plot to illustrate differences between clinical and forensic method

Clinical: *SPE and LC-MS/MS*

Forensic: *LLE and SFC-MS/MS*

- Forensic SFC-MS/MS method generally quantified lower PEth levels than clinical LC-MS/MS method
  - Changes in PM blood could contribute to differences



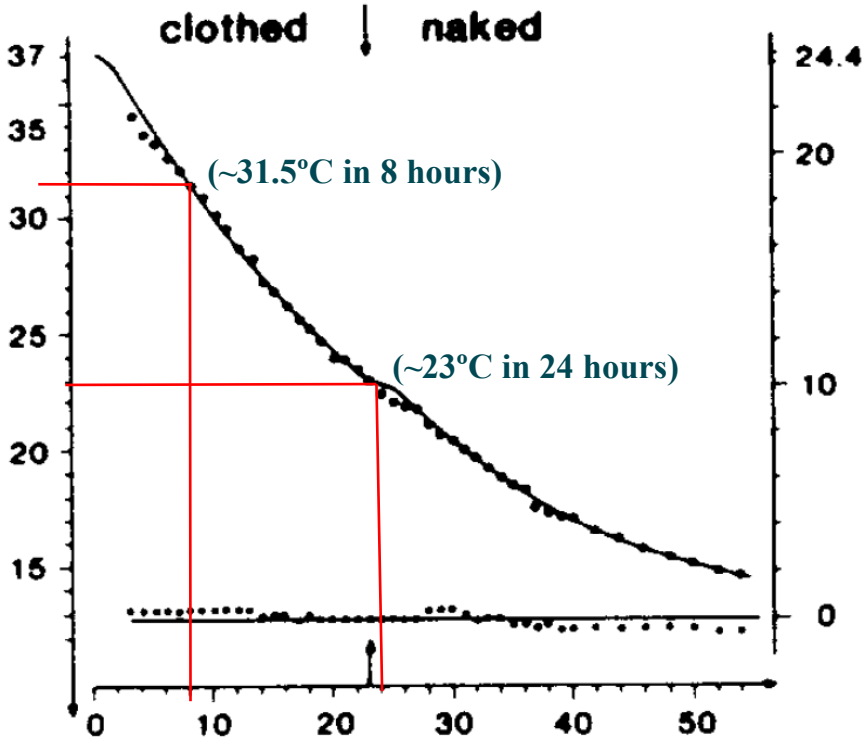
Research Objectives

1. Develop and validate the first SFC-MS/MS method to quantify PEth 16:0/18:1 in PM blood
   - *per ANSI/ASB 036 recommendations and in-house validation protocols*
2. Develop a simple *in vitro* model to simulate PEth 16:0/18:1 formation and degradation in the first 48 hours postmortem

Henssge's Nonogram

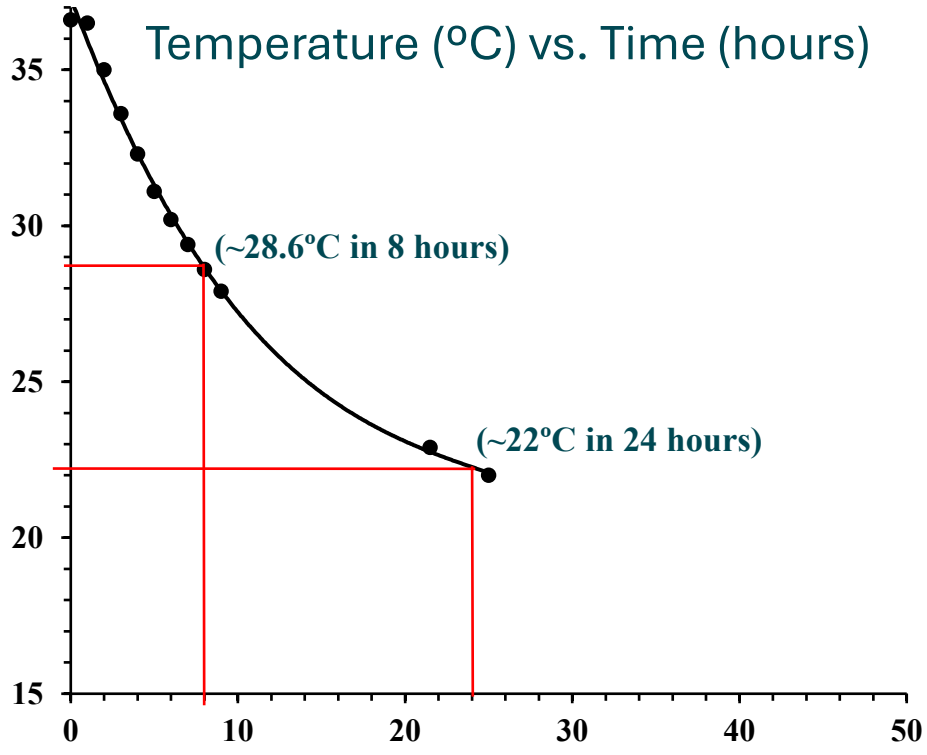
- *Cooling of body as sigmoidal function*
- *Initial plateau before cooling according to Newton's law of cooling*
- *Simplified model offers fair estimates*

