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RESEARCH ARTICLE

Metabolomic Profiles of Sperm Reserves as Potential Fertility Biomarkers for Yoruba Ecotype Chickens

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ABSTRACT

The Yoruba Ecotype Chicken (YEC) is an indigenous Nigerian poultry breed of considerable importance. The metabolomic composition of its sperm reserves remains uncharacterized, with limiting molecular-level understanding of its reproductive biology. The present study was aimed to quantify metabolites in sperm reserves of YEC, map them to annotated metabolic pathways, and integrate them with sperm quality parameters to derive candidate fertility biomarkers. Thirty sexually mature cocks of 25-30 weeks age were used. Semen was collected from these birds via abdominal massage method. Sperm reserves were analyzed using an Agilent 3800 Gas Chromatography with a 4000 MSD and HP-5 Mass Spectrophotometry column. Methyl chloroformate was used for pre-column derivatization. Metabolites were identified via the NIST08/Wiley275 library, mapped to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and integrated with sperm progressive motility, concentration, acrosomal competitive index (ACI), membrane evaluation index (MEI), and morphological abnormalities. Results showed that 16 metabolites were identified across eight biochemical classes. The metabolome was dominated by dl-2-Aminobutyric acid (17.24±12.05%), L-Glutamine (12.50±1.61%), and cholesterol (10.04±0.95%). Metabolites mapped to 11 KEGG-annotated pathways. Sperm quality was excellent, with progressive motility 77.50±1.44%, MEI 80.00±0.58%, ACI 71.75±7.26%, sperm viability 63.09±2.53% and morphological abnormalities 3.00±0.71%. In conclusion, YEC sperm reserve metabolome reflects active energy metabolism, antioxidant defense, and membrane maintenance. L-Glutamine, cholesterol, and dl-2-Aminobutyric acid are proposed as primary biomarkers of sperm reserve quality. This study delivers the first comprehensive GC–MS-based metabolomic profile of sperm reserves in YEC, integrating data with sperm quality parameters to propose a tier-ranked fertility biomarker panel. These findings support conservation and productivity of indigenous poultry, contributing to Zero Hunger through enhanced reproductive efficiency in smallholder systems.

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INTRODUCTION

The Yoruba Ecotype Chicken is an indigenous Nigerian poultry breed found predominantly in the Yoruba-speaking regions of south-western regions of the country (Lasagna *et al.*, 2020). Like other local ecotypes, it has evolved under traditional low-input management systems, conferring notable adaptability to tropical climatic conditions (Ademola *et al.*, 2023, 2024), resistance to endemic diseases, and compatibility with local feed resources (Akintunde and Toye, 2023). Despite its socioeconomic importance to smallholder farmers,

systematic characterization of the reproductive biology of this ecotype, particularly at the molecular and metabolic level, remains limited.

Sperm quality is a critical determinant of fertility and hatchability in poultry (Arif *et al.*, 2025). Standard semen evaluation parameters, including sperm concentration in the ejaculate, motility, acrosomal integrity, membrane integrity, and morphological abnormalities of spermatozoa (Silyukova *et al.*, 2022), provide important proxies for fertilizing capacity but do not reveal the underlying biochemical environment of the sperm reserve (Akintunde *et al.*, 2020, 2021; Ndubuisi-Ogbonna *et al.*, 2022; Tvrdá *et al.*, 2023). In addition, metabolomics, the holistic characterization of small-molecule metabolites that make up the biochemical landscape of a biological system, can be applied to gain insight into the biochemical environment of avian sperm reserves and to develop metabolic signatures that predict fertility outcomes (Li *et al.*, 2025; Akintunde *et al.*, 2025; Cheng *et al.*, 2026).

Gas Chromatography–Mass Spectrometry (GC-MS) is an established metabolomics platform which is very suitable for the analysis of volatile and semi-volatile organic compounds, amino acids, sugars, organic acids and lipid-related metabolites after pre-column derivatization (Fiehn, 2016; Zhang *et al.*, 2024). It has high resolution and sensitivity and is suitable for profiling of complex biological materials like semen.

To our knowledge, there is no published study that describes the metabolomic profile of sperm reserves of Yoruba Ecotype Chicken. Elucidation of the biochemical basis of sperm quality traits, identification of candidate metabolite biomarkers for non-invasive fertility prediction and establishment of baseline metabolomic data for comparative studies among Nigerian and African indigenous poultry ecotypes, are important for understanding this profile. It was hypothesized is that the Yoruba ecotype chicken have a unique metabolomic profile, which is measurable, and correlates with the sperm quality traits (progressive motility, membrane integrity, acrosomal integrity, and sperm morphology), and that a subset of these metabolites may be useful as non-invasive markers of the sperm reserve and quality of Yoruba Ecotype Chicken. Therefore, the present study was planned to characterize the GC-MS metabolome of Yoruba Ecotype Chicken sperm reserves, map detected metabolites to annotated metabolic pathways, integrate findings with measured sperm quality parameters, and propose a tier-ranked biomarker panel for sperm reserve quality assessment.

MATERIALS AND METHODS

Chicken and semen collection: A total of 30 mature, male Yoruba Ecotype Chickens, weighing 1.06±0.29Kg and aged 25-30 weeks, were used for this study. These birds were collected around December, 2025 from the local villages in Ikenne Local Government Area of Ogun State, Nigeria. They were maintained under standard management conditions with *ad libitum* access to water and a commercial breeder mash containing 17.5% crude protein and 2700kcal metabolizable energy. The birds were acclimatized for 28 days before semen collection. Semen samples from these chickens were collected weekly for 4 weeks by the abdominal massage method (Pimprasert *et al.*, 2023). The technique involved restraining the male and gently stroking the back of the bird from behind the wings towards the tail with firm rapid strokes. The male responded with tumescence erection of the phallus, at which time the handler gently squeezed the cloaca, extracting semen through the external papillae of the ductus deferens (vas deferens) and collecting the semen into a container. Since phallus of the bird was located in the same duct as the anus, feed was removed 12h prior to semen collection to prevent fecal contamination of the semen. Semen samples were analyzed per individual cock. Sperm reserves were processed immediately for GC-MS analysis and sperm quality evaluation.

