Fresh Paraformaldehyde Preserves Thrombus Biochemistry

An ATR–FTIR and PCA Investigation of Pulmonary Artery Thrombi

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Abstract

Background Biochemical analyses of pulmonary embolism derived thrombi depend critically on the fixative quality.

Objective To quantify the impact of paraformaldehyde (PFA) shelf life on thrombus molecular integrity using attenuated total reflectance Fourier transform infrared (ATR–FTIR) spectroscopy and principal component analysis (PCA).

Methods Ten pulmonary artery thrombi retrieved during catheter directed mechanical thrombectomy for acute pulmonary embolism were fixed in either aged paraformaldehyde (PFA; 6 months; n=6) or freshly prepared 4% PFA (n=4); an in-vitro plasma clot control received the same fresh PFA. Triplicate ATR–FTIR spectra ($400\,\mathrm{cm}^{-1}$–$4000\,\mathrm{cm}^{-1}$) were collected, pre-processed (rubber band baseline correction, Savitzky–Golay smoothing), and vector normalized to the Amide I band before PCA. Fixative chemistry was assessed by the carbonyl absorbance at $1700\,\mathrm{cm}^{-1}$.

Results Freshly fixed thrombi displayed narrow Amide I ($1650\,\mathrm{cm}^{-1}$) and Amide II ($1540\,\mathrm{cm}^{-1}$) bands with a higher signal-to-noise ratio (SNR; median 41.8) than aged-PFA samples (18.2; $P < 0.01$). Carbonyl absorbance at $1700\,\mathrm{cm}^{-1}$ was markedly higher in fresh PFA. PCA showed clear separation of fresh versus aged specimens and alignment of fresh thrombi with plasma controls across spectral windows.

Conclusions PFA solutions older than three months markedly deteriorate thrombus biochemical fidelity. ATR–FTIR combined with PCA offers a rapid quality-control assay prior to molecular analyses.

Keywords: Clots; Fixation; Infrared Spectroscopy; Pulmonary Embolism

1 Introduction

Pulmonary vascular disease is characterized by tightly coupled disturbances in haemostasis, fibrinolysis, inflammation, and endothelial activation. In idiopathic pulmonary arterial hypertension (IPAH), Kopeć and colleagues demonstrated elevated fibrinolytic activity (tPA and plasmin antiplasmin complexes) alongside increased IL-6 and endothelin-1, with strong interrelations among these pathways, underscoring the biochemical lability of thrombus related markers within the pulmonary circulation [1].

At the tissue scale, the architecture of embolic material in acute pulmonary embolism (PE) can inherently limit fibrinolysis. Scanning electron microscopy of a pulmonary artery embolus revealed tightly packed, compact fibrin networks with relatively fewer platelets centrally and erythrocyte rich peripheral segments, an organization associated with impaired permeability and resistance to lysis [2].

Systemic inflammatory milieus further bias fibrin toward dense, lysis resistant networks. In Chronic Obstructive Pulmonary Disease (COPD), plasma forms clots with reduced permeability, prolonged lysis time, and denser fibrin ultrastructure; these properties correlate with inflammatory burden and are partly amenable to statin therapy [3]. Together, these clinical and biophysical observations motivate careful biochemical preservation and quality control when analysing pulmonary artery thrombi.

Paraformaldehyde (PFA) serves as a polymeric formaldehyde source that creates covalent crosslinks stabilising protein structure; with storage, depolymerisation reduces available aldehydes and may compromise preservation [4]. Fourier transform infrared (ATR–FTIR) spectroscopy provides rapid, label free biochemical readouts spanning proteins, lipids, and nucleic acids [5, 6]. When paired with principal component analysis (PCA), small fixation-driven spectral shifts become detectable [7, 8]. Given that fixative chemistry can dominate pre analytical variance, verifying PFA freshness prior to immersion is essential to preserve diagnostically informative features and ensure reproducible molecular phenotyping of PE thrombi.

The PFA freshness has been studied in brain and tumour biopsies [9], yet its influence on thrombus biochemistry remains poorly characterised. We hypothesised that aged PFA compromises thrombus molecular preservation, producing spectral signatures discernible by ATR–FTIR and PCA.

2 Materials and Methods

2.1 Ethics Approval and Consent

The study was approved by the Ethics Committee of Krakowski Szpital Specjalistyczny im. Św. Jana Pawła II (Protocol #2023-152, 14 February 2023). Written informed consent was obtained from each participant or legal representative.

2.2 Safety Statement

All protocols involving PFA were conducted in certified chemical fume hoods, with lab coats, gloves, and safety glasses. Human specimens were handled under Biosafety Level 2 (BSL-2) precautions. All biological material and chemical waste were decontaminated or autoclaved prior to disposal.

2.3 Patient Cohort and Thrombus Retrieval

Ten pulmonary artery thrombi were retrieved via catheter directed mechanical thrombectomy for acute PE between March 2023 and May 2024 at Krakowski Szpital Specjalistyczny im. św. Jana Pawła II [10, 11]. Cohort characteristics are summarised in Table 1, with full comorbidities in Supplementary Table S1.

