TLC–chemometric analysis reveals putative chemotypic variation in Yucatecan *Clusia* species

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Left to right: Luis Alfredo Herbert-Doctor, Carolina Abril Cordero-Riande, Miriam Monforte-Gonzalez, Ileana Echevarria-Machado, Luis Manuel Peña-Rodríguez, Séverine Derbré.

Carolina Abril Cordero-Riande, PhD student at CICY, combines HPTLC, chemometrics, and dereplication to investigate plant bioactive metabolites. Miriam Monforte-Gonzalez operates CICY's HPTLC equipment and trains students and researchers, while Dr. Ileana Echevarria-Machado supervises the laboratory's research activities. Dr. Séverine Derbré, University of Angers, specializes in ^{13}C NMR dereplication. Dr. Luis Alfredo Herbert-Doctor, UADY, applies metabolomics and dereplication to chemotaxonomic studies. Dr. Luis Manuel Peña-Rodríguez, CICY, develops strategies for extracting, purifying, and identifying bioactive metabolites.

Introduction

Over the last two decades, the study of plant chemotypes and their chromatographic profiles has relied heavily on column-based chromatographic methods such as High-Performance Liquid Chromatography (HPLC), Ultra-Performance Liquid Chromatography (UPLC), and Gas Chromatography (GC) [1-3]. Recently, however, High-Performance Thin-Layer Chromatography (HPTLC), an important analytical alternative for screening crude extracts and obtaining fingerprint profiles of medicinal plants, has been adopted in pharmaceutical, food, and biological research because of its significantly lower operating costs [4-5], minimal and straightforward sample preparation, shorter analysis times, and the possibility of analyzing multiple samples in parallel [6-7].

Modern HPTLC analyses, which include advanced image-capturing devices and powerful scanning instruments that provide highly reproducible densitometric data, can generate large and complex datasets that limit their visual interpretation. To address this, HPTLC chromatographic profiles can be analyzed using chemometric tools such as Principal Component Analysis (PCA) and Hierarchical Clustering Heat-Maps, which enable the efficient exploration of complex data and the detection of subtle chemical differences among chromatographic profiles [8]. This combined strategy has been successfully applied in chemotaxonomic studies to differentiate *Peperomia* [9] and *Clerodendrum* spp. [10], as well as to fingerprint and classify Apiaceae samples [11].

Recently, the occurrence of chemotypes within *Clusia* spp. of the Yucatecan flora (*C. rosea*, *C. flava*, *C. lundellii*, and *C. suborbicularis*) was suggested by significant variations observed in the HPLC chromatographic profiles of bark extracts from individuals within the same population [12-13]. Here, we report on the use of chemometric analyses of TLC chromatographic profiles to investigate possible chemotypic variation in *Clusia* spp. from the Yucatecan flora [14].

Sample preparation

Crude bark extracts and semi-purified fractions were dissolved at 10 mg/mL in dichloromethane–methanol 3:2 (*v/v*). Semi-purified fractions were obtained through a liquid-liquid partition fractionation of the crude bark ethanolic

extracts, resuspending a portion (30 mg) of each crude extract in 30 mL of water-methanol 3:2 (*v/v*) and successively partitioning the resulting suspension with hexane and ethyl acetate [three times each, 2:1 (*v/v*) solvent-to-suspension ratio]. Evaporation of the organic phases under reduced pressure at 40 °C produced the corresponding low- and medium-polarity semi-purified fractions.

Chromatogram layer

TLC aluminum sheets coated with silica gel 60 F_{254} (Merck/Supelco, catalogue No. 1.05554.0001). The original 20 × 20 cm aluminum sheets were cut (10 × 20 cm) before use.

Sample application

Sample solutions (3 µL) were applied as 5.0 mm bands using the Linomat 5 semi-automatic sample applicator. Each plate contained 18 tracks, with a 5.3 mm space between adjacent bands; applications were made 10.0 mm from the left and right edges, and 10.0 mm from the lower edge.

Chromatography

The plates were developed over a migration distance of 70 mm in a Twin Trough Chamber, after 10 min of chamber saturation, using 10 mL of a diethyl ether–hexane 3:2 (*v/v*) mixture, supplemented with 50 µL of formic acid, as eluant. After development, the plates were dried at 75 °C for 9 min using the TLC Plate Heater 3.