Sperm quality evaluation: Standard semen quality parameters were evaluated as follows: Sperm progressive motility was assessed subjectively under light microscopy. Then the semen sample was diluted at room temperature with a Tris-based extender containing tris-hydroxymethyl-aminomethane (2.42g), citric acid (1.35g), glucose (1.0g) and penicillin (0.028g), egg yolk 20mL and distilled water added to make up 100mL. Semen samples were diluted at a ratio of 1:2 (semen: extender) using the prepared Tris-based extender. Sperm concentration and total sperm count were determined using a hemocytometer. Morphological abnormalities (ABN) were determined by bright-field microscopy after eosin–nigrosine staining (≥200 cells per sample).

Progressive sperm motility was determined as described by Hernawati *et al.* (2025). About 5µL of sample was placed directly on a pre-warmed microscope slide, overlaid with a cover slip and assessed for sperm motility using Celestron Penta-View microscope (LCD-44348 by RoHS, China) at 400× magnification. Each sample was viewed twice (2 slides per sample) and five microscopic fields were examined. Only spermatozoa that moved forward in a straight line among the spermatozoa from each microscopic field were counted as progressively motile spermatozoa by three observers. The mean of the five successive evaluations was recorded and percentage progressive sperm motility was calculated as follows:

Progressive sperm motility (%) = Number of progressively motile spermatozoa/total number of spermatozoa examined X 100.

The percentage of spermatozoa with intact acrosomes was determined after the semen samples were stained with Giemsa stain according to the procedure described by Günay *et al.* (2023) and Petričáková *et al.* (2024). Integrity of sperm acrosome, characterized by normal apical ridge or spermatozoa with presence of crescent shaped acrosomes, was considered normal. The percentage of acrosomal competitive index (ACI, %) was calculated as follows:

ACI (%) = Number of spermatozoa with normal apical ridge/number of spermatozoa examined X 100.

Hypo-osmotic swelling test (HOST) described by Elomda *et al.* (2024) was used to determine sperm plasma membrane integrity. This test was done by incubating 10µL semen sample in 100µL of a 100mOsm/L hypo-osmotic solution (7.35g sodium citrate [0.0285M] and 13.5g

fructose [0.075M]) at 37°C for 30 minutes. Then, 0.1mL of the mixture was spread over a warmed slide, overlaid with a cover slip and examined under Celestron PentaView LCD-44348 digital microscope (400× magnification). Sperm with swollen or coiled tails were taken as having intact plasma membrane. At least 200 sperm were examined and percentage of sperm with intact plasma membrane was calculated. Eosin–nigrosine staining was used to evaluate percentages of sperm morphological abnormalities and sperm viability according to the method of Hernawati *et al.* (2025). Spermatozoa that appeared white (unstained cells) were regarded as live (viable), while those that picked up eosin stain (appeared pink) were regarded as dead spermatozoa. Sperm liveability (%) was calculated as follows:

Sperm viability (%) = Number of live sperm/number of sperm examined X 100

GC-MS instrumentation and conditions: Metabolite profiling was carried out using an Agilent 3800 gas chromatograph coupled to a 4000 inert mass selective detector (Agilent Technologies, USA). An Agilent HP-5MS capillary column (30m x 0.25mm × 0.25µm film thickness) was used for chromatographic separation with nitrogen as the carrier gas at an injection volume of 1.0mL. The GC oven temperature program was: initial 50°C for 2min, ramped at 20°C/min to 130°C, then at 2°C/min to 150°C held for 5min, finally ramped at 20°C/min to 230°C. Quadrupole and ion source temperatures were 150°C and 230°C, respectively. Mass spectra were acquired in total ion current (TIC) mode. Metabolite identification used the NIST08/Wiley275 combined spectral library (Fiehn, 2016).

Pre-column derivatization: For pre-column derivatization, semen samples were dissolved in 390µL of 1.0M sodium hydroxide; then 333µL methanol and 67µL pyridine were added and mixed for 5s. Then, 80µL methyl chloroformate was added and stirred for 60s. Amino acid derivatives were extracted with 400µL chloroform, followed by addition of 400µL of 50mM sodium bicarbonate to remove excess reagent (Fiehn, 2016). Metabolite analysis was carried out on undiluted semen samples to avoid interference from extender-derived compounds.

Data processing and analysis: Ten replicate GC-MS runs were treated as biological replicates. Peak area percentages were averaged across replicates and variability was expressed as standard deviation (SD) and coefficient of variation (CV %) using the NumPy library (Harris *et al.*, 2020) implemented in Python (version 3.11; Python Software Foundation, 2023). Hierarchical cluster analysis was performed using the SciPy library (Virtanen *et al.*, 2020), applying Ward linkage with Euclidean distance as the dissimilarity metric (scipy.cluster.hierarchy). All figures, including bar charts, heatmaps, cluster dendrograms, radar plots, bubble plots, and the metabolite–sperm quality integration network, were generated using Matplotlib (Hunter, 2007), with heatmap visualizations additionally styled using Seaborn (Waskom, 2021). Metabolite pathway annotations were assigned by cross-referencing compound names and molecular formulae against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa *et al.*, 2023) and the Human Metabolome Database (HMDB; Wishart *et al.*, 2022). A composite Biomarker Score (BS) was calculated for each metabolite as a weighted sum of three components, each rescaled to a common 1–10 scale: (i) mechanistic relevance to the measured sperm quality parameters (M), scored according to the strength of the documented biochemical pathway linking the metabolite to sperm motility, viability, membrane or acrosomal integrity, or morphology; (ii) literature support (L), scored according to the number and quality of peer-reviewed studies reporting the metabolite as a reproductive or fertility-associated biomarker; and (iii) relative abundance (A), the mean GC-MS peak area (%) for each metabolite, linearly rescaled to a 1–10 range using min–max normalization (Han *et al.,* 2012): $A=1+9\times[(peak_i - peak\_min)/(peak\_max - peak\_min)]$. The composite score was calculated as $BS=(0.4\times M) + (0.4\times L) + (0.2\times A)$. Mechanistic relevance and literature support were weighted equally and given greater combined weight (80%) than raw abundance (20%), consistent with multi-criteria evidence-weighting approaches that prioritize biological plausibility and validation potential over signal intensity alone (Pepe *et al.*, 2001; Berghoff *et al.*, 2013; Thokala *et al.*, 2016). Metabolites were then assigned to one of four tiers using pre-defined thresholds: Primary (★★★★★), BS ≥8.5; Secondary (★★★★☆), 7.0≤ BS <8.5; Supportive (★★★☆☆), 5.0≤ BS <7.0; Exploratory (★★☆☆☆), BS <5.0. The metabolite 1,2-Benzenedicarboxylic acid was excluded from scoring and flagged separately as a probable exogenous (plasticizer-derived) contaminant rather than an endogenous biomarker candidate.