Table 1. Condensed patient demographics for the two fixation batches.

Batch	n	Median age (y)	Female (%)	Diabetes (%)
Aged PFA[a]	6	64.5	33.3	33.3
Fresh PFA[b]	4	62.0	50.0	25.0

[a] Aged PFA = paraformaldehyde solution prepared earlier (previous batch).
[b] Fresh PFA = paraformaldehyde solution prepared immediately before fixation.
Legend: n = number of patients; y = years; percentages are within-batch proportions.

2.4 Baseline Clinical Characteristics and Subgroup Stratification

Table 2 summarises baseline clinical variables (medians) stratified by history of deep vein thrombosis (DVT; DVT^+ vs DVT^-) and by age (55 vs >55 years).

Subgroup definitions. Patients were stratified by (i) history of deep vein thrombosis (DVT) DVT^+ if a prior or concomitant DVT was documented at presentation; DVT^- otherwise and (ii) age dichotomised at 55 years. The 55 year cut point was pre specified to improve comparability between groups in this small cohort and to aid interpretability; it was not used for hypothesis testing.

Table 2. Baseline clinical variables by DVT status and age group (median [IQR]; units indicated).

	DVT status		**Age group**	
Variable	**DVT^+ (n=5)**	**DVT^- (n=5)**	**≤55 y (n=4)**	**>55 y (n=6)**
sPESI	2.00 [1–3]	2.00 [1–3]	2.00 [1–3]	2.00 [1–3]
D-dimer baseline ($ng\,mL^{-1}$)	10 685 [9 500–12 000]	4 102 [3 800–4 500]	9 010 [8 000–10 500]	6 958 [5 500–7 800]
Fibrinogen baseline ($g\,L^{-1}$)	3.81 [3.50–4.10]	3.13 [3.00–3.40]	3.48 [3.30–3.60]	3.47 [3.40–3.60]
hs-Troponin ($ng\,L^{-1}$)	0.04 [0.03–0.05]	0.04 [0.03–0.05]	0.04 [0.03–0.05]	0.06 [0.04–0.08]
NT-proBNP ($pg\,mL^{-1}$)	5 642 [4 500–6 800]	2 294 [2 000–2 700]	5 202 [4 800–5 900]	3 968 [3 500–4 500]
hsCRP ($mg\,L^{-1}$)	68.0 [50–90]	114.6 [100–130]	120.2 [100–140]	80.2 [60–100]
Lactate ($mmol\,L^{-1}$)	1.90 [1.6–2.2]	1.20 [1.0–1.5]	1.80 [1.5–2.0]	1.60 [1.4–1.8]

Legend: DVT^+ = documented prior or concomitant deep vein thrombosis; DVT^- = no such history; n = group size; values are median [IQR]; units as shown. Abbreviations: sPESI = simplified Pulmonary Embolism Severity Index; hsCRP = high-sensitivity C-reactive protein; NT-proBNP = N-terminal pro–B-type natriuretic peptide; hs-Troponin = high-sensitivity cardiac troponin.

2.5 Fixative Preparation and Quality Control

Fresh paraformaldehyde (PFA). Paraformaldehyde powder (4 g) was dissolved in 100 mL 0.01 N Phosphate-buffered saline (PBS) at 60 °C with gentle stirring; a few drops of 2 N sodium hydroxide (NaOH) aided dissolution without exceeding 65 °C. Once clear, the pH was adjusted to 7.2–7.4 with 1 M hydrochloric acid (HCl). The solution was sterile-filtered (0.22 µm) and stored at 4 °C (30 days) or −20 °C (90 days).

Aged PFA. An identical solution stored at room temperature for six months.

QC Metrics. Each batch followed the standard operating procedure in Table 3; carbonyl absorbance at 1700 cm^{-1} was measured by ATR–FTIR.

Table 3. Fixative quality control standard operating procedure.

Step	Measurement	Acceptance Criterion	Rationale
1	Visual inspection	Clear, colorless	Initial degradation screen
2	ATR–FTIR carbonyl peak	Absorbance $\geq$ 0.25	Free aldehyde content
3	pH at 25 °C	7.2–7.4	Optimal cross-linking
4	Shelf-life	90 days	Empirical integrity limit

2.6 Sample Fixation and Storage

Each thrombus was fixed for 24 h at 4 °C in either aged PFA (Batch I, n=6) or fresh PFA (Batch II, n=4), washed (3 × 10 min PBS), and stored at 4 °C for up to three months. Chemical composition and spectra for all thrombi and PFA solutions are presented in Fig. 1, with anonymized labels corresponding to each specimen.

2.7 Plasma Clot Control

Platelet-poor plasma from the Regional Centre for Blood Donation and Blood Treatment in Kraków was thawed and mixed with a thrombin working solution in Tris-HCl buffer. After incubation at 37 °C for 30 min to 60 min, clots were washed, fixed in freshly prepared 4% PFA for at least 2 hours at room temperature or overnight at 4 °C, then rinsed three times (10 minutes each) with Tris-HCl buffer. Dehydration was performed in a vacuum desiccator (relative humidity ≈ 2%) over anhydrous calcium sulfate. Mass was monitored until stable (4 days) prior to ATR–FTIR analysis.