Post-chromatographic derivatization

The plates were immersed in phosphomolybdic acid reagent, prepared using 20 g of phosphomolybdic acid and 2.5 g of cerium (IV) sulfate (Sigma-Aldrich, product No. 359009, CAS No. 13590-82-4) in 500 mL of 5% (*v/v*) sulfuric acid. Derivatization was performed using the Chromatogram Immersion Device 3, with an immersion speed of 3 cm/s and an immersion time of 2 s. The plates were dried with cold air for 30 s and then heated at 75 °C for 9 min using the TLC Plate Heater 3.

Densitometry

Absorbance measurements were performed at 470 nm using the TLC Scanner 4 and *winCATS*, with slit dimensions of 5.00 mm × 0.20 mm, a scanning speed of 20 mm/s, and a data resolution of 100 µm/step.

Data treatment

Peak data were exported from *winCATS* as a PDF file and transferred manually to an Excel matrix, which in turn was saved as a CSV file. The start R_F values and peak areas were used to construct the data matrix, and relative intensities were calculated from the peak areas. Peaks from different profiles were manually aligned according to their R_F values, using a tolerance of ±0.02. The resulting data matrix was imported into MetaboAnalyst 5.0, normalized by sum, and Pareto-scaled prior to PCA.

Results and Discussion

A set of 36 TLC chromatographic and densitometric profiles (nine samples in total, four replicate applications per sample) was generated from the crude bark extracts of *C. flava* (three samples), *C. suborbicularis* (two samples), *C. rosea* (two samples), and *C. lundellii* (two samples); representative profiles are shown in Figure 1.

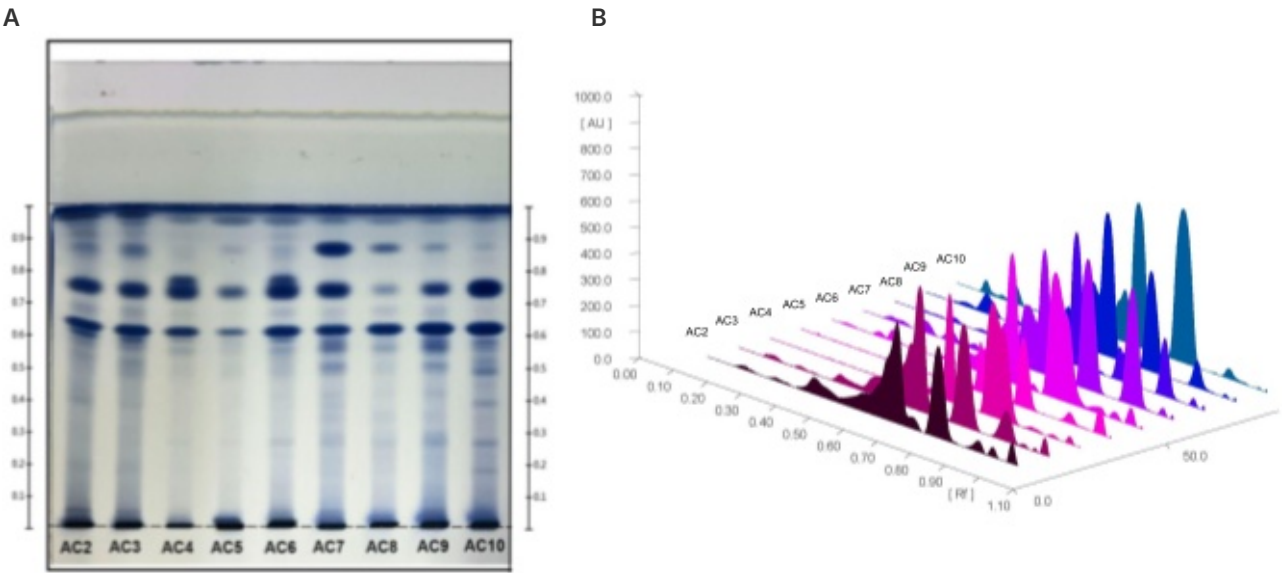


Figure 1. Representative TLC chromatographic profiles recorded under white light after derivatization (A), and corresponding densitometric profiles recorded at 470 nm (B), of the crude bark extracts of C. suborbicularis (AC2 and AC3), C. flava (AC4, AC5 and AC6), C. rosea (AC7 and AC8), and C. lundellii (AC9 and AC10).

