Supplementary Information

Ehrlich chromogenic reaction based merging-zone flow injection analysis for accurate and rapid determination of total indole phytohormones in plant resources

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1. Detection method

1.1. Manual sampling method

2.00 mL of different concentrations of 5-HT standard solutions were accurately transferred to a 10 mL volumetric flask. Subsequently, 5.00 mL of Ehrlich's reagent was added and then mixed thoroughly. After 1 hour of reaction at room temperature, the solution was diluted to the volume with 0.1 mol/L HCl and then mixed thoroughly. The absorbance at 591 nm was measured using UV-Vis spectrophotometry. A calibration curve was established based on the absorbance-concentration relationship (5-300 mg/L).

1.2. HPLC method

The column, Waters XBridge C18 (250 mm × 4.6 mm, 5 μm) was used. The mobile phase was consisted of 2% acetic acid-water solution (A) and methanol (B). The gradient elution program is shown in Table S1, with a flow rate of 0.5 mL/min, a detection wavelength of 281 nm, and an injection volume of 10 μL.

Table S1 Gradient elution procedure for HPLC analysis.

Time (min)	A (%)	B (%)
0	60	40
15	0	100
15.01	45	55
25	45	55

2. Results

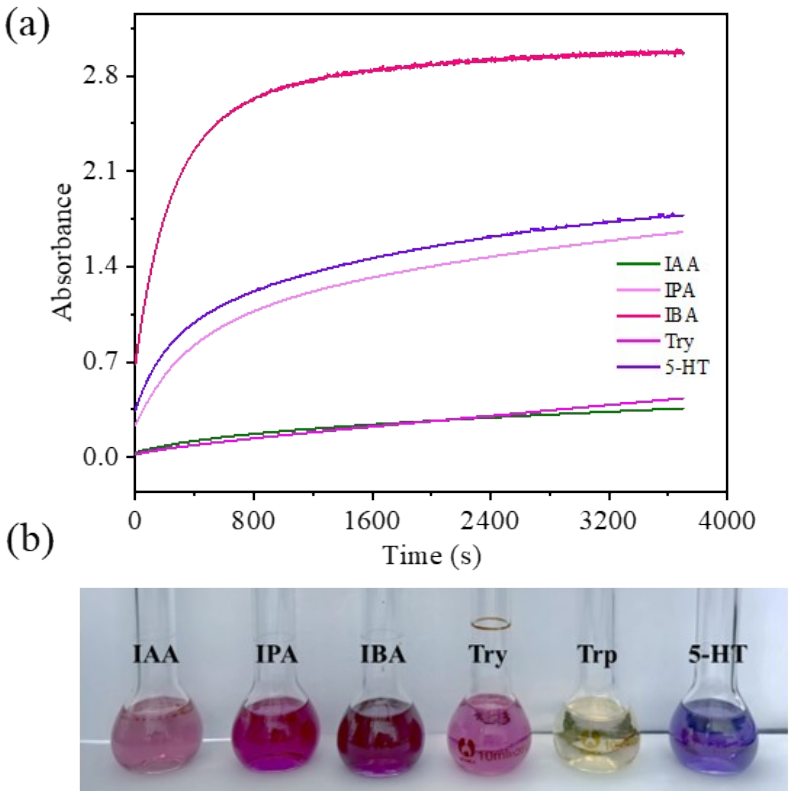

Fig. S1 (a) Colorimetric reaction kinetic curves of five indole compounds obtained using manual sampling; (b) Photo images of six indole compounds reaction mixture with the Ehrlich reagent under daylight.