2.8 ATR–FTIR Spectroscopy

Dried samples ($\approx$ 10 mg) were ground, placed on a diamond ATR crystal (Nicolet 6700), and scanned ($400\,\mathrm{cm}^{-1}$–$4000\,\mathrm{cm}^{-1}$, 128 scans, $4\,\mathrm{cm}^{-1}$ resolution). Spectra were averaged, atmospheric-compensated, rubber-band corrected, smoothed (Savitzky–Golay, 2nd order, 9-point window), and vector-normalized to the Amide I band.

Deep Vein Thrombosis subgroup. Thrombi were classified as originating from patients with a documented history of lower limb DVT (DVT^{+}) or without such history (DVT^{-}); spectra were averaged in OriginLab 2025.

Age-stratified subgroup. Patients were split at 55 years (55 vs >55) and spectra averaged.

2.9 Principal Component Analysis

PCA was conducted in OriginLab 2025 (OriginLab Corporation, Northampton, USA) on three spectral regions: the biochemical fingerprint window ($900\,\mathrm{cm}^{-1}$ to $1800\,\mathrm{cm}^{-1}$), the protein-associated Amide I+II region ($1480\,\mathrm{cm}^{-1}$ to $1800\,\mathrm{cm}^{-1}$), and the lipid-rich CH-stretch window ($2750\,\mathrm{cm}^{-1}$ to $3010\,\mathrm{cm}^{-1}$). All spectra were vector-normalized to Amide I prior to analysis. Components with eigenvalues ≥ 1 (Kaiser criterion) were retained. Score plots (PC1 vs PC2) were shown with a 95% Hotelling's T^2 ellipse.

2.10 Statistical Analyses

All analyses were performed in Python (SciPy 1.13). For each sample, spectral quality was quantified as signal to noise ratio (SNR), computed as the Amide I peak height ($\approx 1650\,\mathrm{cm}^{-1}$) divided by the root mean square (RMS) of baseline noise estimated over a flat, absorption free window (e.g., $1900\,\mathrm{cm}^{-1}$ to $2000\,\mathrm{cm}^{-1}$) after preprocessing. SNR is summarised as median [IQR]. Because SNR and clinical variables exhibited non Gaussian features (histograms, Q–Q plots; Shapiro–Wilk tests), between batch differences (fresh vs aged PFA) were assessed using two tailed Mann Whitney U tests. Monotonic associations between SNR and clinical markers were evaluated with Spearman's rank correlation (ρ) using exact P values and 95% bootstrap confidence intervals. All tests were two-sided with $\alpha = 0.05$; no imputation was applied, and ties used SciPy defaults.

3 Results

3.1 PFA Quality Analysis

3.1.1 Fixative Chemical Stability

To assess PFA chemical stability, fresh and aged PFA solutions were analysed by ATR–FTIR. They exhibited markedly different carbonyl signatures (Fig. 1A,B). Fresh PFA produced a strong absorbance at 1700 cm^{-1} (0.31 a.u.), reflecting abundant free aldehyde groups essential for cross-linking. In contrast, aged PFA displayed a greatly diminished carbonyl peak (0.09 a.u.), indicating >70% loss of reactive species following prolonged storage. Additional broadening and baseline drift between 1650 cm^{-1} and 1800 cm^{-1} suggest polymerisation and oxidation.