TLC–chemometric profiling

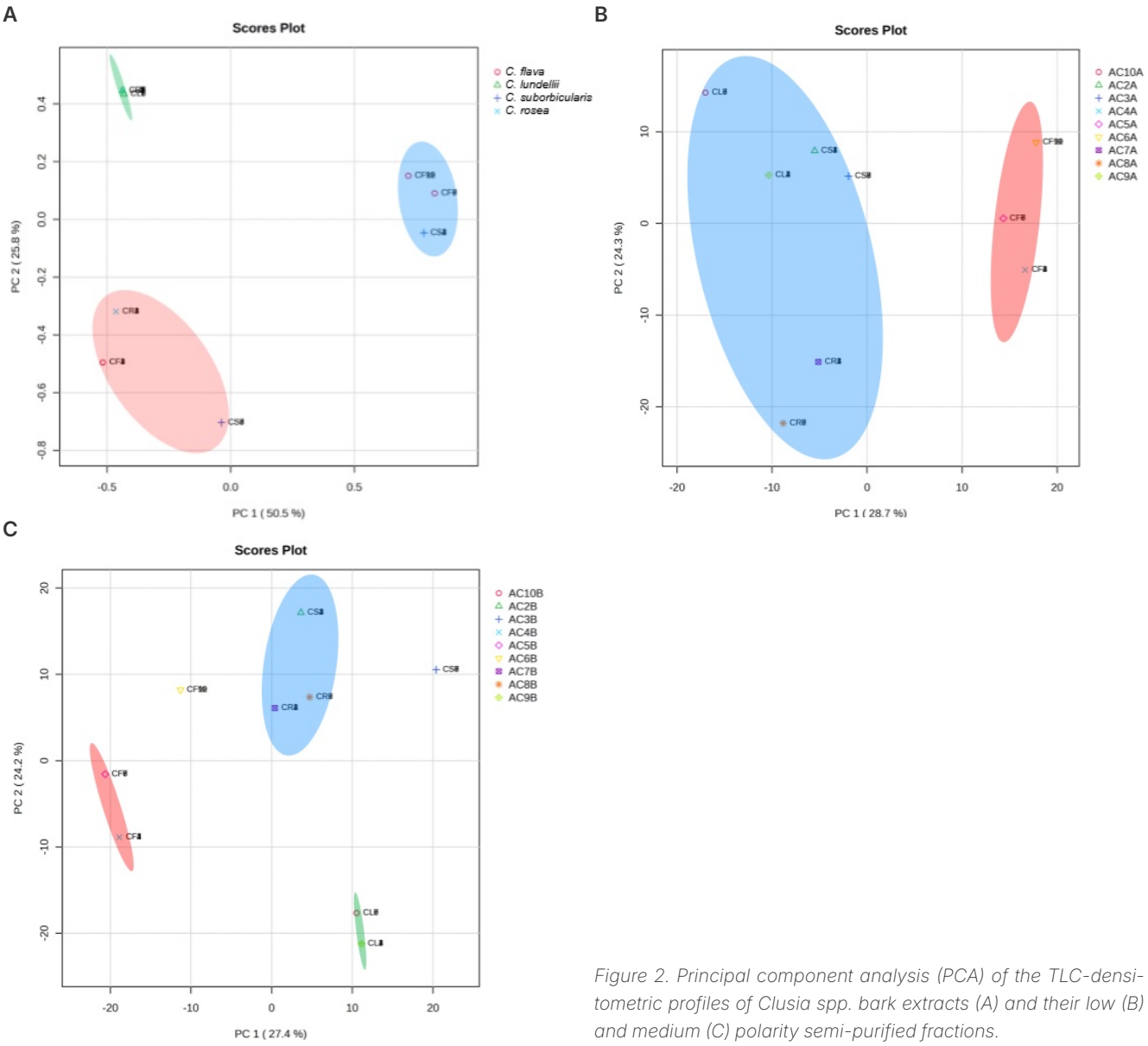


Figure 2. Principal component analysis (PCA) of the TLC-densitometric profiles of Clusia spp. bark extracts (A) and their low (B) and medium (C) polarity semi-purified fractions.