Henssge's cooling curve



***in vitro* model**

Temperature (°C) vs. Time (hours)



Assumptions:

1. *Core body temp. at time of death is approx. 37°C*
2. *Body temperature PM would reach ambient (22°C) in 24 hours*

BAC 0 g/L and heparinized

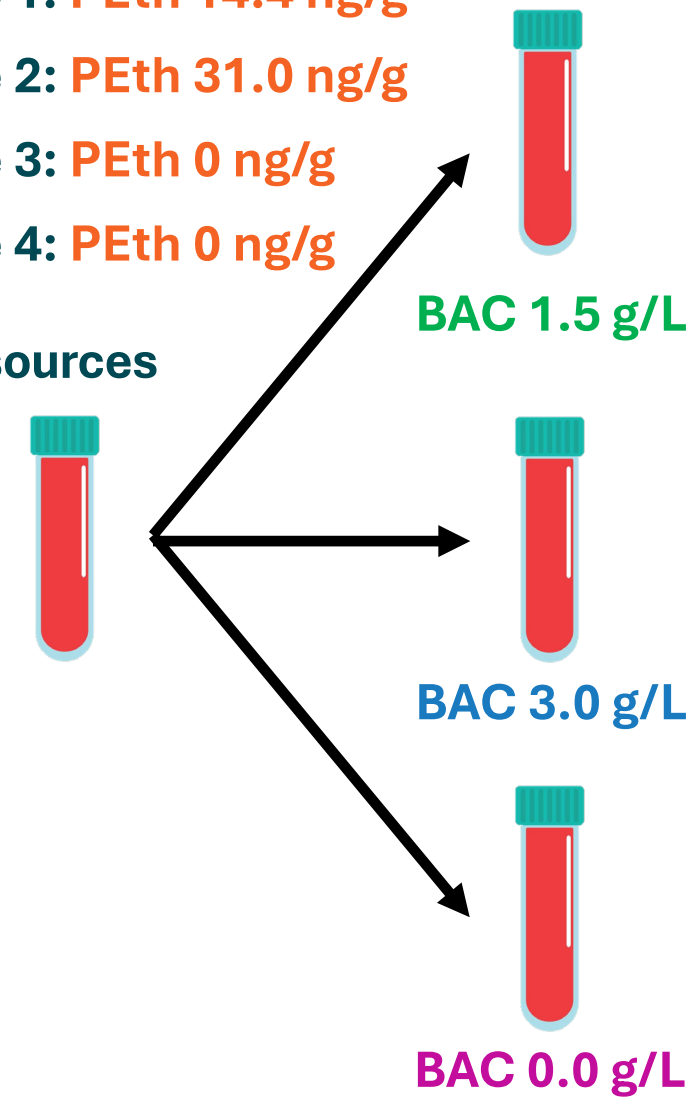
Source 1: PEth 14.4 ng/g

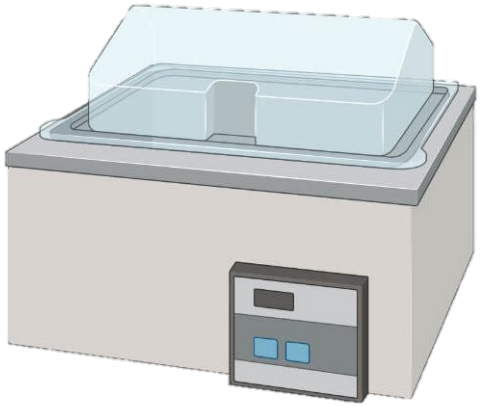
Source 2: PEth 31.0 ng/g

Source 3: PEth 0 ng/g

Source 4: PEth 0 ng/g

4 sources



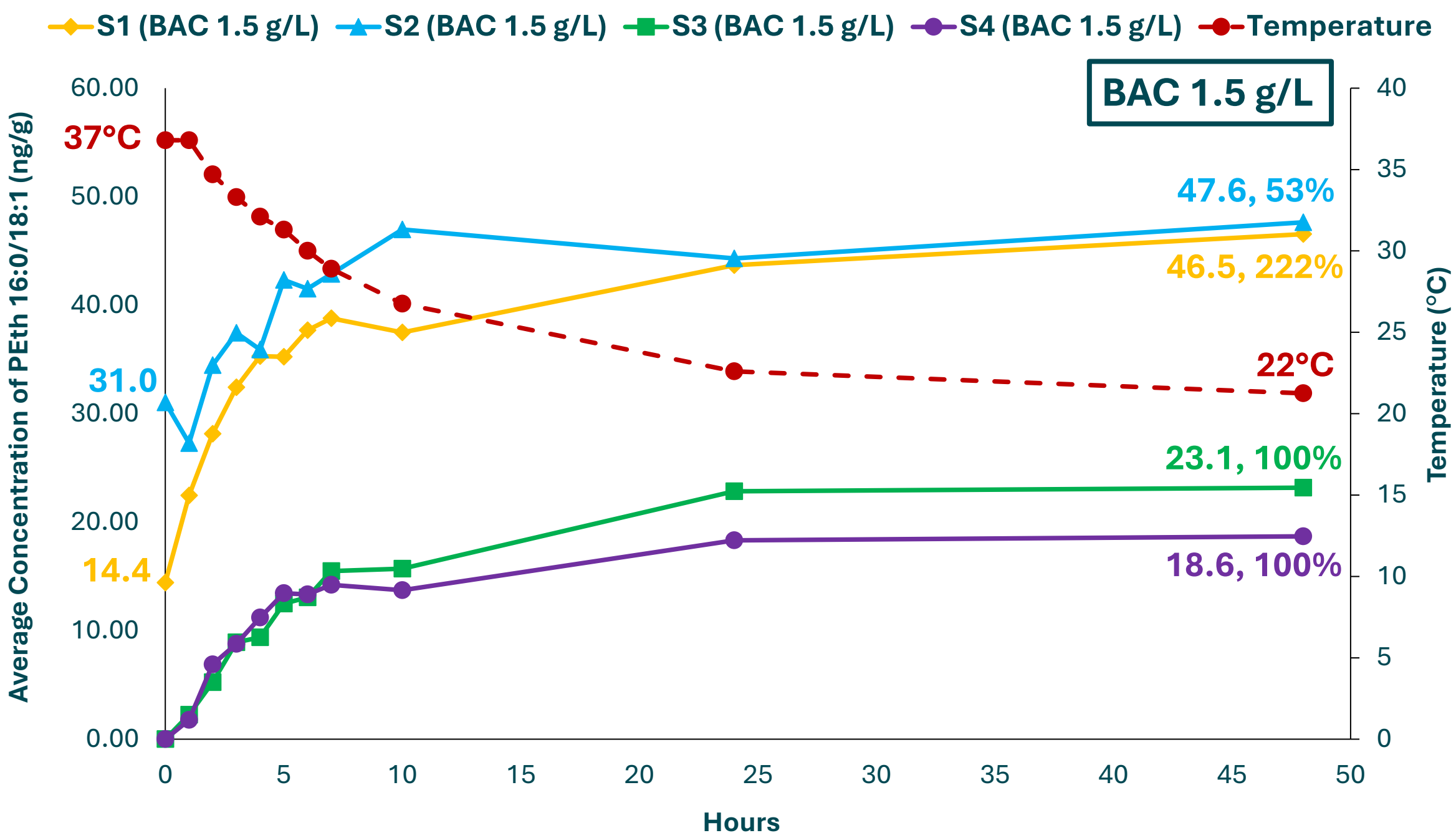


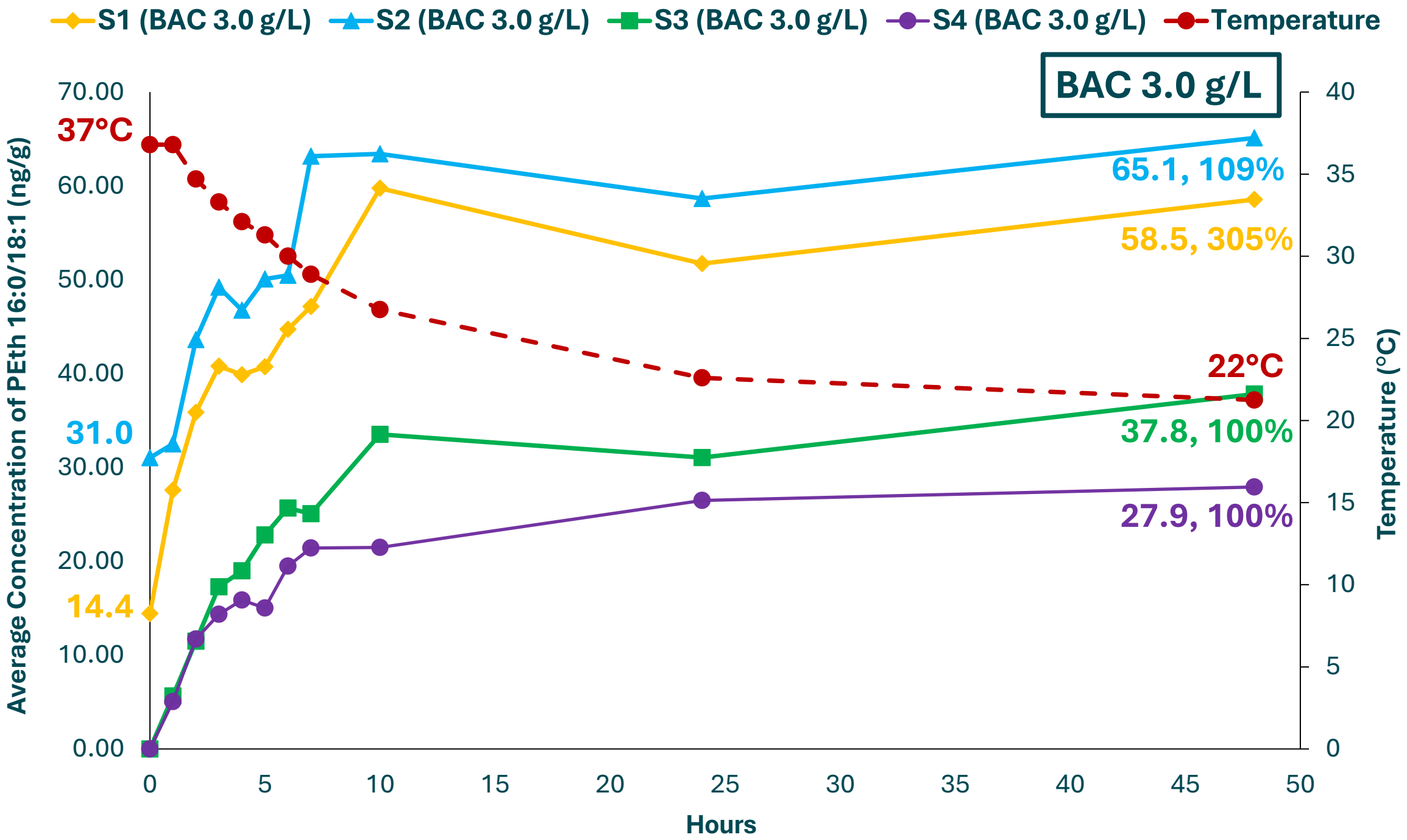
- *Set at 37°C for 1 hour*
- Turned off with lid on
- Left to cool back down to room temp. (22°C) over time

x2

Aliquot in duplicate per each source

- Once an hour for *7 hours*
- After *24 hours*
- After *48 hours*
- Recorded temperature during each sampling


