

Chemical Mapping of *Crocus sativus* L. Floral Parts for Assessing Compositional Consistency and Auto-Adulteration in Saffron-Based Botanicals

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Abstract

Phytochemical variability complicates the quality assessment of botanicals, both in powdered and extract form, particularly when authenticated reference materials are limited. Saffron (*Crocus sativus* L.) used both as a spice and as a food supplement ingredient, exhibits marked compositional differences across floral parts, making quantitative evaluation of compositional balance essential for quality control and authentication. Saffron-based botanicals were profiled using LC–TWIMS–HRMS and evaluated within a floral part-anchored multivariate framework. Sixty-eight metabolites were tentatively identified across stigma, petal, and stamen reference parts and nine commercial botanicals. Principal component analysis of log-transformed data revealed a dominant stigma–petal compositional gradient. Botanicals were positioned relative to this gradient using projections onto anatomical centroids, and robustness was evaluated through compound-level and macro-class resampling. Residual analysis allowed to define the components not explained by the primary gradient. Most botanicals remained closely aligned with the stigma reference profile, while a subset showed reproducible displacement toward petal-associated chemical patterns driven by coordinated variation in flavonoids and anthocyanins relative to crocins, suggesting a lower ingredient purity.

These findings demonstrate that floral parts-anchored chemical mapping combined with structured multivariate modelling may enable the detection of compositional deviations consistent with auto-adulteration of saffron-based botanicals under conditions representative of routine quality assessment.

1. Introduction

Despite the increasing global demand for plant-derived health products, quality control remains inconsistent, largely due to the absence of robust and enforceable authentication and standardization protocols capable to properly deal with phytochemical variability [1, 2]. While authentication is relevant for assuring the identity of the botanical used, standardization is intended to ensure known and reproducible concentrations of bioactive compounds which are responsible for the product's claimed health benefits. For both, however, multiple studies regularly reveal discrepancies between labeled and actual content [3–6]. Such inconsistencies can lead to diminished efficacy and, in certain cases, safety concerns. Above all, these phenomena make the work of companies manufacturing plant-based food supplements and health products more challenging. Requiring them, for example, to carry out continuous and extensive quality controls on the botanical ingredients purchased for their formulations.

The variability in plant materials can be caused by multiple interacting factors, including intraspecific genetic differences, environmental conditions during cultivation, timing and methods of harvest, post-harvest handling, and the choice of extraction or processing techniques [7]. Additionally, food fraud, such as the intentional adulteration or substitution of raw materials, may worsen the problem, especially in high-value botanicals [8, 9]. A specific subset in the field of authentication is auto-adulteration, which refers to the addition of undeclared parts of the same plant (e.g., leaves in fruit-based material), thereby lowering quality and misleading buyers [10].

Within such context, saffron (*Crocus sativus* L.) represents a clear example where such variability and potential for adulteration are of particular concern, as the unique use of flower stigma makes its price high and its manufacture challenging (ISO 21983:2019(en) Guidelines for the harvesting, transportation, separation of stigma, drying and storage of saffron before packing).

As their spice and common food products counterparts, saffron-based food supplements are typically marketed as being derived exclusively from the stigmas of the plant, as these are the parts traditionally recognized for their high value and bioactive potential. In this regard, the stigma's therapeutic and sensory properties are attributed primarily to its carotenoid derivatives (e.g., crocins), monoterpene aldehydes (e.g., picrocrocin), and volatile compounds (e.g., safranal) [11, 12]. For instance, apocarotenoids, mainly present in the stigma, are associated with neuroprotective and antidepressant actions [13–15]. In fact, saffron food supplements are mainly employed as natural alternatives of selective serotonin reuptake inhibitors (SSRIs) to reduce depressive and anxiety symptoms [16, 17]. While these hypotheses fueled the market of saffron as a food supplement, such claims have yet to be confirmed, indeed this botanical is still on hold according to the European Food Safety Authority (EFSA) [18].