RESULTS

Mean ± SD values for quality parameters of Yoruba Ecotype Chickens semen indicated that these chickens produced semen of excellent quality. The mean ejaculatory volume was 0.77±0.01mL. The semen samples exhibited high progressive sperm motility (77.50±1.44%), substantial sperm concentration of 2.65±0.09 $\times10^9$ mL^{-1}, and total sperm count per sample of 2.05±0.07 $\times10^9$, while sperm viability was 63.09±2.53%. Values of the ACI and MEI were 71.75±7.26 and 80.00±0.58%, respectively, which were good values of acrosomal and membrane integrity. The level of morphological abnormalities was low (3.00±0.71%), which indicated a high quality of semen.

Table 1 shows the mean ± SD values for GC-MS metabolite profile of Yoruba Ecotype Chicken sperm reserves. Sixteen metabolites were found in the sperm reserves of Yoruba Ecotype Chicken in 10 replicates of GC-MS run. The most abundant metabolite was dl-2-Aminobutyric acid (17.24±12.05%), followed by L-Glutamine (12.50±1.61%), Cholesterol (10.04±0.95%), L-Pyroglutamic acid (7.77±0.80%), Myo-Inositol (7.00±3.48%), and Glucose (6.14±0.84%). Metabolites identified included representatives from nine biochemically distinct classes: amino acids (3), sugars (3), polyols (2), lipids/sterols (2), aromatic acids (2), one representative each of glycoside, nucleoside, terpenoid and flavonoid (Table 1).

Table 1: GC-MS metabolite profile of Yoruba Ecotype Chicken sperm reserves (mean ± SD)

Peak	Compound	Formula	MW	Mean±SD (%)	CV (%)	m/z	Class
1	dl-2-Aminobutyric acid	$C_4H_9NO_2$	103	17.24±12.05	69.90	41,58,103	Amino acid
2	L-Pyroglutamic acid	$C_5H_7NO_3$	129	7.77±0.80	10.30	41,84,129	Amino acid
3	L-Glutamine	$C_5H_{10}N_2O_3$	145	12.50±1.61	12.90	41,84,145	Amino acid
4	Inosine	$C_{10}H_{12}N_4O_5$	268	2.39±1.13	47.30	54,136,258	Nucleoside
5	Glucose	$C_6H_{12}O_6$	180	6.14±0.84	111.40	43,73,180	Sugar
6	D-Ribose	$C_5H_{10}O_5$	150	5.91±0.65	112.50	43,73,150	Sugar
7	Melibiose	$C_{12}H_{22}O_{11}$	342	3.72±0.18	31.70	41,97,342	Sugar
8	Octyl-β-D-glucopyranoside	$C_{14}H_{28}O_6$	292	4.63±0.18	90.30	57,60,292	Glycoside
9	Myo-Inositol	$C_6H_{12}O_6$	180	7.00±3.48	49.70	43,73,180	Polyol
10	Sorbitol	$C_6H_{14}O_6$	182	5.12±0.02	109.80	43,73,182	Polyol
11	1,2-Benzenedicarboxylic acid	$C_8H_6O_4$	166	5.31±0.04	121.10	43,78,166	Aromatic acid
12	4-Hydroxybenzoic acid	$C_7H_6O_3$	138	1.70±0.27	15.90	65,121,138	Aromatic acid
13	N-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	5.34±0.02	19.10	43,73,256	Fatty acid
14	Cholesterol	$C_{27}H_{46}O$	386	10.04±0.95	29.40	43,105,386	Sterol
15	Caryophyllene	$C_{15}H_{24}$	204	1.82±0.43	23.60	41,93,204	Terpenoid
16	Kaempferol, TMS derivative	$C_{27}H_{44}O_6Si_4$	576	3.41±0.09	116.10	57,119,576	Flavonoid

MW = Molecular weight; CV = Coefficient of variation; m/z = Major mass spectral fragments.

The metabolome was clustered hierarchically with Ward linkage and Euclidean distance, to show the within-replicate structure (Fig. 1). The dendrogram divided the metabolites into two main groups. The first cluster consisted mainly of carbohydrate-related and polar metabolites such as glucose, D-ribose, sorbitol, and octyl β-D-glucopyranoside, whereas cholesterol and L-glutamine were close to this cluster. The second group consisted of a more diverse series of compounds, like amino acid derivatives, organic acids, lipids and secondary metabolites. However, dl-2-aminobutyric acid was a separate outlier, together with the cluster, at a higher linkage distance, suggesting less similarity with other metabolites.

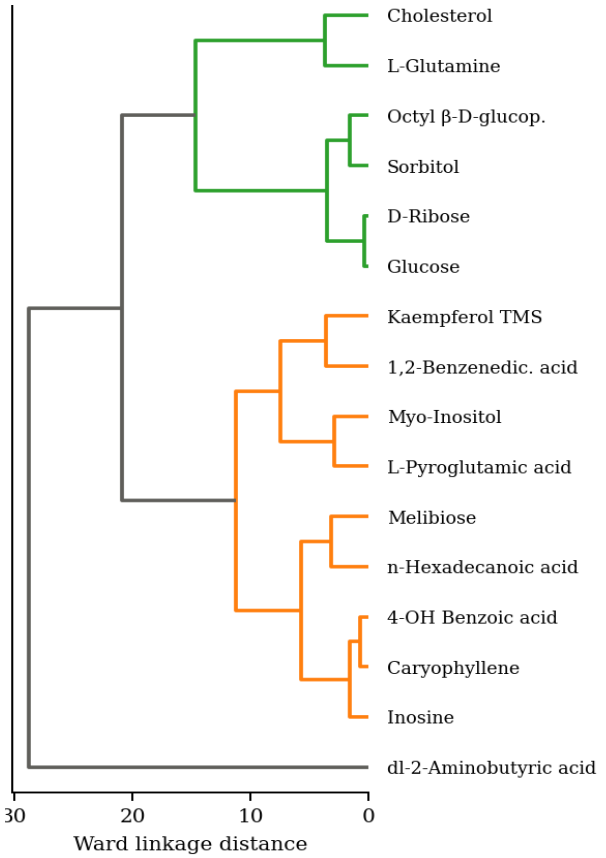

Fig. 1: Hierarchical cluster analysis of GC-MS metabolites in Yoruba Ecotype Chicken sperm reserves. Left panel: dendrogram (Ward linkage; Euclidean distance).