S1 (BAC 1.5 g/L)
S2 (BAC 1.5 g/L)
S3 (BAC 1.5 g/L)
S4 (BAC 1.5 g/L)
Temperature
BAC 1.5 g/L
Average Concentration of PEth 16:0/18:1 (ng/g)
Temperature (°C)
Hours
37°C
22°C
47.6, 53%
46.5, 222%
23.1, 100%
18.6, 100%
31.0
14.4
60.00
50.00
40.00
30.00
20.00
10.00
0.00
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
35
40
45
50


S1 (BAC 3.0 g/L)
S2 (BAC 3.0 g/L)
S3 (BAC 3.0 g/L)
S4 (BAC 3.0 g/L)
Temperature
BAC 3.0 g/L
37°C
22°C
65.1, 109%
58.5, 305%
37.8, 100%
27.9, 100%
31.0
14.4
Average Concentration of PEth 16:0/18:1 (ng/g)
Temperature (°C)
Hours
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
40
35
30
25
20
15
10
5
0
0
5
10
15
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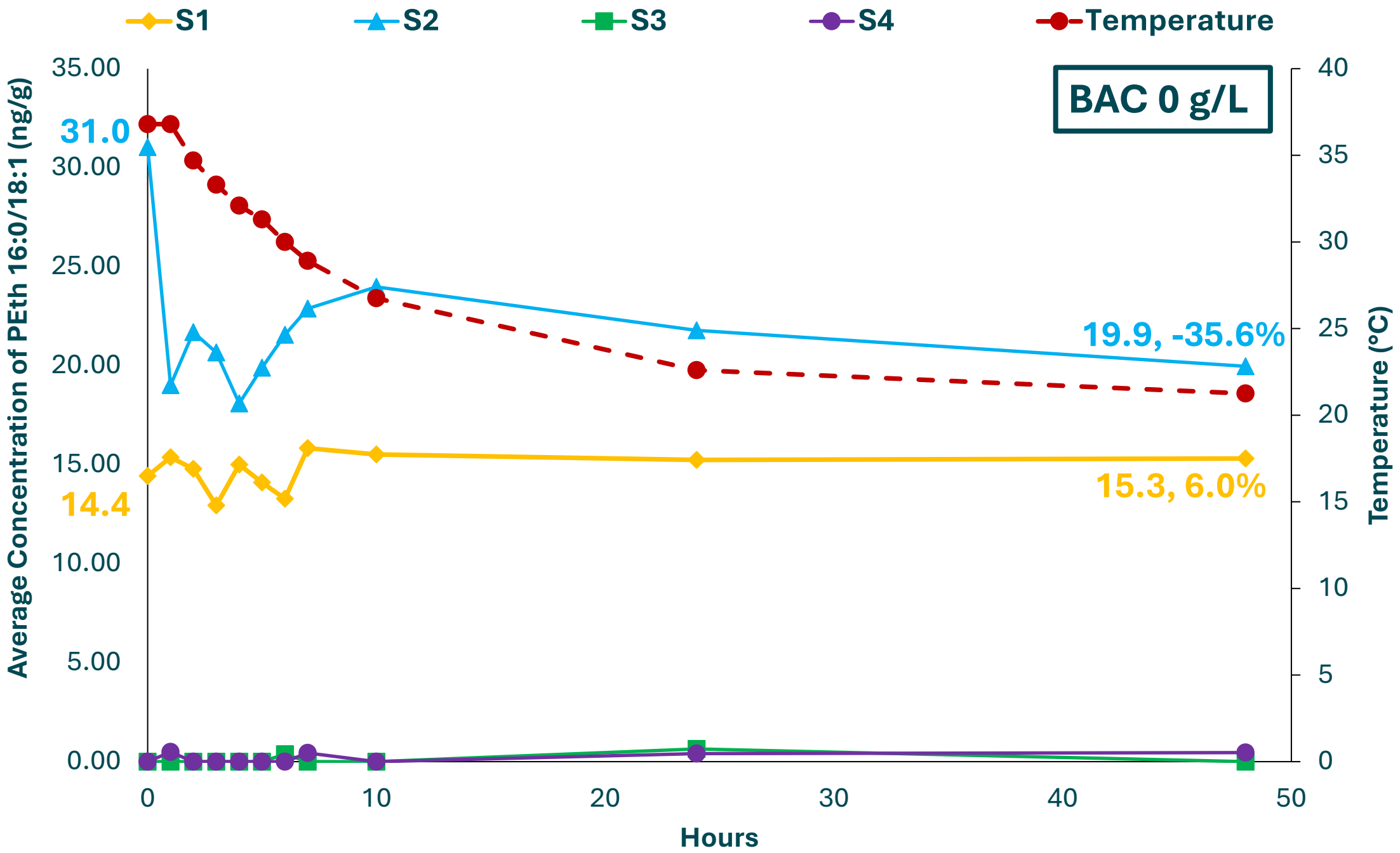
25
30
35
40
45
50