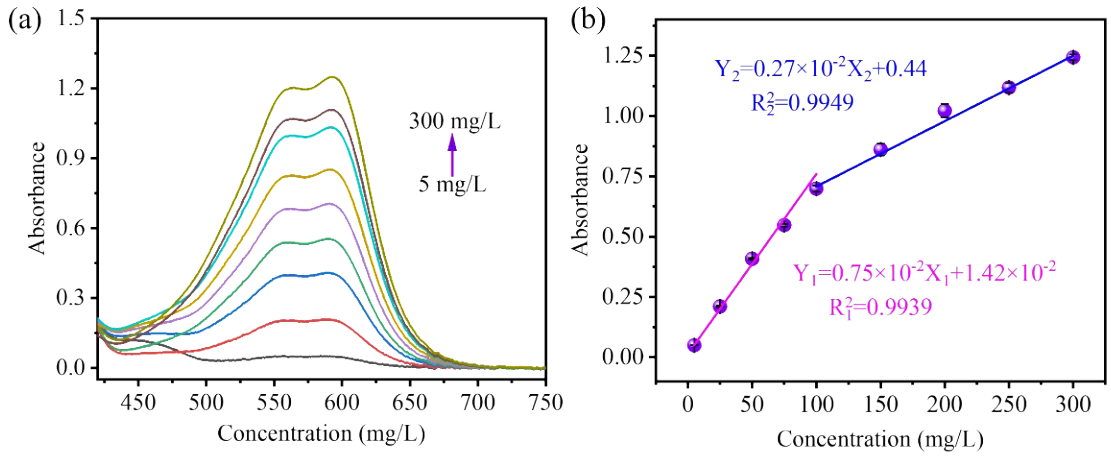

Fig. S2 (a) Visible absorption spectra of reaction products at different concentrations of 5-HT (5-300 mg/L); (b) Corresponding calibration curve.

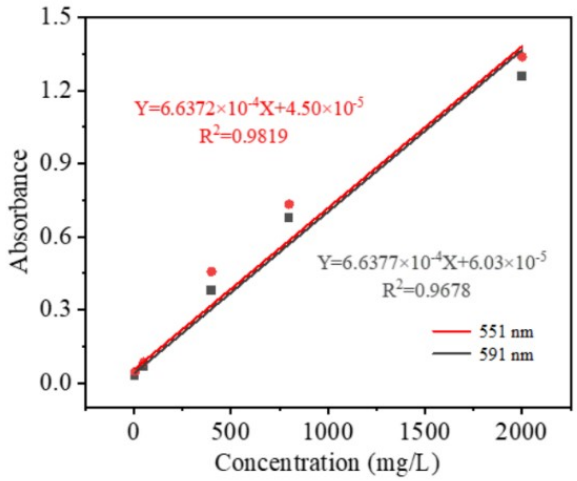

Fig. S3 Effect of measurement wavelength on the linearity of the calibration curve. Carrier temperature=85°C, RC length=6 m, V_S=200 μL, V_R=300 μL, C_R=40 g/L, RC temperature=60°C, and flow rate=1.75 mL/min.