Moreover, recent studies have highlighted that the *Crocus sativus* L. petals, commonly regarded as a lower-cost by-product, also contain significant levels of bioactive compounds [19–21]. For instance, some flavonoids present in the petal contribute to antioxidant and anti-inflammatory properties [22–24]. Given its prized value as both a spice and a food coloring agent, saffron has a long and articulate history of adulteration, more often performed by adding foreign plant material as bulking agent in ground stigmas and extracts, in order to increase volume and weight of commercial lots [25, 26]. At the commercial level, fraudulent practices can be addressed through the assessment of saffron purity and authenticity, as well as through the determination of its quality grade. The latter is commonly certified using a combination of UV spectroscopic measurements of picrocrocin, safranal, and crocin, as established in ISO 3632-2 and its technical specification, ISO 3632/TS. However, its limited reliability for the detection of plant-derived foreign matter has been highlighted showing that the ISO/TS 3632-2 method is not capable to detect addition of up to 10–20%, w/w, of foreign plant matter [27]. At the same time, economic pressure and the peculiar manual separation of stigma from other saffron floral parts has led to the widespread practice of auto-adulteration, performed by adding other components of the same plant such as styles, stamens, tepals to the stigmas [28]. Because auto-adulterants are taxonomically identical to the declared species, species-level tests alone are insufficient. Therefore, detection relies on techniques that are sensitive to tissue type or compositional differences such as time-consuming microscopic evaluation or peculiar molecular analysis including epigenetic or tissue-specific genetic signatures, which may be however unsuitable for plant extracts, leaving space for detailed phytochemical or metabolomic approaches [28, 29] .

Phytochemical profiling through liquid chromatography coupled to mass spectrometry (LC – MS), particularly high-resolution mass spectrometry (HRMS), has proven to be a suitable approach for assessing the composition of botanicals, with a particular focus on their authenticity and quality [30, 31].

In practice, however, authenticity assessment of botanical is often constrained by the limited availability of well-characterized reference materials. In many real-world scenarios, only a small number of authenticated samples are accessible, preventing the development of fully supervised classification models. Under these conditions, analytical strategies must rely on approaches capable of identifying compositional deviations or inconsistencies rather than on strict class prediction.

Therefore, this study aimed to define a characteristic phytochemical profile of the main floral parts of *Crocus sativus* L. using liquid chromatography–traveling wave ion mobility spectrometry–high-resolution mass spectrometry (LC–TWIMS–HRMS), applied to a limited set of raw materials used for saffron-based botanicals, and to evaluate their compositional variability in a structured manner.

The objective was to evaluate the suitability of an analytical workflow capable of detecting compositional deviation within an auto-adulteration hypothesis relative to an internal benchmark under realistic small-sample conditions, where only a limited number of authenticated references are available [32–35]. Under such constraints, the compositional evaluation should rely on internal structured representations of the chemical space where deviations could be interpreted relative to biologically meaningful anchors, instead of using predefined sample classification methods.

2. Materials and methods

2.1. Reagents and Chemicals

Reagents used for sample preparation and LC–TWIMS–HRMS analysis included LC – MS grade methanol and acetonitrile from Merck (Darmstadt, Germany), and formic acid (99%) supplied by Carlo Erba Reagents (Milan, Italy). A Milli-Q system (Millipore Corporation, Bedford, MA, USA) was used to obtain purified water.

2.2. Samples

In the present study, nine samples (S1-S9) constituting the raw material used in the production of saffron-based food botanicals were analyzed. The samples were kindly provided by Bios Line, Padova, Italy. According to product labeling, all of them derived from the stigmas of *Crocus sativus* L., except for one sample (S7), which contained the whole flower and another (S9) in which the presence of flowers is uncertain. In addition, six in-house *Crocus sativus* L. reference plant materials were also analyzed to support compositional sample interpretation and comparison: two petals (n = 2; P1, P2) and two stamens (n = 2; St1, St2) obtained from Zaf Zaf, Cartignano, Italy; one pure stigma (n = 1; S10) and a mix of saffron floral by-products (n = 1; P3) provided by PRIMA-SAFFROMFOOD project partners. Detailed sample information is provided in Table 1.