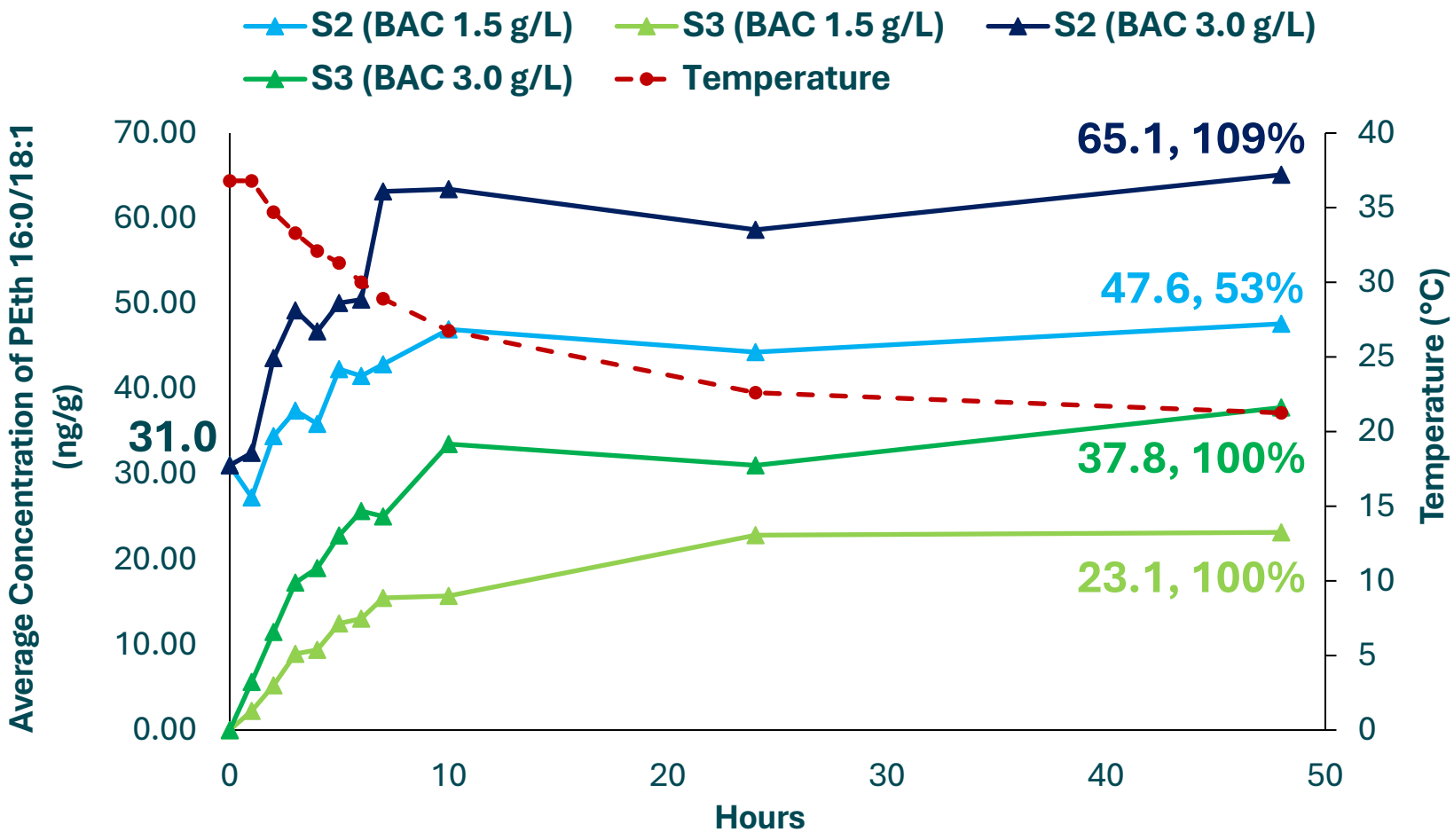

S1
S2
S3
S4
Temperature
BAC 0 g/L
Average Concentration of PEth 16:0/18:1 (ng/g)
Temperature (°C)
Hours
31.0
14.4
19.9, -35.6%
15.3, 6.0%
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50

Results

1. Formation occurs and decreases at lower temperatures, levels before reaching room temperature, and with minimal PEth formation after 10 hours
   - Simultaneous formation and degradation of PEth 16:0/18:1
2. PEth formation occurs in an ethanol concentration-dependent manner
   - Statistically significant ($\alpha=0.05$) difference between BAC 1.5 g/L and 3.0 g/L groups
3. Results from our study aligns with previous findings from Schröck *et al.* (2018) study of *in vitro* PEth formation at 37°C for 7 hours