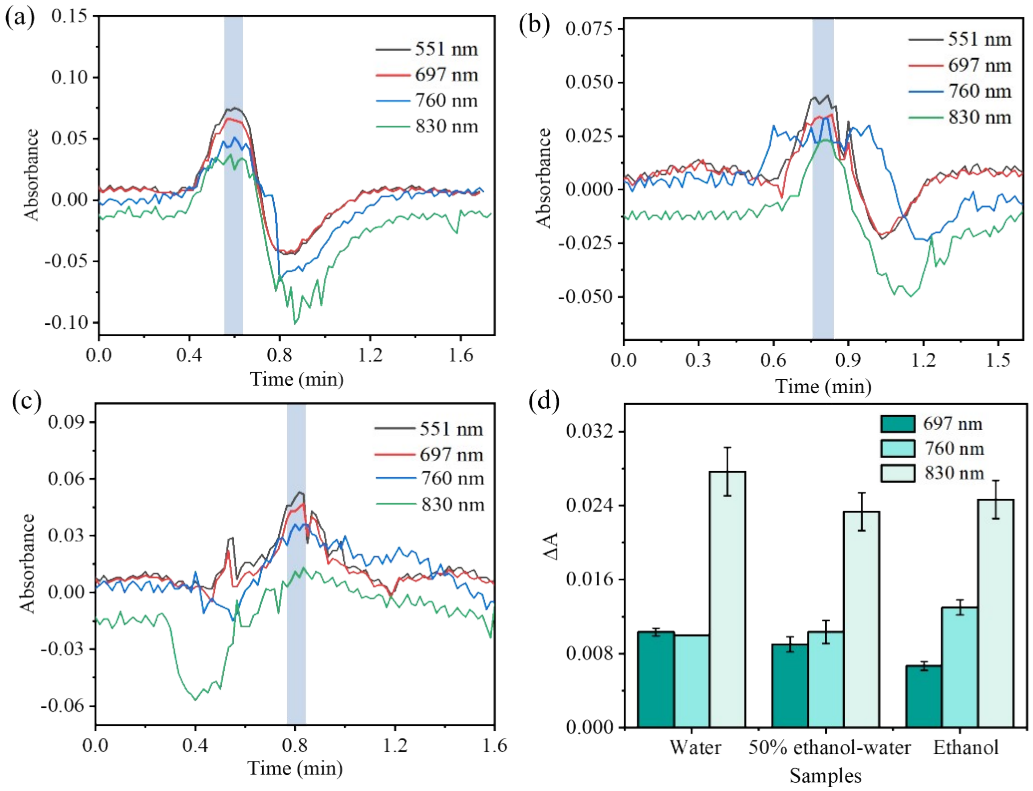

Fig. S4 FIA response curves for different solvents: (a) Water; (b) 50% ethanol-water (v/v); (c) Ethanol; (d) Comparison of absorbance differences (ΔA) for samples in different solvents at different reference wavelengths. Detection wavelength=551 nm, carrier temperature=85°C, RC length=6 m, V_S=200 μL, V_R=300 μL, C_R=40 g/L, RC temperature=60°C, and flow rate=1.75 mL/min.

A 0.1 g/L potassium permanganate solution, for its stability, inertness, and characteristic absorbance at 525 nm, was used as a tracer to quantify the system dispersion coefficient. The procedure involved first measuring the absorbance (A_0) by injecting the tracer directly into the flow cell, bypassing the FIA manifold. Subsequently, potassium permanganate solution was drawn into the sample loop (200 μL) and injected into the water-filled flow path (where both C1, C2 and the reagent loop were filled with water), then transferred to the detector to obtain the dispersed absorbance (A). The dispersion coefficient (D) was calculated using the formula, $D = A_0/A$. As shown in Fig. S5, the D value exhibits linear correlation with the square root of the reaction coil length, yielding a correlation coefficient R^2 of 0.9626.

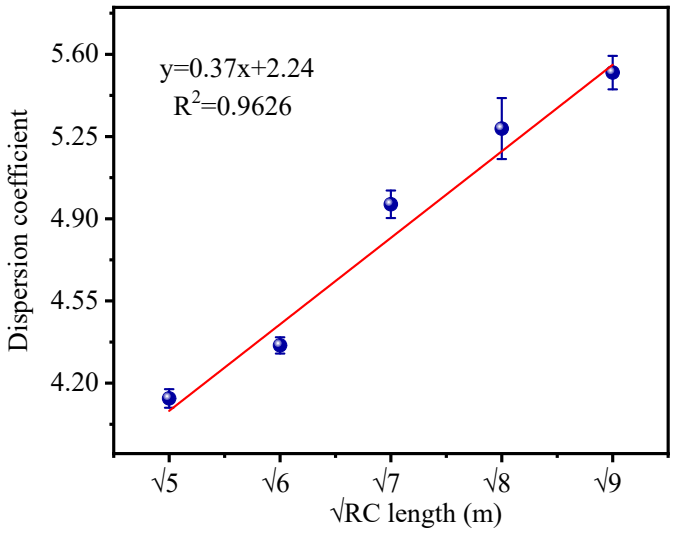

Fig. S5 Effect of RC length (5-9 m) on dispersion coefficient with V_S=200 μL, and flow rate=1.75 mL/min.

A 0.1 g/L potassium permanganate solution was employed as tracer to determine the overlapping pattern of the sample and reagent. Both the C1 and C2 carriers and the reagent loop were filled with water to ensure no other components interfered within the system. The tracer solution was drawn into the sample loop (200 μL) and injected into the aforementioned water-filled flow path. Absorbance signals were recorded at 525 nm to acquire the peak profile of the sample loop. Under the same flow conditions, the tracer solution was drawn into the reagent loop (20 μL), the injection procedure was repeated, and the peak profile of the reagent loop was recorded. Both curves were imported into the same absorbance-time plot to synchronize the onset of their peaks.

Results are shown in the Fig. S6. The peak height of the sample loop is significantly higher than that of the reagent loop, attributable to different injection volumes and consistent with the concentration-absorbance relationship governed by the Lambert-Beer law. The near-identical timing of peak onset for both curves indicates that the sample and reagent solutions reached the merging point and detector almost simultaneously. Upon curve superimposition, the sample loop's peak shape completely envelops the reagent loop's peak (exhibiting a broad peak enveloping a narrow peak phenomenon), confirming that the overlap pattern is sample enveloping reagent.