Although the chromatographic profiles of the different species appeared broadly similar on initial observation and shared several components, chemometric analysis of the densitometric profiles yielded a PCA model in which the first five principal components together accounted for 99.1% of the total variance (PC1: 50.5%; PC2: 25.8%; PC3: 12.3%; PC4: 6.6%; PC5: 3.9%). The PC1 versus PC2 score plot showed three apparent groupings (Figure 2A). The first included two extracts of *C. flava* (AC4, from Tixcacalpupul, and AC5, from Punta Laguna) and one extract of *C. suborbicularis* (AC3, from Sian Ka'an). The second included the remaining *C. suborbicularis* extract (AC2, also from Sian Ka'an), one *C. rosea* extract (AC7, from Mérida), and the third *C. flava* extract (AC6, also from Punta Laguna). The third comprised both *C. lundellii* extracts (AC9 and AC10, from Calakmul) and the second *C. rosea* extract (AC8, also from Calakmul). These results showed marked chemical differences between individuals from the same populations, particularly for *C. flava* and *C. suborbicularis*, consistent with previous findings [13], and provided additional support for the possible occurrence of chemotypic variation within Yucatecan *Clusia* species.

Interestingly, the apparent groupings did not correspond strictly to either species identity or collection locality. Extracts from individuals belonging to the same species and population, such as AC2 and AC3 for *C. suborbicularis* and AC5 and AC6 for *C. flava*, occupied different regions of the PCA score plot, whereas extracts from different species sometimes grouped together. This indicates that species identity alone did not explain the observed chemical variation. However, given the limited number of individuals examined, these groupings should be considered exploratory rather than definitive evidence of stable chemotypes. Additional individuals and independent collections would be required to distinguish genetic variation from environmental, developmental, or temporal effects.