The sperm reserve metabolome was cross-referenced with the KEGG database and the HMDB, the metabolites were mapped to 11 annotated pathways (Fig. 2). Fig. 2A shows each pathway as a proportion of the total number of pathways, while Figure 2B shows the rank of each pathway from most to least contributing, where amino acid metabolism was the top functional class. The most abundant functional class (~37.50% cumulative mean) was amino acid metabolism (KEGG maps00250 and 00471) with dl-2-Aminobutyric acid, L-Glutamine and L-Pyroglutamic acid being the most abundant components. Steroid biosynthesis (map00100) was found to be 10.04%, inositol phosphate signaling (map00562) was found to be 7.00%, and glycolysis/gluconeogenesis (map00010) was found to be 6.14%. The combined abundance of all the metabolites related to the carbohydrate related pathways constituted about 20.9% of the total metabolite abundance. Kaempferol TMS represented the flavonoid antioxidant pathway (map00941) which contributed 3.41% (Table 2).

Table 2: Metabolic pathway annotation of Yoruba Ecotype Chicken sperm reserve metabolites (KEGG cross-referenced)

Pathway	KEGG map	Metabolites	Mean (%)	Sperm parameter indicators
Amino acid metabolism	map00250, map00471	dl-2-Aminobutyric acid, L-Glutamine, L-Pyroglutamic acid	37.51	Motility, viability
Glycolysis/TCA cycle	map00010	Glucose	6.14	Motility, sperm count
Pentose phosphate pathway	map00030	D-Ribose	5.91	ACI, DNA integrity
Inositol phosphate signaling	map00562	Myo-Inositol	7.00	Motility, ACI, capacitation
Steroid biosynthesis	map00100	Cholesterol	10.04	MEI, membrane fluidity
Fatty acid biosynthesis	map00061	N-Hexadecanoic acid	5.34	MEI, acrosomal membrane
Fructose/sorbitol metabolism	map00051	Sorbitol	5.12	Osmotic regulation
Galactose metabolism	map00052	Melibiose	3.72	Carbohydrate reserve
Flavonoid biosynthesis	map00941	Kaempferol TMS	3.41	ABN reduction, ROS defense
Purine metabolism	map00230	Inosine	2.39	ACI, nucleotide recycling
Terpenoid backbone	map00900	Caryophyllene	1.82	Anti-inflammatory, ABN

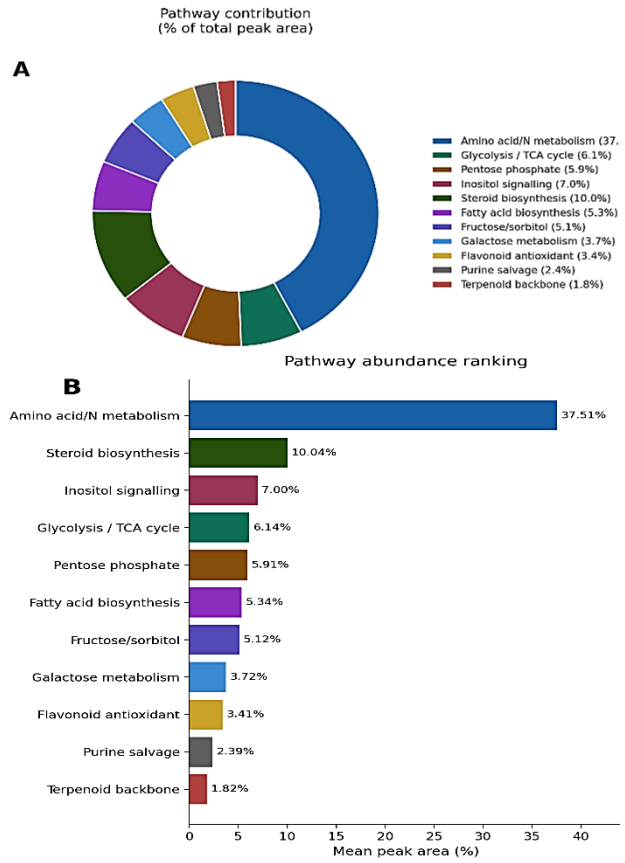

Fig. 2: Metabolic pathway contribution in Yoruba Ecotype Chicken sperm reserves. A: Proportional donut chart (% of total peak area). B: Ranked horizontal bar chart. Colors correspond to biochemical class.

The sperm quality radar (Fig. 3) shows a balanced fertility phenotype with a relatively strong score for the sperm morphological normality (97.0%), MEI (80.0%), and progressive motility (77.50%). Associations between metabolites and sperm quality parameters are presented in the integration network (Fig. 4), with mechanistic associations put in perspective. High levels of L-glutamine and glucose were correlated with high progressive motility of the sperm, which were used as energy supplies for ATP production in the flagellum. Myo-Inositol was associated with sperm concentration by PKC-mediated cascades. D-Ribose was also shown to support the high ACI through the pentose phosphate pathway for replenishment of the nucleotide pool. The high abundance of cholesterol regulating membrane fluidity was mechanistically consistent with the activity of Kaempferol TMS, which was associated with ABN.