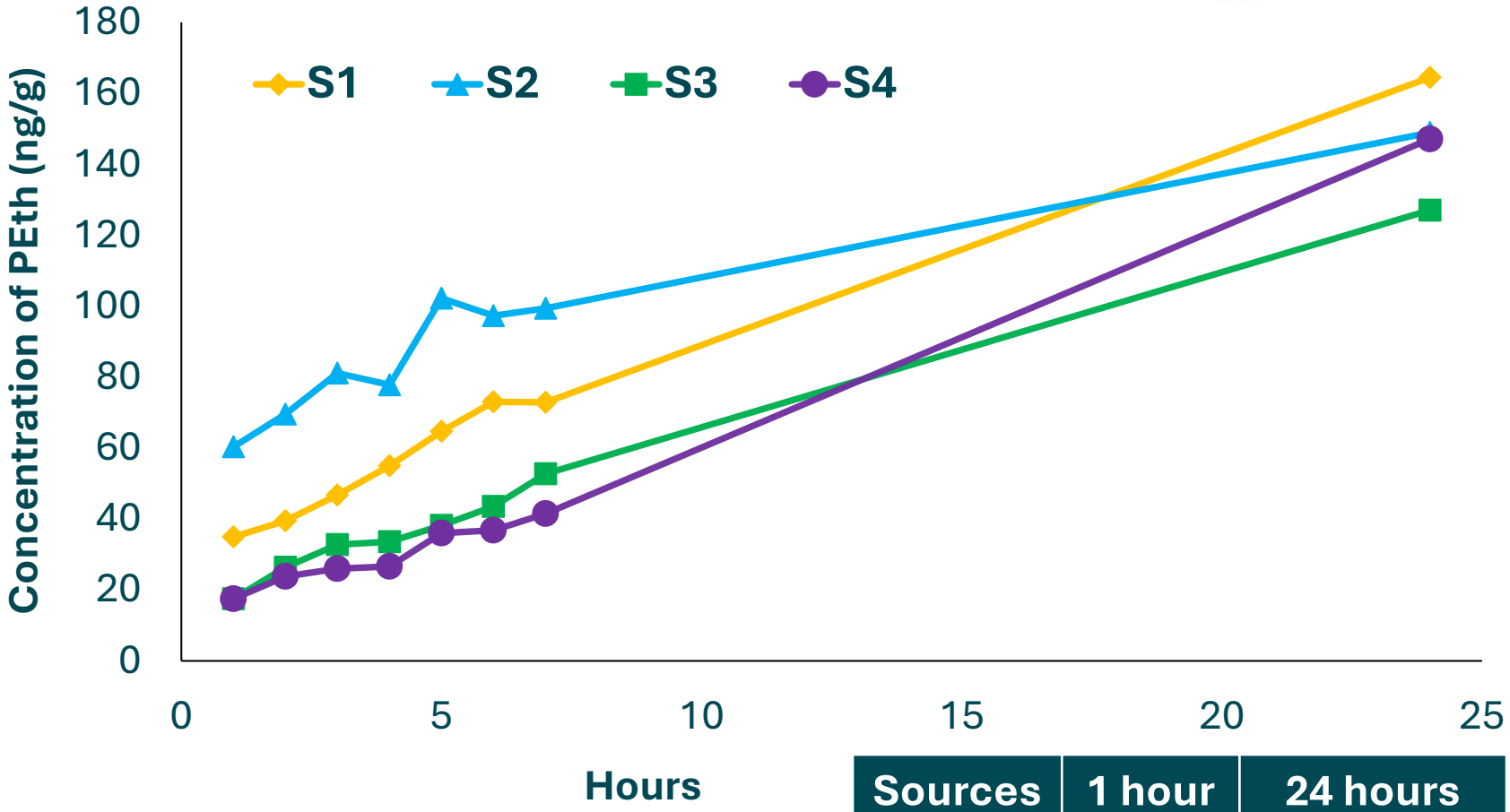
Conclusions

- At temperatures below 37°C, phospholipase D remains active at a lower rate and continues to produce PEth
  - Interindividual variability in PEth formation
- Temperature is a rate-limiting factor
  - Limited BAC decline (5.2-22.6% decrease)



Follow-Up

- Same 4 blood sources fortified to BAC 3.0 g/L and incubated at 37°C for 24 hours
- Continuous PEth formation with higher final PEth concentrations
- Consistent with findings by Schröck et al. (2018)



Sources	1 hour (ng/g)	24 hours (ng/g)
1	34.9	164.4
2	60.3	148.9
3	17.4	127.0
4	17.4	147.1

Summary

- Measured PEth not an accurate representation of exact levels at time of death
- Cases with high BAC and delayed sample collection are more prone to PM PEth formation
  - Extra considerations needed if decedent's body has been left at elevated temperatures