Table 1
Description of saffron samples and reference materials included in the study

Sample ID	Sample Type	Plant Part Declared	Source	Origin	Notes
S1	Raw material	Stigmas	Company	Spain	Purified water-based extract
S2	Raw material	Stigmas	Company	Spain	Standardized to 30% polyphenols, 7.5% crocins, and 3% safranal,
S3	Raw material	Stigmas	Company	Spain	Extract standardized to a minimum of 10% Crocins and 2% Safranal
S4	Raw material	Stigmas	Company	France	Extract standardized to contain $\geq 3\%$ Crocins and 2% Safranal
S5	Raw material	Stigmas	Company	Spain	Purified water-based extract
S6	Raw material	Stigmas	Company	N/A	Extract, no further information given
S7	Raw material	Whole flower	Company	N/A	Powdered plant material standardized to $\geq 0.3\%$ Safranal and $\geq 0.8\%$ Crocins
S8	Raw material	Stigmas	Company	Iran	Unprocessed bulk saffron powder, not standardized
S9	Raw material	Stigmas	Company	N/A	Extract, produced with whole flowers
S10	Reference	Pure stigma	PRIMA-SAFFROMFOOD	Spain	In-house reference
P1	Reference	Petals	Company	Italy	In-house reference
P2	Reference	Petals	Company	Italy	In-house reference
P3	Reference	Floral by-products (mixed)	PRIMA-SAFFROMFOOD	Spain	Mix of petals, stamens, and residues
St1	Reference	Stamens	Company	Italy	In-house reference
St2	Reference	Stamens	Company	Italy	In-house reference

2.3. Sample preparation

For each sample, 10 mg of powdered material were accurately weighed into a 2 mL Eppendorf tube. Then, 1.5 mL of methanol:water (80:20, v/v) solution were added to initiate a solid-liquid extraction (SLE).

The tubes were subsequently vortexed for 1 min to ensure homogeneous mixing, followed by ultrasonication for 30 min to enhance the extraction efficiency. The samples were then centrifuged at 5000 rpm for 30 min at 4°C and the resulting supernatant was filtered using 0.22 µm polytetrafluoroethylene (PTFE) filters. Finally, the extract was diluted at a 1:3 (v/v) dilution ratio in water:acetonitrile (95:5, v/v) and transferred into an LC–TWIMS–HRMS injection vial. Samples were extracted in duplicate.

Moreover, a quality control (QC) sample, a pool of all the samples analyzed, was prepared to control the LC–TWIMS–HRMS instrumental performance. A solvent blank, composed of water:acetonitrile (95:5, v/v) was also prepared to monitor potential carryover. Both QC and solvent blank were periodically injected in the sample sequence.

2.4. Phytochemical profiling through LC–TWIMS–HRMS

The analysis of the samples was performed using an ACQUITY UPLC I-Class system coupled to a VION IMS QTOF mass spectrometer (Waters, Wilmslow, UK), equipped with an electrospray ionization (ESI) source. The instrumental setup was controlled using the UNIFI software (Waters), which was also employed for data acquisition and processing.

The injection volume was 2 µL for each sample, kept at 10°C in the autosampler. Then, the chromatographic separation was carried out under reversed-phase conditions, using an ACQUITY BEH C₁₈ column (100 mm × 2.1 mm, 1.7 µm particle size) (Waters) maintained at 40°C. The mobile phase consisted of water (eluent A) and acetonitrile (eluent B), both acidified with 0.1% (v/v) formic acid. The separation was achieved using a gradient elution at a constant flow rate of 0.35 mL·min⁻¹. The optimized chromatographic conditions included an initial composition of 5% B, maintained for 1 min, followed by a linear gradient to 100% B in 9 min. This composition was held for 3 min before returning to initial conditions over 1 min. The LC system was re-equilibrated for 4 min, leading to a total analysis time of 18 min.