The metabolite abundance, mechanistic relevance and published literature support were used to create a tier-ranked biomarker panel (Table 3). Three Primary (★★★★★) biomarkers included dl-2-Aminobutyric acid, L-Glutamine, and cholesterol. Five Secondary (★★★★☆) biomarkers included L-Pyroglutamic acid, Myo-Inositol, glucose, D-Ribose, and kaempferol TMS. The five Supportive (★★★☆☆) biomarkers were N-Hexadecanoic acid, Sorbitol, Octyl β-D-glucopyranoside, Inosine and Caryophyllene. Two metabolites (Melibiose and 4-Hydroxybenzoic acid) were kept as Exploratory (★★☆☆☆) metabolites for further validation. Component mechanistic-relevance, literature-support and abundance scores, the resulting composite Biomarker Score, and the corresponding tier for each metabolite are presented in Table 4. 1,2-Benzenedicarboxylic acid was identified as an exogenous contaminant. The biomarker relevance plot is shown in Figure 5. The metabolites in the upper-right quadrant (dl-2-Aminobutyric acid, L-Glutamine and Cholesterol) were the highest-priority biomarker candidates. The inter-replicate CV% for each metabolite is shown in Fig. 6, where the metabolites with the lowest CV% (L-Pyroglutamic acid, L-Glutamine and 4-Hydroxybenzoic acid) were the most analytically stable metabolites and thus most suitable for use in biomarker development.

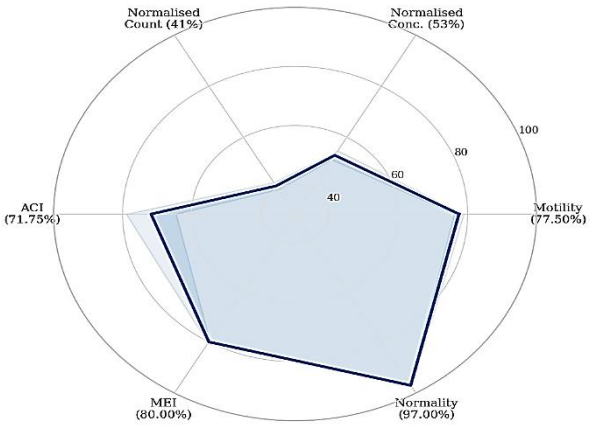

Fig. 3: Sperm quality radar plot for Yoruba Ecotype Chickens. Concentration and Count values are normalized to a 0–100 scale for display; all other axes represent actual percentage values. Shaded band represents ± SEM.

Table 3: Proposed biomarker panel for Yoruba Ecotype Chicken sperm reserve quality (tier-ranked)

S/N	Metabolite	Mean ± SD %	Sperm parameter	KEGG	Tier
1	dl-2-Aminobutyric acid	17.24±12.05	Motility; catabolism index	map00250	★★★★★ Primary
2	L-Glutamine	12.50±1.61	Motility, viability, count	map00471	★★★★★ Primary
3	Cholesterol	10.04±0.95	MEI, membrane fluidity	map00100	★★★★★ Primary
4	L-Pyroglutamic acid	7.77±0.80	Motility, TCA energy	map00250	★★★★☆ Secondary
5	Myo-Inositol	7.00±3.48	Motility, ACI, capacitation	map00562	★★★★☆ Secondary
6	Glucose	6.14±0.84	Motility, sperm count	map00010	★★★★☆ Secondary
7	D-Ribose	5.91±0.65	ACI, DNA integrity	map00030	★★★★☆ Secondary
8	Kaempferol TMS	3.41±0.09	ABN reduction, ROS defense	map00941	★★★★☆ Secondary
9	N-Hexadecanoic acid	5.34±0.02	MEI, acrosomal membrane	map00061	★★★☆☆ Supportive
10	Sorbitol	5.12±0.02	Osmotic balance	map00051	★★★☆☆ Supportive
11	Octyl-β-D-glucopyranoside	4.63±0.18	Membrane stability	map00600	★★★☆☆ Supportive
12	Inosine	2.39±1.13	ACI, purine salvage	map00230	★★★☆☆ Supportive
13	Caryophyllene	1.82±0.43	ABN, anti-inflammatory	map00900	★★★☆☆ Supportive
14	Melibiose	3.72±0.18	Carbohydrate reserve	map00052	★★☆☆☆ Exploratory
15	4-Hydroxybenzoic acid	1.70±0.27	Minor antioxidant	map00362	★★☆☆☆ Exploratory
16	1,2-Benzenedicarboxylic acid	5.31±0.04	Possible contaminant	Not endogenous	⚠ Flagged

⚠ Flagged for follow-up investigation.

Table 4: Biomarker Score (BS) worksheet showing the mechanistic relevance (M), literature support (L), and normalized abundance (A) component scores underlying the tier assignments (BS = 0.4M + 0.4L + 0.2A)

S/N	Metabolite	M (1–10)	L (1–10)	A (1–10)	BS	Tier
1	dl-2-Aminobutyric acid	9	9	10.00	9.20	★★★★★ Primary
2	L-Glutamine	9	9	7.26	8.65	★★★★★ Primary
3	Cholesterol	9	10	5.83	8.77	★★★★★ Primary
4	L-Pyroglutamic acid	8	8	4.52	7.30	★★★★☆ Secondary
5	Myo-Inositol	8	8	4.07	7.21	★★★★☆ Secondary
6	Glucose	8	8	3.57	7.11	★★★★☆ Secondary
7	D-Ribose	8	8	3.44	7.09	★★★★☆ Secondary
8	Kaempferol TMS	8	9	1.99	7.20	★★★★☆ Secondary
9	N-Hexadecanoic acid	7	7	3.11	6.22	★★★☆☆ Supportive
10	Sorbitol	6	6	2.98	5.40	★★★☆☆ Supportive
11	Octyl-β-D-glucopyranoside	6	6	2.70	5.34	★★★☆☆ Supportive
12	Inosine	7	6	1.40	5.48	★★★☆☆ Supportive
13	Caryophyllene	7	6	1.07	5.41	★★★☆☆ Supportive
14	Melibiose	5	6	2.17	4.83	★★☆☆☆ Exploratory
15	4-Hydroxybenzoic acid	5	5	1.00	4.20	★★☆☆☆ Exploratory
16	1,2-Benzenedicarboxylic acid	—	—	—	—	⚠ Flagged (contaminant, excluded)

M=mechanistic relevance score (1–10); L=literature support score (1–10); A=normalized relative abundance (1–10); BS=composite Biomarker Score. Tier thresholds: Primary, BS ≥8.5; Secondary, 7.0–8.49; Supportive, 5.0–6.99; Exploratory, BS <5.0. 1,2-Benzenedicarboxylic acid was excluded from scoring as a probable exogenous contaminant.