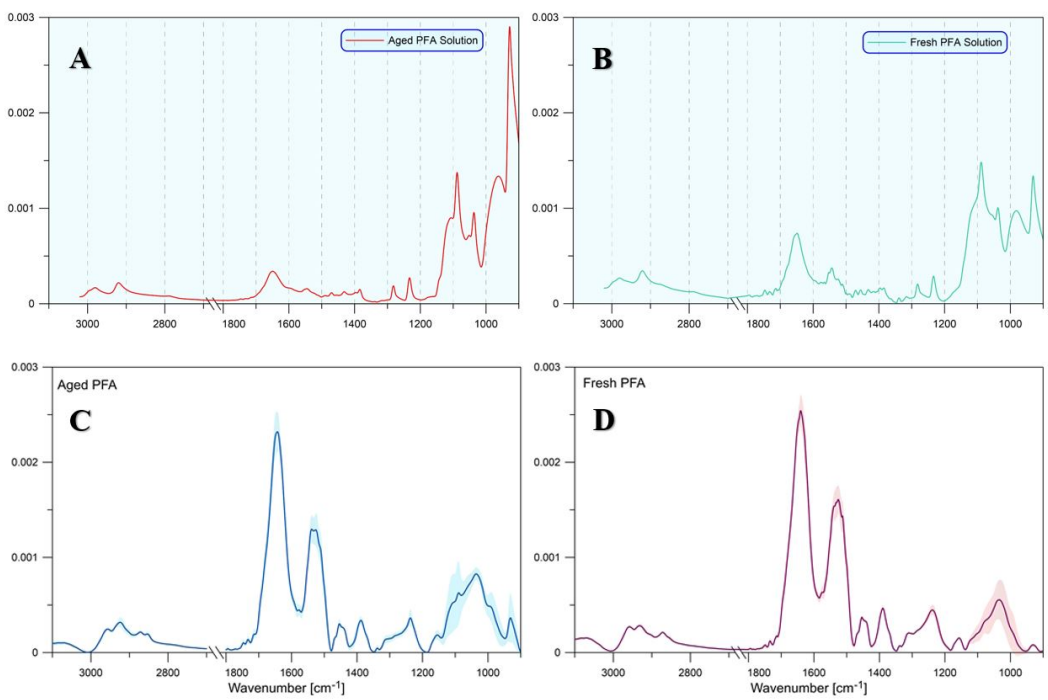

Figure 1. **ATR–FTIR of paraformaldehyde (PFA) solutions and averaged spectral profiles. (A) Aged PFA.** The carbonyl band near 1700/cm is diminished and broadened with baseline drift, indicating aldehyde depletion. **(B) Fresh PFA.** The carbonyl band is strong and well defined on a flat baseline, indicating preserved chemistry. **(C) Aged PFA (mean ±SD).** Across 1800/cm to 1650/cm, the carbonyl absorbance is lower with greater variability; modest fingerprint features appear at 1200/cm to 1000/cm. **(D) Fresh PFA (mean ±SD).** The carbonyl is higher and sharper with tighter variability; only minor fingerprint features are present. Overall, fresh PFA retains reactive carbonyls, whereas aged PFA shows characteristic degradation signatures.

3.1.2 Averaged Spectral Profiles of Fixed Thrombi

To assess whether PFA quality alters thrombus chemistry, we applied the same ATR–FTIR analysis to thrombi fixed in aged versus fresh PFA. Quantitative analysis of averaged spectra revealed systematic differences between fixation conditions across major biochemical regions (Fig. 1C,D), demonstrating the molecular consequences of PFA degradation on preservation quality.

Aged-PFA Group (n=6). Thrombi fixed in degraded PFA (specimens 1EDU, 1DKU, 1EA, 1FDA, 1CFL, 1BM1) showed compromised protein preservation with broad Amide I peaks centered at $1649 \pm 3\,\mathrm{cm}^{-1}$. The broadening reflects disrupted hydrogen bonding networks and altered secondary structure due to inadequate cross-linking [9, 12].

Fresh-PFA Group (n=4). Thrombi preserved in fresh PFA (specimens 1GIN, 1PAN, 1ZAN, 1ZMA) exhibited sharp Amide I peaks at $1652 \pm 1\,\mathrm{cm}^{-1}$ and well defined Amide II bands at $1540 \pm 2\,\mathrm{cm}^{-1}$, consistent with preserved protein conformation and effective aldehyde mediated cross-linking.

3.1.3 Chemometric Discrimination by PCA (Fixative freshness)

The composite PCA figure (Fig. 2) summarises three windows. (A) Fingerprint ($900\,\mathrm{cm}^{-1}$ to $1800\,\mathrm{cm}^{-1}$): PC1 69.0% (PC2 14.7%) separates aged-PFA (negative PC1) from fresh-PFA and plasma controls (positive PC1). (B) Amide I+II ($1480\,\mathrm{cm}^{-1}$ to $1800\,\mathrm{cm}^{-1}$): discrimination sharpens (PC1 78.0%, PC2 11.5%), indicating predominant protein-band perturbation. (C) Lipid CH-stretch ($2750\,\mathrm{cm}^{-1}$ to $3010\,\mathrm{cm}^{-1}$): separation persists (PC1 80.5%, PC2 15.6%), consistent with CH_2/CH_3 degradation when aldehydes are depleted. Plasma controls align with fresh-PFA.