TLC–chemometric profiling

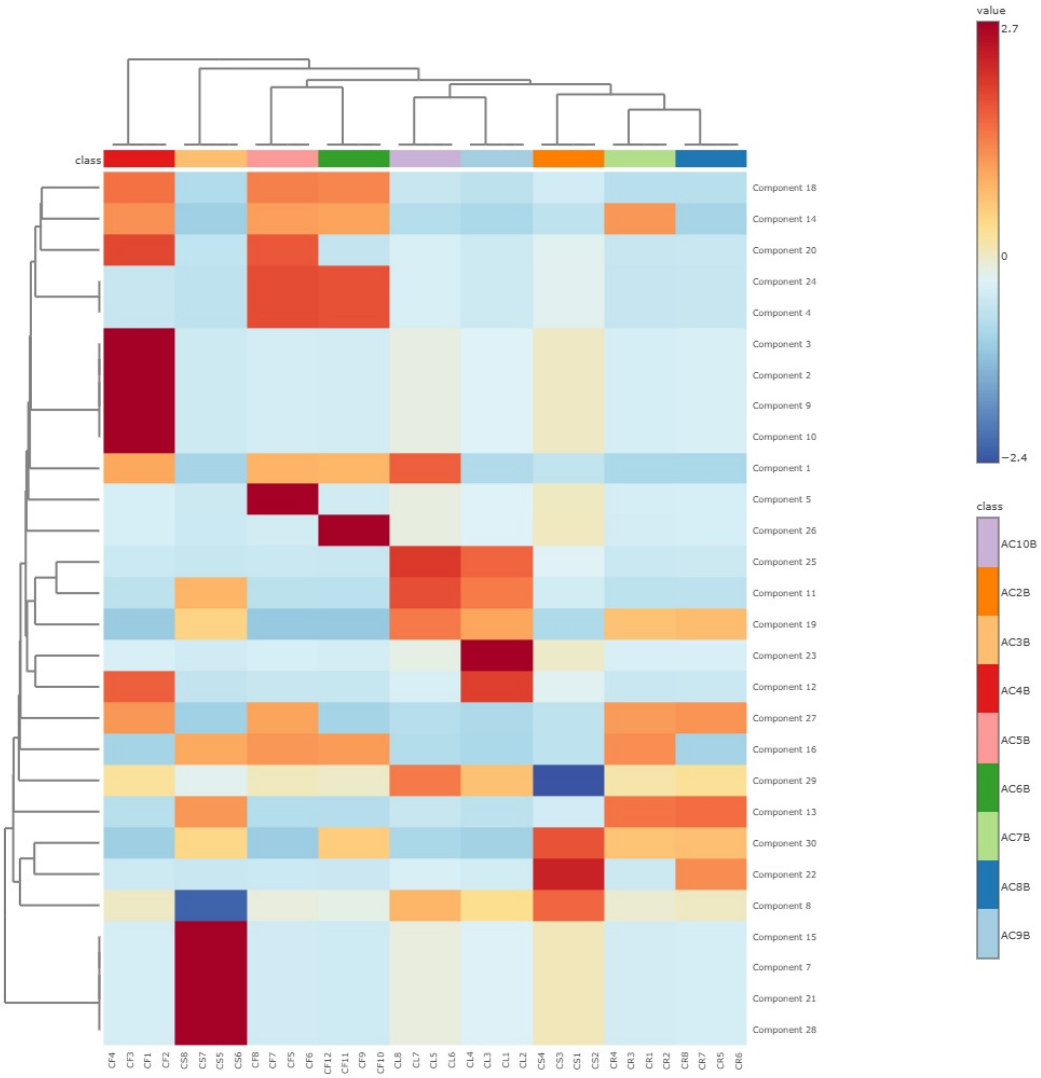


Figure 3. Hierarchical Clustering Heat-Map of the TLC-densitometric profiles of Clusia spp. medium polarity semi-purified fractions (C. suborbicularis: CS; C. flava: CF; C. rosea: CR; C. lundelli:CL). The heat-map color scale represents autoscaled relative abundances (Z-score) of chromatographic R_F features, ranging from blue (lower than average levels) to red (higher than average levels). Hierarchical clustering was performed using Euclidean distance and Ward's linkage method, illustrating sample relationships and intraspecific metabolic variations that suggest potential chemotypes within C. suborbicularis and C. flava.

To determine which polarity fractions contained the chemical features contributing to the observed differences, each crude extract was suspended in a water-methanol mixture and fractionated by liquid–liquid partition to obtain the corresponding low-polarity (hexane, A) and medium-polarity (ethyl acetate, B) fractions (see sample preparation for details). Chemometric analysis of the TLC–densitometric profiles of the low-polarity fractions produced a PCA model in which the first five principal components explained 87.2% of the total variance (PC1: 28.7%; PC2: 24.3%; PC3: 16.6%; PC4: 9.8%; PC5: 7.8%). In the PC1 versus PC2 score plot, the low-polarity fractions of *C. lundellii*, *C. rosea*, and *C. suborbicularis* occupied the same main region (Figure 2B), while the *C. flava* fractions formed a separate group. This suggests that the first three species had broadly similar chromatographic profiles in this fraction.

For the medium-polarity fractions, the first five principal components accounted for 90.2% of the total variance (PC1: 27.4%; PC2: 24.2%; PC3: 17.6%; PC4: 12.4%; PC5: 8.6%). The PC1 versus PC2 score plot showed three apparent groupings (Figure 2C): the first comprised both *C. lundellii* fractions (AC9B and AC10B, from Calakmul); the second included both *C. rosea* fractions (AC7B, from Mérida, and AC8B, from Calakmul) and one *C. suborbicularis* fraction (AC2B, from Sian Ka'an); and the third included two *C. flava* fractions (AC4B, from Tixcacalpupul, and AC5B, from Punta Laguna). One *C. flava* fraction (AC6B, from Punta Laguna) and one *C. suborbicularis* fraction (AC3B, from Sian Ka'an) fell outside these groupings, indicating differences from their respective same-species counterparts, which were also reflected in the hierarchical clustering heat map (Figure 3).

Taken together, these findings show that the main intraspecific chromatographic differences associated with possible chemotypic variation in *Clusia* were most apparent in the medium-polarity fractions.

The fractionation step provided complementary information on the distribution of the observed chemical variation. While the low-polarity fractions mainly differentiated *C. flava* from the other species, the medium-polarity fractions retained the clearest differences between individuals of *C. flava* and *C. suborbicularis* collected from the same populations. Fractionation therefore helped simplify the complex crude-extract profiles and localized the main intraspecific differences within a narrower polarity range. Nevertheless, TLC–densitometric analysis and PCA alone do not identify the individual metabolites responsible for these differences, and further targeted characterization would be required.

Conclusions

TLC fingerprinting combined with chemometric analysis differentiated bark extracts of *C. suborbicularis* and *C. flava* collected from the same populations. Analysis of the semi-purified fractions showed that the main chemical differences were associated with the medium-polarity fractions. These results demonstrate the value of TLC as a rapid screening tool for investigating intraspecific chemical variation. In the complete study, the TLC–chemometric analysis was complemented by ^{13}C NMR-based dereplication of selected medium-polarity fractions, which provided tentative annotations of several differential terpenoids [14].

References

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