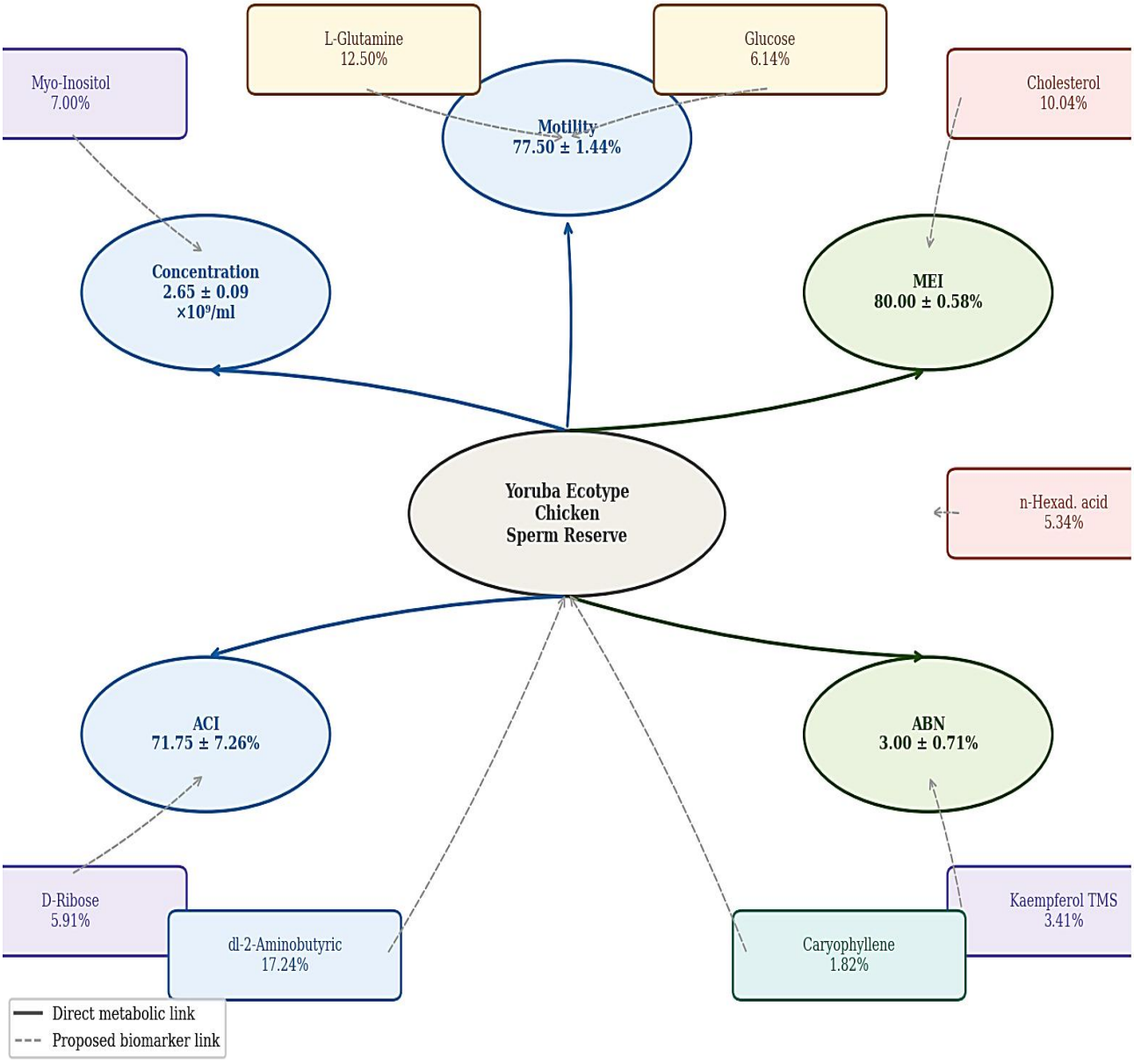

Fig. 4: Metabolite–sperm quality integration network for Yoruba Ecotype Chicken sperm reserves. Ellipses represent sperm quality parameters; rectangles represent key metabolites. Solid arrows indicate direct metabolic relationships; dashed arrows indicate proposed biomarker associations.