As mentioned, the chromatographic system was coupled to the TWIMS–HRMS system through an ESI source, operating in positive and negative modes. Each sample was injected twice to acquire data in both ESI modes. The ionization was conducted using a capillary voltage of 1.5 kV (positive mode) and 2.0 kV (negative mode), with a source temperature of 120°C and a desolvation temperature of 650°C. The sample cone and source offset voltages were established at 40 and 80 V, respectively. The desolvation gas (nitrogen) flow rate was maintained at 950 L·h⁻¹, while the cone gas (nitrogen) was set to 50 L·h⁻¹.

TWIMS parameters consisted of a nitrogen flow rate of 25 mL·min⁻¹, wave velocity of 300 m·s⁻¹, and wave height of 15 V. Regarding the HRMS acquisition, the quadrupole time-of-flight (QTOF) mass analyzer was operated using the sensitivity analyzer mode and the high definition MS^E (HDMS^E) acquisition mode, establishing a mass range of *m/z* 100–1,200. In particular, the HDMS^E mode is a data independent acquisition (DIA) mode that acquires scans at low- (6 V) and high-collision energies (ramp

from 27 to 45 V). Furthermore, the TWIMS–HRMS system was externally calibrated – m/z and $^{TW}CCS_{N_2}$ calibration – using the Major Mix IMS/ToF Calibration Kit (Waters) for both ESI polarities. Besides, leucine-enkephalin was infused at $15\ \mu\text{L}\cdot\text{min}^{-1}$ as the LockSpray solution ($50\ \text{pg}\cdot\mu\text{L}^{-1}$) for real-time m/z and $^{TW}CCS_{N_2}$ correction.

LC–TWIMS–HRMS data were processed following a suspect screening approach, using the UNIFI software (Waters). In this line, distinct compounds previously found in *Crocus sativus* L. stigmas and petals (shown in **Table S1**), collected from both online databases – such as Phytohub (*PhytoHub*) – and previous studies, were screened [37, 37–48]. Metabolite identification followed the Schymanski et al. confidence level scheme and, therefore, mass error < 5 ppm, isotopic pattern match error < 20%, and MS² spectral similarity, were established as filtering criteria [49].

2.5 Statistical analysis and data processing

All statistical analyses were conducted in Python (v3.11) using NumPy, pandas, SciPy, scikit-learn, and Matplotlib. Analyses were performed at the run level unless otherwise specified. All scripts used for data processing, modelling, robustness assessment, and figure generation are publicly available in a dedicated GitHub repository : <https://github.com/chidal63/saffron-directional-fingerprinting>.

Aligned compound-level abundance matrices were constructed across samples. Zeros introduced during compound alignment were treated as missing values and imputed using a half-minimum strategy within each run. Although imputation may influence low-abundance features, its impact on overall structure was assessed through sensitivity analyses using alternative strategies. Sensitivity to alternative imputation schemes (zero baseline and 5th-percentile-based imputation) was assessed and rank stability was quantified using Spearman correlation (Supplementary Table S2). Compound intensities were transformed using $\log_{10}(1 + x)$ prior to multivariate analysis. This transformation reduces the dominance of highly abundant crocin signals and enables secondary compound classes to contribute more effectively to the multivariate structure.

Reference floral part characterization

Reference samples were grouped into stigma, petals, and stamen. For each sample, compound abundances were summed by chemical class and converted to relative abundances (within-sample sum = 1). Group-level profiles were obtained by averaging relative abundances within each part group and visualized as a bubble plot (Fig. 1a).

Compound overlap among stigma, petals, and stamen was evaluated using a presence–absence approach. A compound was considered present in a floral part if detected in at least two samples of that tissue group. Intersection patterns were visualized using an UpSet-style representation (Fig. 1b).

Floral part-enrichment assessment

Part-specific enrichment was quantified using Cliff's δ effect size, comparing each floral component against the pooled set of the other reference parts on $\log_{10}(1 + x)$ -transformed intensities. Compounds exceeding a predefined effect size threshold were ranked and visualized (Fig. 2).