- *in vitro* modelling cannot be directly extrapolated to fully replicate complex physiological processes that occur within the human body
  - Larger sample size needed
- Findings reinforce key considerations in the 2022 Consensus of Basel on PEth interpretation
  - Necessity to account for relevant, case-specific factors

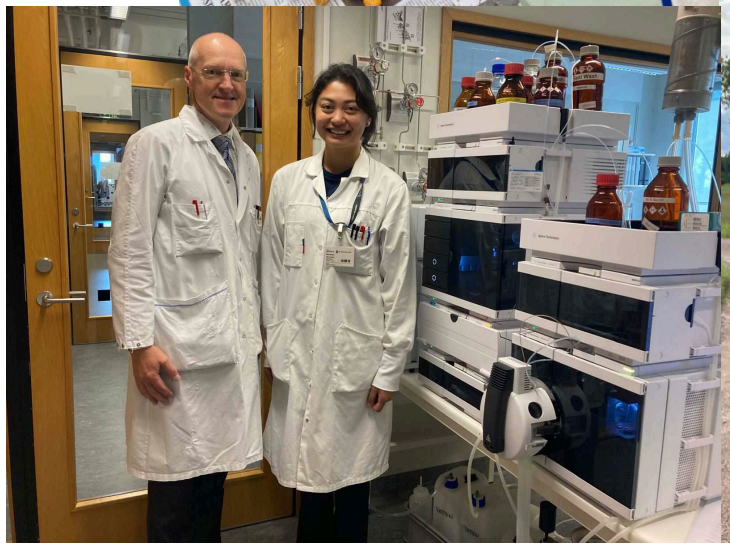
Acknowledgements

- Markus Roman
- Dr. Britni Skillman
- Dr. Michael Truver
- Dr. Robert Kronstrand

RÄTTSMEDICINALVERKET

Sam Houston State University

INSTITUTE FOR FORENSIC RESEARCH TRAINING AND INNOVATION



Coming soon on the Journal of Analytical Toxicology!

Title: Quantification of Phosphatidylethanol 16:0/18:1 in blood using Supercritical Fluid Chromatography-Tandem Mass Spectrometry

Questions?

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