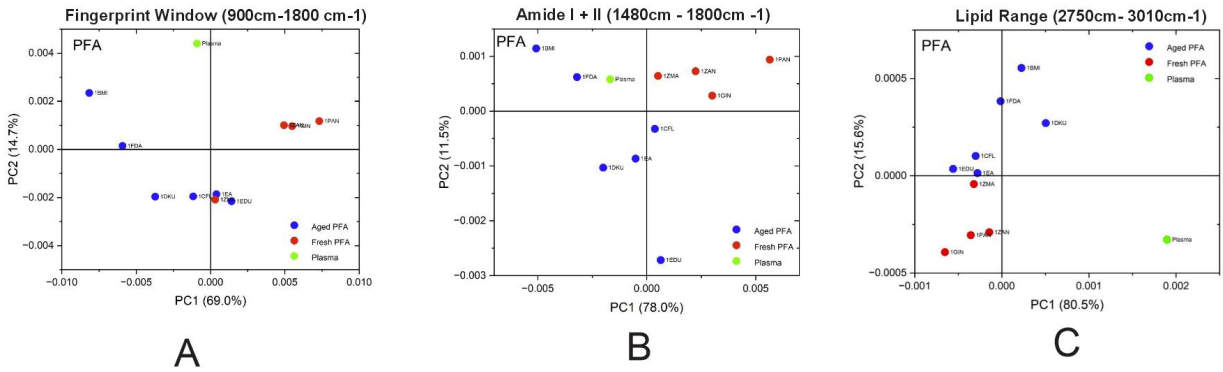

Figure 2. **Principal component analysis (PCA) of attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra. (A) Fingerprint region (900/cm to 1800/cm).** Principal component 1 (PC1) explains 69.0% of the variance and principal component 2 (PC2) explains 14.7%; aged paraformaldehyde (PFA; blue) separates from fresh PFA (red) and plasma (green). **(B) Amide I and II region (1480/cm to 1800/cm).** PC1 explains 78.0% and PC2 11.5%; the increased discrimination highlights protein sensitivity to fixative freshness. **(C) Lipid C–H stretch region (2750/cm to 3010/cm).** PC1 explains 80.5% and PC2 15.6%; separation persists, driven by CH_2/CH_3 stretching contributions.

3.1.4 Signal-to-noise analysis

Fresh-PFA thrombi showed a median SNR of 41.8 versus 18.2 for aged-PFA thrombi ($P < 0.01$), a more than two fold difference exceeding inter-patient variability.

3.2 Spectral Comparison by DVT History

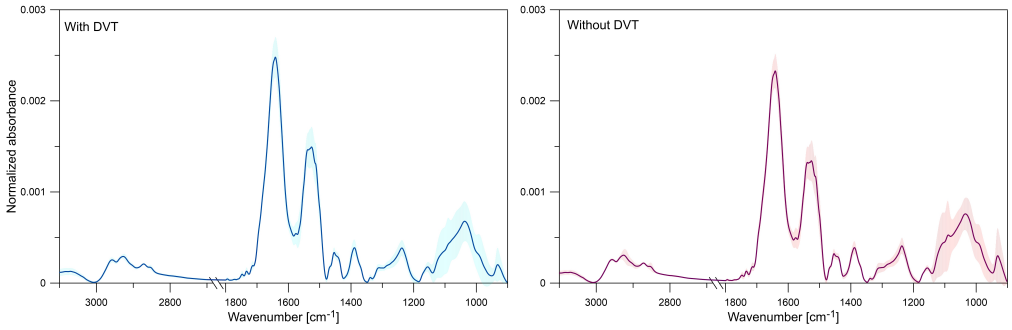

Figure 3. Averaged ATR–FTIR spectra grouped by history of deep-vein thrombosis (DVT). Black trace = DVT^+ thrombi ($n = 5$); cyan = DVT^- thrombi ($n = 5$). Shaded bands denote $\pm$ 1 SD.

Across 900 cm^{-1} to 3100 cm^{-1}, DVT^+ and DVT^- traces were virtually superimposable: no Amide I/II shifts or lipid-to-protein ratio changes; phosphate at 1080 cm^{-1} was unchanged. Median SNR did not differ (40.9 vs 40.6; $P = 0.10$).

3.3 Spectral Comparison by Age Group

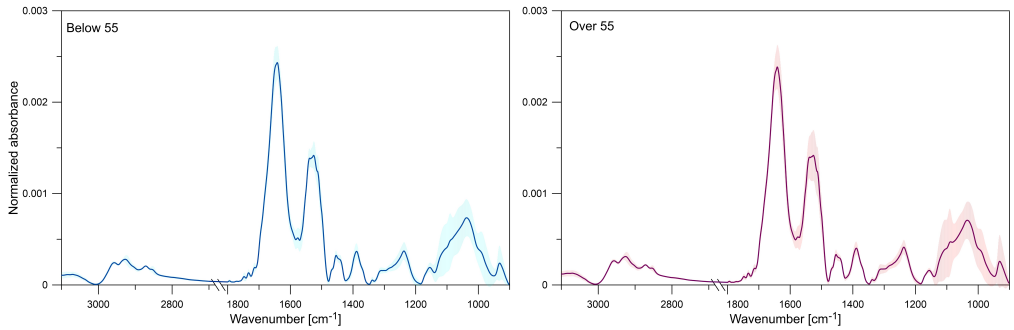

Figure 4. Averaged ATR–FTIR spectra grouped by age. Cyan trace = patients 55 y ($n = 4$); magenta = patients > 55 y ($n = 6$). Shaded bands denote ± 1 SD.

Younger (55 y) and older (>55 y) cohorts showed near complete spectral overlap with indistinguishable SNR (40.3 vs 40.2; $P = 0.97$); no age-related clustering was seen in PCA. This absence of age-driven separation aligns with reports that age-related lipid metabolism shifts are subtle and context dependent [13, 14].

3.3.1 Chemometric Discrimination by PCA (DVT vs Age, composite)

The composite PCA summary (Fig. 5) contrasts deep vein thrombosis (DVT) history and age across three spectral windows using identically pre-processed, Amide I-normalised spectra. In every window, scores from the clinical subgroups are extensively intermixed with similar dispersion, indicating that subgroup membership does not explain the sample variance once fixative freshness is controlled.