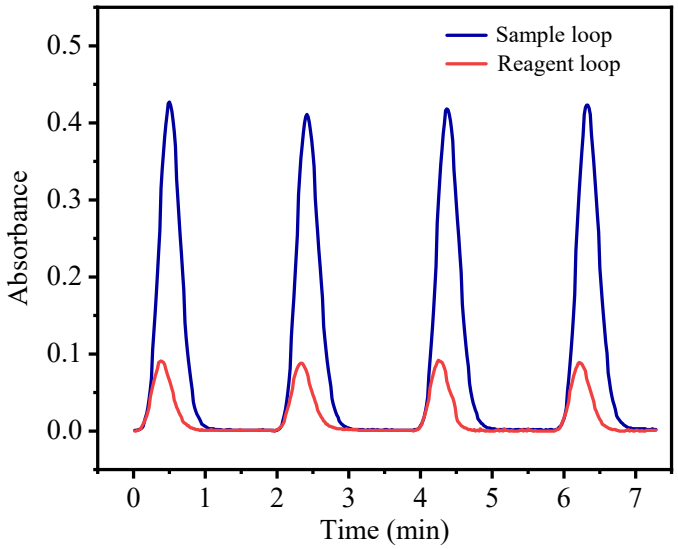

Fig. S6 Peak profile curves for the sample loop (200 μL) and reagent loop (20 μL).

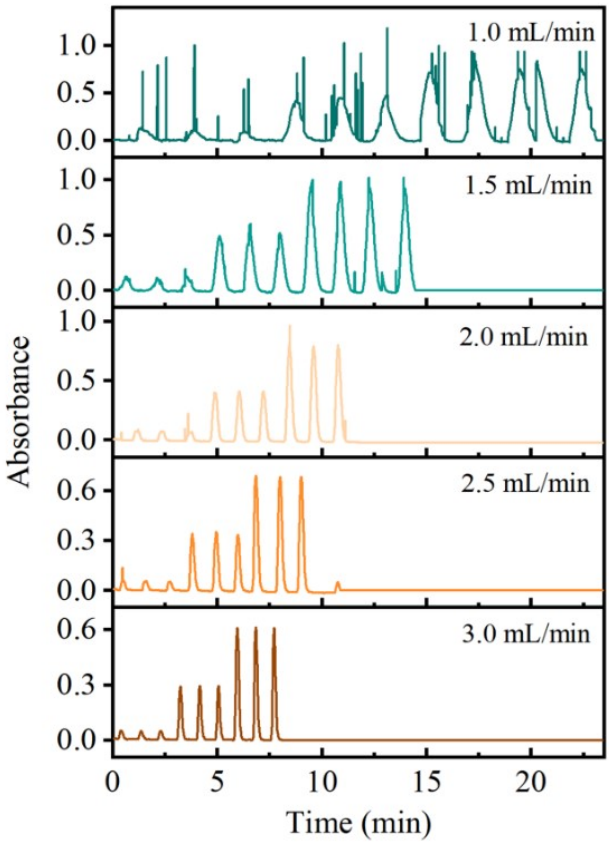

Fig. S7 Effect of flow rate (1-3 mL/min) on peak shape with 5-HT concentration=100, 500 and 1000 mg/L, carrier temperature=85°C, RC length=7 m, V_S=200 μL, V_R=20 μL, C_R=250 g/L, and RC temperature=70°C.