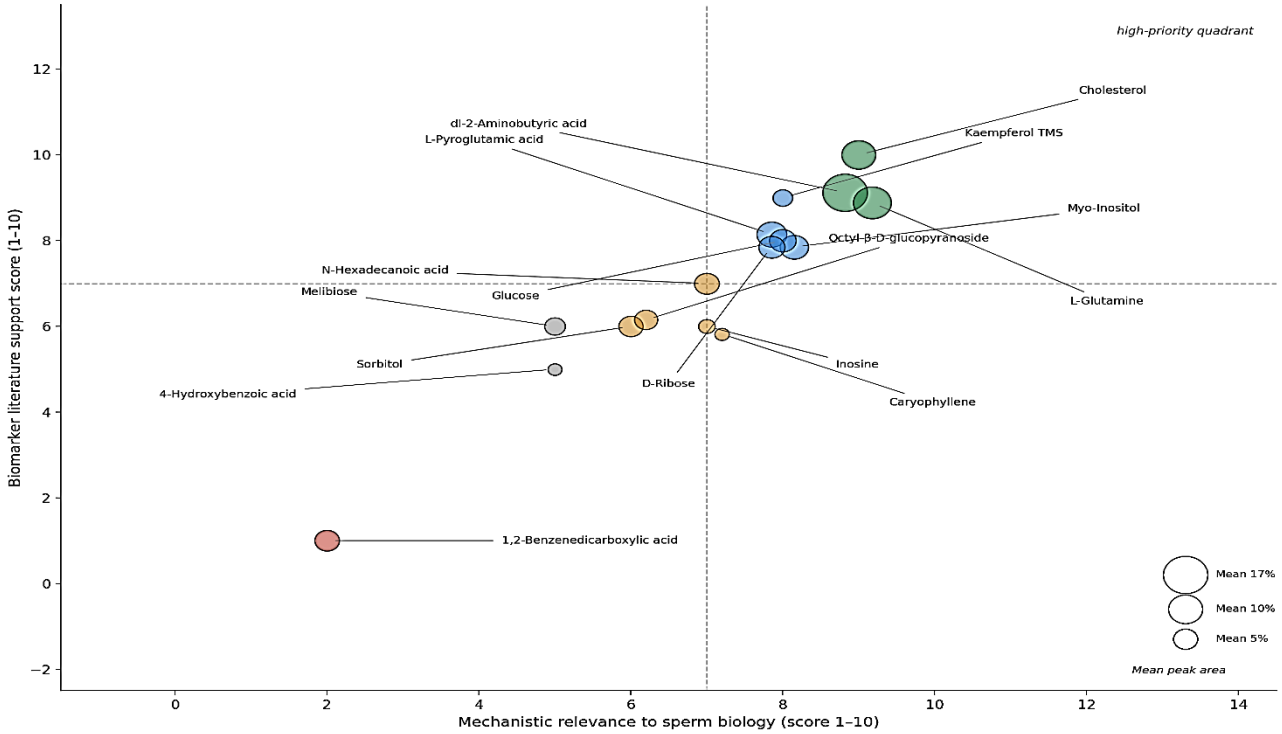

Fig. 5: Biomarker relevance plot for Yoruba Ecotype Chicken sperm reserve metabolites. Bubble area is proportional to mean peak area (%). The X-axis represents mechanistic relevance to sperm biology (1–10) and the y-axis represents biomarker literature support (1–10). Upper-right quadrant = high-priority candidates.

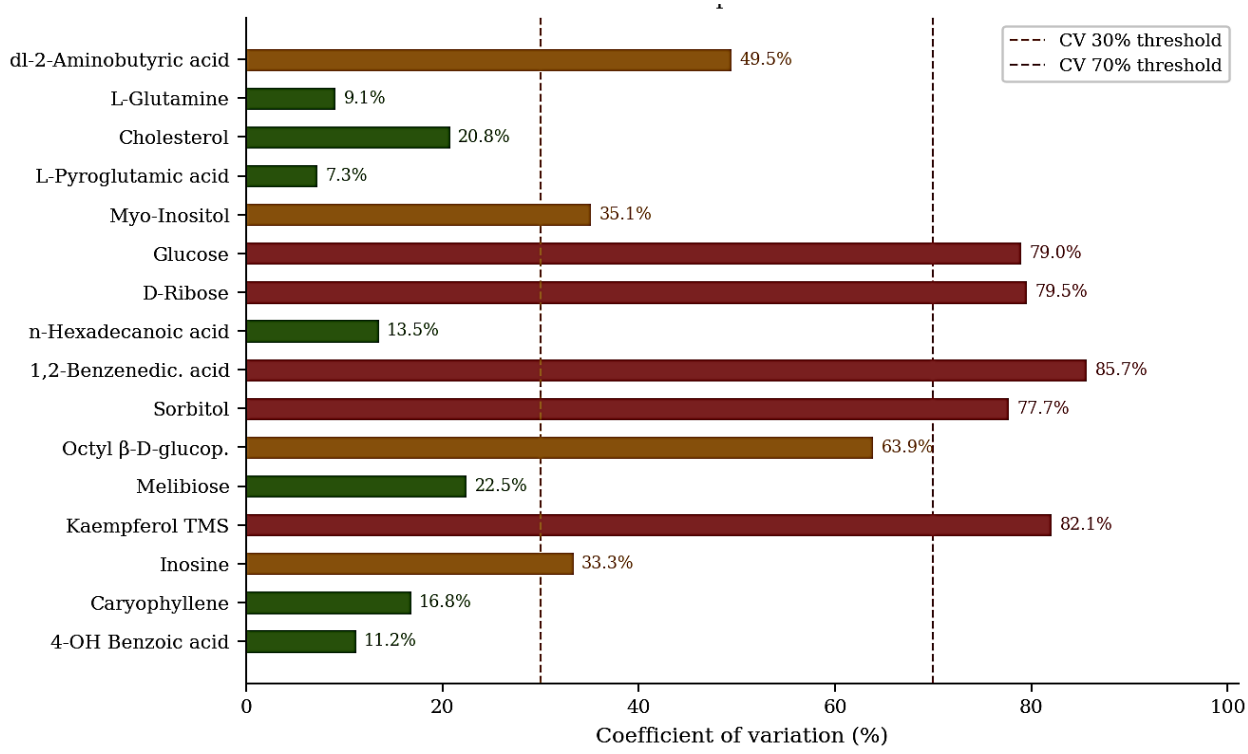

Fig. 6: Inter-replicate coefficient of variation (CV %) for each identified metabolite in Yoruba Ecotype Chicken sperm reserves. Dashed lines indicate 30% and 70% CV thresholds. Green = CV <30% (stable); amber = 30–70%; red = >70% (high inter-replicate variability).

DISCUSSION

A metabolome of all amino acids, sterols, sugars and polyols was detected in the Yoruba Ecotype Chicken sperm reserves, which is a general type of profile typical of the metabolic constituents that are essential in sustaining the viability of sperm in the male reproductive tract that is energetically demanding and redox sensitive. Nine biochemical classes of metabolites were identified with 16 metabolites for the first time for this indigenous Nigerian poultry ecotype and also as a basis for comparative study.

The most significant part of the sperm reserve metabolome (~37.50%) was related to amino acid metabolism. The abundance of the single metabolite was dl-2-Aminobutyric acid (17.24±12.05%), a non-proteinogenic amino acid recognized as a marker of glutathione biosynthesis and oxidative stress response (Irino *et al.*, 2016; Shrestha *et al.*, 2025). The high abundance of dl-2-Aminobutyric acid metabolite is probably due to active antioxidant cycling through the trans-sulphuration pathway. A high CV (69.90%) among replicates indicates a dynamic metabolic flux and not a static structural role. L-Glutamine is a versatile nitrogen carrier and energy substrate supporting anaplerotic TCA cycle entry, with high abundance (12.50±1.61%) and a low CV (12.90%), indicating constitutive stability. L-Pyroglutamic acid represents an important intermediate linking glutamate and proline metabolism to the TCA cycle.