DVT comparison (left column). In the fingerprint region (900/cm to 1800/cm; PC1 = 69.0%, PC2 = 14.7%), DVT^+ (n=5) and DVT^- (n=5) occupy overlapping areas with no axis-wise trend. Restricting to the protein bands (Amide I+II, 1480/cm to 1800/cm; PC1 = 78.0%, PC2 = 11.5%) tightens the cloud yet maintains full overlap. In the lipid C–H stretch window (2750/cm to 3010/cm; PC1 = 80.5%, PC2 = 15.6%), patient samples cluster near the origin without DVT-related separation, while plasma controls tend to lie at positive PC1.

Age comparison (right column). Age-stratified scores (55 y, n=4 vs >55 y, n=6) similarly intermix across the fingerprint, protein, and lipid windows, with comparable within-group dispersion and no monotonic gradient along PC1 or PC2.

Overall. Across windows, neither prior DVT nor age yields separable clusters when fixative freshness is standardised, consistent with the axis-wise variance shown on each plot.

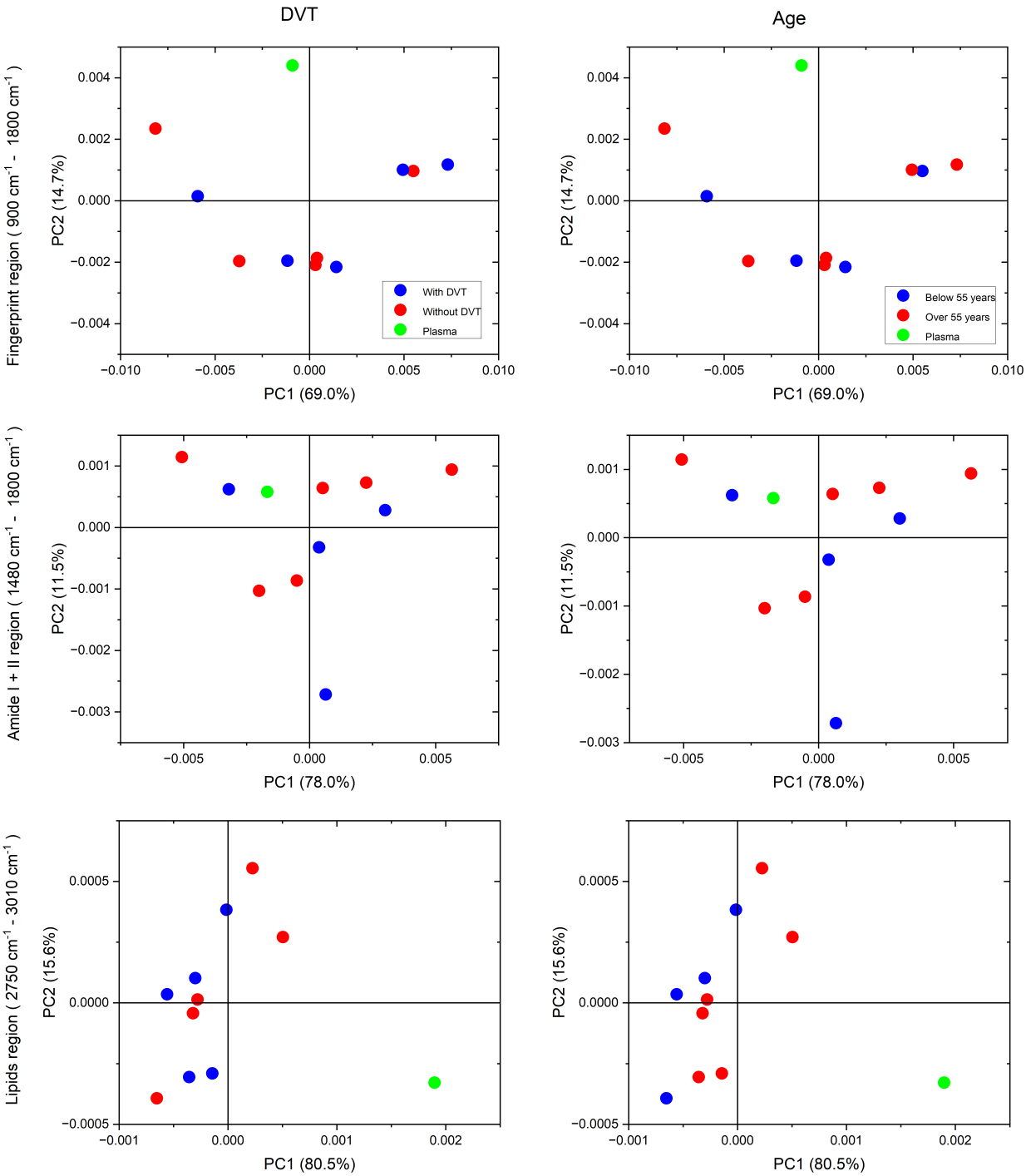

Figure 5. **Composite principal component analysis (PCA) comparing DVT history and age across spectral windows.** *Left column (DVT comparison).* Blue = DVT$^+$ (n=5); red = DVT$^-$ (n=5); green = plasma control. *Right column (age comparison).* Blue = $\leq$55 years (n=4); red = >55 years (n=6); green = plasma control. Rows correspond to the fingerprint region (900/cm to 1800/cm), the Amide I+II region (1480/cm to 1800/cm), and the lipid C–H stretch region (2750/cm to 3010/cm). The percentages of variance explained by PC1 and PC2 are shown on the axes; scores largely overlap across groups.

