Evaporation and reconstitution

Combined supernatants were evaporated on a Büchi Rotavapor R‑114/R‑124 with a ≤ 45 °C water bath under a gentle vacuum. Residues were then transferred in volume to 2 mL amber LC vials (three MeOH rinses of the round‑bottom used to recover residue), given a brief N_2 blow down to near dryness and quantitatively given 1.00 mL methanol to standardize analyte/mL solvent. We did not use membrane filtration to avoid adsorption losses.

Scytonemin is a lipophilic with poor aqueous solubility; low‑temperature, organic extraction is standard (e.g., 1:1 MeOH:EtOAc at 4 °C), followed by evaporation and analysis under organic phases (HPLC‑PDA/MS) (Proteau et al., 1993; Rastogi & Incharoensakdi, 2014). Again, because acetone can drive imine formation, we used an ketone‑free, mildly acidified system to prioritize native scytonemin recovery and spectral fidelity (Larson et al., 2025).

UPLC–DAD–MS

Instrumentation

Analyses were performed on a Waters ACQUITY H‑Class UPLC with a Quaternary Solvent Manager (QSM), FTN autosampler, photodiode‑array detector (PDA), and a Xevo TQ‑MS triple quadrupole (MassLynx 4.1). Column: ACQUITY Premier CSH C18, 150 × 2.1 mm, 1.7 µm, with VanGuard FIT guard.

Chromatographic conditions

Mobile phases: methanol (A) and acetonitrile (B). Gradient at 0.35 mL min^{-1}: 0.00 min, 30 % B; 15.00 min, 20 % B; 15.50 min, return to 30 % B; re‑equilibrate to 25.00 min. Column temperature 35 °C; autosampler 4 °C. Injection volume 5 µL (FTN). Needle wash 10/90 (v/v) acetonitrile/water; post‑inject wash 6 s; purge solvent water.

PDA acquisition. 0–15.00 min, 190–500 nm full‑scan at 2 Hz, 1.2 nm optical resolution. For quantification/spectral checks, single‑wavelength chromatograms were extracted at 384, 430, 450, and 460 nm with background correction.

Mass spectrometry

Electrospray (ESI) in positive and negative modes with common source settings: capillary 2.55 kV, source 130 °C, desolvation 450 °C, cone gas 50 L h^{-1}, desolvation gas 950 L h^{-1}, collision-gas 0.21–0.22 mL min^{-1}. Inter-scan delay 0.005 s; polarity/mode-switch delay 0.020 s. Divert valve: waste 0.00–0.50 min, MS 0.50–15.00 min, waste > 15.00 min.

MS functions (0.00–15.00 min; 55 ms dwell per channel).

• Function 1 (ES^+, SIM) — *Reduced scytonemin*: m/z 547.50 $[M+H]^+$, cone 48 V.

• Function 2 (ES^+, MRM) — *MV-chlorophyll a (± epimers)*: 893.50 → 555.40 and 893.50 → 615.50; cone 44 V; collision 26 V.

• Function 3 (ES^-, MRM) — *Oxidized Scytonemin*: 543.30 → 394.20 and 543.30 → 486.30; cone 56 V; collision 42 V.

Data processing. SIR/MRM spike-removal was enabled; chromatograms were smoothed with a 3-scan window (one pass). PDA and MS acquisitions covered 0.00–15.00 min.

β-carotene did not receive an MS validation as it was not recoverable from this methodology.

Analytical standards and calibration

External standards were scytonemin (aablocks CAS : 152075-98-4 Batch No: AAB25-706074-1), β-carotene, and monovinyl-chlorophyll a (MV-chl a) were also leveraged however due to the extraction procedure being optimized for scytonemin we did not have a robust sample of successful extractions and quantifications to include those anchoring points in analysis. Stock solutions and sample extracts were protected from light and stored cold per manufacturer guidance. On each run, freshly diluted working solutions spanning the analytical range (seven levels) were injected.

All quantitative readouts were anchored to validated linear calibrations of the form:

$$concentration\ (mg{\cdot}mL^{-1}) \;=\; \beta 0 \;+\; \beta 1{\times}AUC,$$

with β0 (mg·mL^{-1}) the intercept and β1 (mg·mL^{-1}·AUC^{-1}) the slope. For biomass-normalized reporting, analyte abundance was scaled to dry weight (DW) as:

$$mg{\cdot}gDW^{-1} \;=\; (\beta 0 \;+\; \beta 1{\times}AUC){\times}VextractmDW.$$

Here, $Vextract$ is the extract volume (mL) and mDW is the dry mass of the thallus aliquot (g). This normalization was applied to both DAD/PDA-based and chromatogram-integrated calibrations and carried through all quantitative summaries.