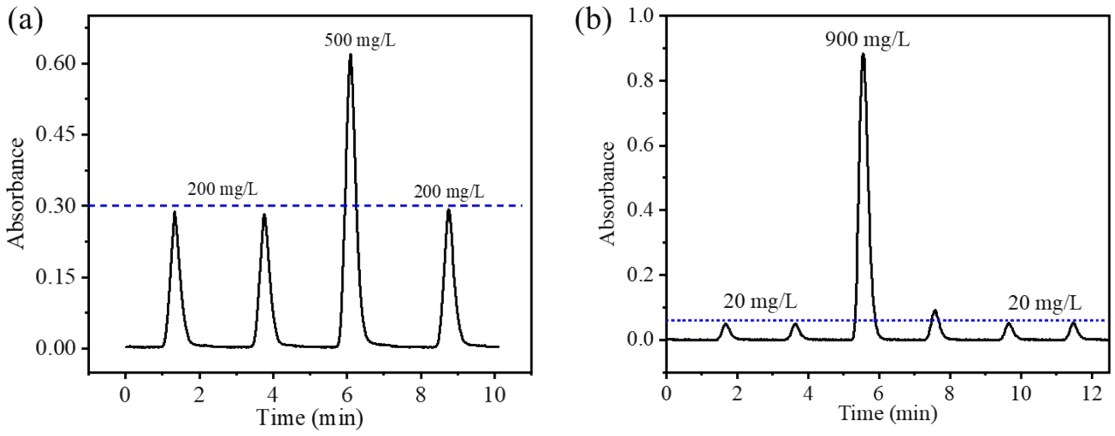

Fig. S8 Carryover effect in sequential sample analysis using high and low concentration samples: (a) 200 mg/L and 500 mg/L; (b) 20 mg/L and 900 mg/L.

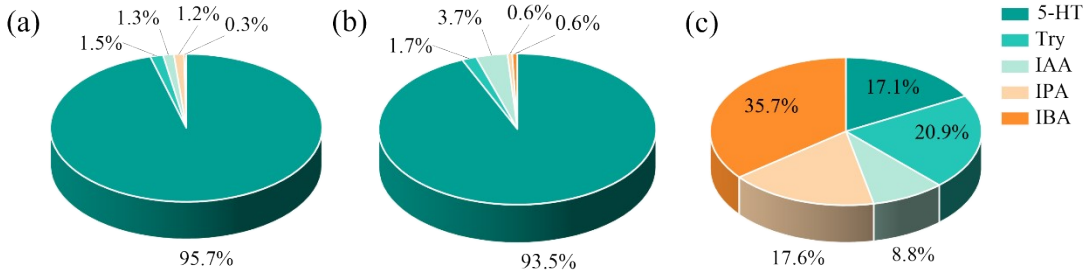

Fig. S9 Relative distribution of five indole hormones in different plant samples (percentage peak area): (a) Banana peel; (b) Peganum harmala L.; (c) Safflower seed meal (I).

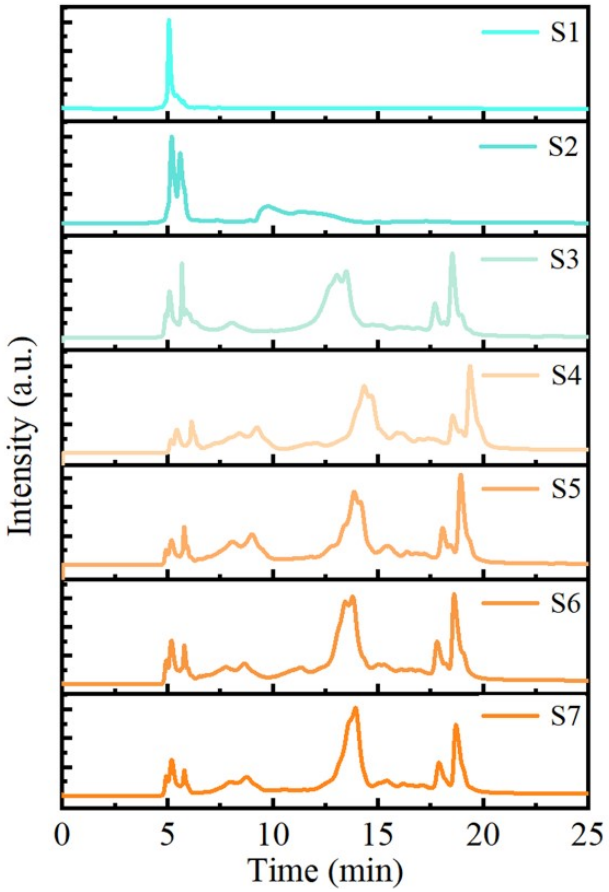

Fig. S10 HPLC chromatograms of seven plant extracts. (S1: Banana peel; S2: Peganum harmala L.; S3: Safflower seed (I); S4: Safflower seed (II); S5: Safflower seed meal (I); S6: Safflower seed (III); S7: Safflower seed meal (II)).