3.4 Clinical Parameter Correlation with Spectral Quality

Spearman analysis showed no meaningful association between SNR and routinely collected markers including simplified Pulmonary Embolism Severity Index (sPESI), baseline D-dimer, fibrinogen, high-sensitivity troponin (hs-troponin), N-terminal pro–B-type natriuretic peptide (NT-proBNP), lactate, high-sensitivity C-reactive protein (hsCRP), or neutrophil-to-lymphocyte ratio (NLR) (all $|\rho| < 0.25$, $P > 0.20$). Median [IQR] values by fixative batch appear in Table 4. Fixative freshness remained the sole significant driver of SNR (fresh PFA: 41.8 vs aged PFA: 18.2; $P < 0.01$).

Table 4. Key clinical variables and attenuated total reflectance Fourier transform infrared (ATR-FTIR) signal-to-noise ratio by fixative batch (median [IQR]).

Variable	**Fresh paraformaldehyde (PFA; $n = 4$)**	**Aged PFA ($n = 6$)**
sPESI	2.0 [1–3]	2.0 [1–3]
D-dimer baseline ($\mathrm{ng\,mL^{-1}}$)	10 712 [9 800–11 500]	5 691 [4 500–6 800]
Fibrinogen baseline ($\mathrm{g\,L^{-1}}$)	3.59 [3.50–3.70]	3.48 [3.30–3.60]
hs-Troponin ($\mathrm{ng\,L^{-1}}$)	37.5 [30–50]	172 [140–210]
NT-proBNP ($\mathrm{pg\,mL^{-1}}$)	2 284 [1 800–2 500]	7 570 [6 500–8 500]
hsCRP ($\mathrm{mg\,L^{-1}}$)	110.5 [90–130]	91.3 [70–110]
NLR	4.51 [3.8–5.2]	5.43 [4.5–6.2]
Lactate ($\mathrm{mmol\,L^{-1}}$)	1.60 [1.4–1.8]	1.80 [1.6–2.0]
SNR	**41.8 [38–45]**	**18.2 [15–22]**

Legend: Data are median [interquartile range, IQR]. PFA = paraformaldehyde; ATR-FTIR = attenuated total reflectance Fourier transform infrared (spectroscopy); SNR = signal-to-noise ratio; sPESI = simplified Pulmonary Embolism Severity Index; hsCRP = high-sensitivity C-reactive protein; NT-proBNP = N-terminal pro–B-type natriuretic peptide; hs-Troponin = high-sensitivity cardiac troponin. Units as shown.

Table 5. Signal-to-noise ratio (SNR) by deep vein thrombosis (DVT) status and by age group (median [IQR]); P values from two-tailed Mann–Whitney U tests.

(A) DVT status			
Group	n	**SNR**	P **value**
DVT^+	5	40.9 [38–43]	0.10
DVT^-	5	40.6 [39–42]	
(B) Age group			
Group	n	**SNR**	P **value**
$\leq$55 y	4	40.3 [39–41]	0.97
>55 y	6	40.2 [38–42]	

Legend: SNR = signal-to-noise ratio; DVT^+ = documented prior or concomitant deep vein thrombosis; DVT^- = no such history; n = group size. P values are from two-tailed Mann–Whitney U tests comparing groups within each panel. Values are reported as median [IQR].

Median SNR did not differ by DVT history (40.9 vs 40.6; $P = 0.10$) or age (55 y vs >55 y: 40.3 vs 40.2; $P = 0.97$). Consistently, composite PCA (Fig. 5) shows extensive overlap across all windows; plasma controls cluster tightly. Thus, once fixative freshness is standardised, neither prior DVT nor age produces detectable biochemical shifts in thrombus spectra.

4 Discussion

4.1 Principal Findings

Across fingerprint (900 cm^{-1} to 1800 cm^{-1}), protein (Amide I+II; 1480 cm^{-1} to 1800 cm^{-1}) and lipid (CH-stretch; 2750 cm^{-1} to 3010 cm^{-1}) windows, fixative freshness specifically, preservation of reactive aldehyde groups was the dominant determinant of thrombus ATR–FTIR integrity. Aged 4% PFA (>3 months) generated broad Amide envelopes, attenuated CH_2/CH_3 bands and a two fold SNR loss, whereas freshly prepared PFA conserved sharp protein and lipid-associated peaks. PCA verified this chemistry driven separation: PC1 explained 69% (fingerprint), 78% (protein) and 80% (lipid) of total variance.

Consistent with these observations, the aged fixative failed the pre specified quality control criteria its carbonyl absorbance at 1700 cm^{-1} fell below 0.25 a.u. and the solution exceeded the 90 day shelf life confirming its unsuitability for reliable biochemical preservation.