PCA and directional modelling

Principal component analysis (PCA) was performed on run-level profiles after feature-wise standardization. To quantify displacement toward petals, each sample was projected onto the direction defined by the centroids of stigma and petal reference parts.

Two complementary projections were calculated: i) a projection in the full transformed feature space (t_{feature}); ii) a projection in the reduced PCA space (t_{PCA}). This approach implicitly assumes that sample profiles can be approximated as linear combinations of tissue-specific compositional patterns, allowing projection onto anatomically defined directions to capture dominant axes of variation.

Petal centroids were defined using anatomical petal references (P1 and P2), while external petal-like samples (P3) were excluded from centroid computation to prevent distortion of the directional axis (Fig. 4). Robustness of directional estimates was evaluated using compound subsampling and macro-class block resampling. Confidence intervals and stability metrics are reported in Supplementary Figure S1. Despite the limited number of reference samples, centroid stability was indirectly supported by the resampling procedures applied at the compound and macro-class level.

Residual non-stigma decomposition

To characterize compositional components not captured by the stigma–petals gradient, each sample profile was decomposed into a directional component and an orthogonal residual component.

The magnitude of the residual component was expressed as a fraction of total displacement from the stigma reference. Residual alignment toward a whole-flower proxy—defined as the mean of petal (P1–P2) and stamen reference profiles—was quantified using cosine similarity.

An evidence metric was defined as the absolute cosine similarity between the sample residual and the whole-flower residual. This metric reflects the extent to which deviations from the stigma–petals gradient resemble a whole-flower compositional pattern (Fig. 5).

Sensitivity analysis

Robustness of directional estimates was evaluated using compound subsampling and macro-class block resampling (Supplementary Figure S1). Sensitivity to imputation strategy was assessed using alternative imputation schemes (Supplementary Table S2). Despite expected variability at the feature level, rank stability across imputation schemes indicates that the main directional trends are not driven by imputation-specific artefacts.

3. Results

3.1. Metabolite distribution across *Crocus sativus* L. flower parts (stigmas, petals, and stamens)

Phytochemical profiling of *Crocus sativus* L. was performed using LC–TWIMS–HRMS under the conditions described in Section 2.4. As summarized in Table S1, 68 metabolites were tentatively identified across all analyzed samples, including saffron-based botanicals and reference flower parts (stigmas, petals, and stamens), based on a suspect screening approach. A total of 49 compounds were assigned to Level 2 (probable structure) and 19 to Level 3 (tentative candidate).

Among the annotated metabolites, two major phytochemical classes were represented: carotenoids and phenolic compounds. Twenty-one crocins were detected either as deprotonated ions $[M - H]^-$ in negative ESI mode or as sodium adducts $[M + Na]^+$ in positive ESI mode. Forty-five phenolic compounds were annotated, comprising 9 anthocyanins, 29 flavonols, 5 flavones, 1 flavanone, and 1 flavanone. Anthocyanins were observed as molecular ions $[M]^+$, whereas most other flavonoids were detected predominantly as deprotonated ions $[M - H]^-$. Isorhamnetin was detected as $[M + H]^+$, and its derivatives isorhamnetin 3,7-diglucoside and isorhamnetin 3-O-sophoroside were better ionized as $[M + Na]^+$.

Picrocrocin and picrocrocinic acid were also tentatively identified among the detected metabolites. Safranal was not detected under the applied LC–TWIMS–HRMS conditions. As saffron adulteration trends highlighted the recurrent presence of specific plants such as *Carthamus tinctorius* and *Calendula officinalis* as bulking agents, a screening was performed to detect the eventual presence of uncommon plant metabolites during untargeted analysis, but with negative results [25, 26].

Before evaluating the chemical composition of saffron-based botanicals, the metabolomic profiles of individual *Crocus sativus* flower parts (stigma, petal, and stamen) were examined. Reference samples S10, St1, St2, P1, and P2 were used for this purpose. Sample P3 was excluded at this stage because it consists of a mixture of the floral by-products and could not be associated with a single anatomical tissue.