Glucose and D-Ribose were the primary carbohydrate constituents of the sperm reserve. Glucose is the preferred exogenous substrate for avian and caprine sperm motility, providing ATP via glycolysis in the sperm principal piece (Setiawan *et al.*, 2021, 2024). D-Ribose, produced via the pentose phosphate pathway (map00030), is the structural backbone of ATP, NADPH, and nucleic acids, supporting sperm DNA integrity and antioxidant NADPH regeneration. Myo-Inositol, a well-established mediator of sperm capacitation via PKC activation and calcium mobilization in humans (Vazquez-Levin and Verón, 2020; Osman *et al.*, 2023; Ghaemi *et al.*, 2024; Salehi *et al.*, 2024), supports the high ACI (71.75±7.26%) observed in the present study. Sorbitol functions as an osmolyte for intracellular volume regulation and as a cryoprotectant (Mohammadi *et al.,* 2021; Khaeruddin *et al.*, 2024; Arif *et al.*, 2025). During glycolysis, glucose, fructose or sorbitol is converted into pyruvate with a net gain of 2 adenosine triphosphate (ATP) molecules (Van de Hoek *et al.*, 2024).

Cholesterol was the most abundant lipid-class metabolite (10.04±0.95%), reflecting its critical structural role in the sperm plasma membrane. In avian sperm, the membrane cholesterol to phospholipid ratio governs fluidity, which determines resistance to cold shock and osmotic stress (Gautier and Aurich, 2022; Stanishevskaya *et al.*, 2023; Castro *et al.*, 2025). The high MEI value (80.00±0.58%) observed in this study is consistent with an abundant cholesterol pool sustaining membrane integrity across the sperm reserve. N-Hexadecanoic acid (palmitic acid) is the most common saturated fatty acid in avian sperm acrosomal phospholipid bilayers (Gautier and Aurich, 2022), with a low CV (19.10%) indicating its constitutive membrane structural role.

Kaempferol TMS is the trimethylsilyl derivative of the flavonol Kaempferol (Parveen *et al.*, 2023), a potent ROS scavenger with documented anti-apoptotic and DNA-protective effects in male germ cells of rats (Adeyi *et al.*, 2024; Molayousefian *et al.*, 2024; Tvrdá *et al.*, 2026). Kaempferol alleviates bisphenol A reproductive toxicity in rats (Molayousefian *et al.*, 2024), and exhibits cytoprotective and membrane-stabilizing effects in bovine sperm (Baňas *et al.*, 2024). It also inhibits lipid peroxidation of sperm membranes and attenuates 8-hydroxy-2'-deoxyguanosine formation in sperm DNA, directly contributing to reduced sperm morphological abnormalities. The low sperm morphological abnormalities rate (3.00±0.71%) recorded in this study is consistent with effective Kaempferol-mediated oxidative protection. Caryophyllene, a selective CB2R agonist, exerts anti-inflammatory and anti-oxidative effects in reproductive tissues (Ricardi *et al.,* 2024; Wang *et al.*, 2024; Olopade *et al.*, 2025; Alsirhani *et al.*, 2026), contributing to a low-inflammation microenvironment conducive to sperm viability.

1,2-Benzenedicarboxylic acid (phthalic acid) is a plasticizer derived from phthalate esters and is not annotated as an endogenous avian metabolite in HMDB or the KEGG animal metabolome. Its high inter-replicate CV (121.10%) further indicates non-biological variability. It is excluded from the biomarker model and flagged for follow-up investigation via blank matrix analysis and additional biological replicates.

The proposed three-tier biomarker panel captures the three dominant metabolic axes of the sperm reserve: nitrogen catabolism/antioxidant activity (dl-2-Aminobutyric acid), energy substrate availability (L-Glutamine), and membrane structural integrity (cholesterol). As illustrated in Figure 6, metabolites that have the lowest CV% (L-Pyroglutamic acid, 7.3%, L-Glutamine, 9.1% and 4-hydroxybenzoic acid, 11.2%) are the most analytically reliable for quantitative biomarker development. To accurately describe inter-individual metabolic variation, future studies should expand the number of biological replicates and validate the panel over other ecotypes and seasons; correlations between the concentration of metabolites and hatchability should be used to build quantitative predictive models.

Conclusions: This study reports for the first time the comprehensive GC-MS based metabolomic characterization of sperm reserve of Yoruba Ecotype Chicken. Sixteen metabolites were identified and mapped to 11 KEGG-annotated metabolic pathways, which indicate that amino acid catabolism, sterol synthesis, and carbohydrate energy metabolism dominate the metabolome. The hierarchical cluster analysis identified two main groups of metabolites. Mechanistic concordances between the identified metabolites and the excellent progressive sperm motility, membrane integrity, acrosomal integrity, viability and low morphological abnormalities in this ecotype were observed. A tier-ranked biomarker panel was proposed, suggesting that dl-2-Aminobutyric acid, L-Glutamine, and cholesterol may be used as primary biomarkers. The results add to the basic knowledge of reproductive biochemistry of Nigerian indigenous poultry and serve as a reference for comparison with other breeds in the future, for the prediction of subsequent fertility and development of conservation strategies for the Yoruba Ecotype Chicken. However, the semen collected from the experimental cocks was not used for artificial insemination of hens to confirm the correlation between the metabolites and the fertility results.

Authors contribution: This work was carried out in collaboration among all authors. AOA and IM conceived the idea and designed the study. AOA executed the experiment and analyzed the samples and the data. All

authors (AOA, IM, LCN, KIA, OSW, IUK, LM and YP) interpreted the data, critically reviewed the manuscript for important intellectual contents and approved the final version.

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Conflict of interest statement: The authors declare that they have no conflicts of interest.

Data availability statement: GC-MS raw chromatographic data and all output files are available from the corresponding author upon request.

Generative AI statement: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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