4.2 Impact on Specific Molecular Classes

Aged PFA, depleted of >70% of free aldehydes, cross-links inefficiently; thrombi exhibit broadened Amide I/II envelopes and baseline drift, consistent with altered secondary structure [9, 15].

The lipid window showed the greatest PC1 variance (80.5%), reflecting erythrocyte membrane and platelet-derived lipid content. Degraded fixative (via methanol/formic acid) can extract lipids, reducing CH_2/CH_3 intensity; this is concordant with lipidomics links to thrombus biology [16, 17, 18, 19].

Intermediate sensitivity (PC1 $\approx$ 69%) arises from concurrent perturbations to protein, lipid, and carbohydrate bands.

4.3 Clinical Implications

A 1 min attenuated total reflectance–Fourier transform infrared (ATR–FTIR) scan of the fixative, combined with a carbonyl absorbance threshold ($\geq$ 0.25 arbitrary units [a.u.]), effectively identifies degraded paraformaldehyde (PFA) before immersion, thereby preventing artefacts in protein, lipid, and mixed bands. Given that spectra are unaffected by age, deep vein thrombosis (DVT) history, or clinical severity, a chemistry-centred quality control (QC) protocol standardises sample quality across centres without the need for demographic stratification. Fresh PFA maintains labile fibrin structures and membrane lipids, thereby minimising proteolytic and extraction artefacts that could complicate quantitative mass spectrometry and imaging workflows.

4.4 Limitations

Sample size was modest (ten thrombi; six aged-PFA, four fresh) and evaluated a single PFA concentration (4%). Only one fixation temperature and a single ATR platform were used.

4.5 Comparison with Previous Work

Fixation-related artefacts similar to those observed here have been reported in brain and tumour tissues [7, 9]. To our knowledge, this study provides the first systematic evaluation of such artefacts in pulmonary artery thrombi from patients with PE. Our observations align with the established understanding that dense fibrin architectures impede fibrinolysis [2].

4.6 Future Directions

Building on these results, ongoing work will (i) assess microwave-enhanced fixation to shorten immersion times, (ii) test alternative fixatives such as glyoxal and zinc formalin, and (iii) integrate partial least squares discriminant analysis (PLS-DA) to predict fixative age directly from raw attenuated total reflectance Fourier transform infrared (ATR–FTIR) spectra. Together, these efforts aim to refine fixation protocols, minimise spectral distortions, and broaden the applicability of vibrational spectroscopy for accurate biochemical characterisation of thrombi. Supplementing these studies with complementary methods using positronium lifetime spectroscopy and secondary ion mass spectrometry (SIMS) will allow for the assessment of the nanostructure of the fibrin clot [20, 21].

5 Conclusions

Fixative chemistry, not clinical status, dominates pre-analytical variance in molecular thrombus profiling. A QC workflow combining fixative carbonyl assessment with ATR–FTIR PCA preserves clinically informative protein and lipid features and enables reliable, multicentre discovery science. Key points:

1. Paraformaldehyde stored ≥ 3 months loses $>70\%$ of reactive aldehydes, yielding broad Amide envelopes, attenuated lipid bands and a two fold SNR drop.

2. PCA of fingerprint, protein or lipid windows cleanly separates aged from fresh fixations (PC1 up to 80% variance).

3. A rapid QC workflow (carbonyl absorbance ≥ 0.25 a.u. plus PCA verification) safeguards molecular integrity irrespective of patient age, DVT history or clinical severity.

4. Adopting this workflow will harmonise multi centre clot biorepositories and enhance the reproducibility of proteomic, lipidomic and imaging studies.

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Ethics, Transparency, and Reproducibility

- **IRB Approval:** Krakowski Szpital Specjalistyczny im. Św. Jana Pawła II, Protocol #2023-152.
- **Informed Consent:** Obtained from all participants or legal representatives.
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Supplementary Information

Table S1 – Detailed Patient Comorbidities and Anonymized Sample Codes

Sample Code	Sex	Age	Batch	Key Comorbidities / Notes
1EDU	F	39	Aged PFA	DVT (left leg); recent brain-tumour surgery; two C-sections; thyroidectomy; rheumatoid arthritis; Sjögren's syndrome; URT infection
1DKU	M	65	Aged PFA	Immobilisation after tibia fracture
1EA	M	83	Aged PFA	Hypertension; hypercholesterolaemia; HFrEF; type 2 diabetes; hip arthroplasty
1FDA	F	26	Aged PFA	Post-partum; family history of chronic venous disease and DVT
1CFL	M	29	Aged PFA	DVT; deep venous insufficiency; family history of early DVT-related death
1BM1	F	76	Aged PFA	Nicotine addiction; hysterectomy and oophorectomy for cancer
1GIN	M	51	Fresh PFA	Total-hip prosthesis; suspected thrombophilia
1PAN	M	63	Fresh PFA	Hypertension; immobilisation; DVT; head injury
1ZAN	F	71	Fresh PFA	Immobilisation; DVT; hypercholesterolaemia
1ZMA	F	64	Fresh PFA	Hypertension; Diabetes