Figure 1a shows the relative distribution of the major phytochemical classes across the three tissue groups. Flavonoids contributed predominantly to the petal and stamen profiles, with a smaller relative contribution in stigma. Crocins were strongly enriched in the stigma reference material and contributed only minorly to petals and stamens. Anthocyanins contributed predominantly to the petal profile, showed a low relative contribution in stamens, and were not observed in stigma samples in the class-level summary.

Compound overlap among flower parts is reported in the UpSet plot (Fig. 1b). Most annotated metabolites were detected in at least two parts, while only a smaller number of compounds were specific of a precise part. Four tentatively identified compounds were not observed in any of the analyzed reference tissues.

Given the substantial compound overlap across floral parts, enrichment analysis was performed to identify metabolites most strongly associated with each one. Enrichment was quantified using Cliff's δ , comparing samples from the target part against samples from the other reference parts on $\log_{10}(1 + x)$ intensities. Only positively enriched compounds above the reporting threshold were retained (Fig. 2).

The stigma reference material was characterized by higher levels of apocarotenoids, including picrocrocin and multiple crocin isomers. Stamen samples showed enrichment of flavonols, predominantly isorhamnetin and its glycosides together with quercetin and luteolin derivatives. Petal samples were characterized by higher levels of glycosides and anthocyanins, including quercetin glycosides and petunidin, pelargonidin, and malvidin derivatives.

These floral part-related profiles provide the reference chemical framework used in the subsequent directional analyses.

A heatmap was generated to visualize compound-level patterns across samples, including botanicals (S1–S9), references comprising stigma (S10), petals (P1–P2), stamen (St1–St2) and a mixed petal–stamen sample (P3) (Fig. 3). To emphasize relative differences across samples, abundances were standardized at the compound level. Hierarchical clustering separated petal and stamen references from samples grouped within the stigma-associated branch, which included the stigma reference and the majority of botanicals, resulting in two main groups at the column dendrogram level. However, within the botanicals branch, samples displayed varying distances to S10, indicating internal compositional heterogeneity.

At the feature level, the row dendrogram revealed three groups of metabolites displaying coordinated abundance patterns across samples. A set of features showing elevated relative abundance in S10 and in several botanicals corresponded to annotated crocins (e.g., trans-4-GG, trans-3-Gg and multiple cis crocin isomers). Additional apocarotenoid markers, including picrocrocin and picrocrocinic acid, followed similar patterns. In contrast, features showing higher relative abundance in petal and stamen references corresponded to phenolic compounds, particularly anthocyanins (e.g., delphinidin, cyanidin, petunidin and malvidin derivatives). Between these dominant patterns, an intermediate feature group corresponded primarily to flavonol glycosides, including kaempferol, quercetin and isorhamnetin derivatives, which displayed heterogeneous abundance across both sample groups.

Within the botanicals group, S1, S2, S5 and S6 were positioned in closer proximity to S10 and exhibited abundance patterns comparable to the stigma reference across crocin-associated features. Conversely, S3, S4, S7, S8 and S9 showed increased relative abundance of phenolic feature clusters that were more prominent in petal and stamen references, resulting in intermediate positioning within the column dendrogram relative to S10. Among these, S7 displayed the most pronounced deviation, characterized by relatively weak signals across the crocin-associated feature set together with clearer signals from annotated phenolic compounds, including flavonol glycosides (e.g., kaempferol and quercetin derivatives) and anthocyanins (e.g., delphinidin and petunidin glycosides), distinguishing it from the more crocin-aligned botanicals.

The clustering pattern was consistent with the differential enrichment trends shown in Fig. 2, where crocin-associated metabolites were higher in stigma and stigma-proximal botanicals, whereas flavonoid- and anthocyanin-associated metabolites were more abundant in petal and stamen references. This structured variation across samples is further explored using directional modelling.

3.2 Directional placement of botanicals within the tissue chemical space

The descriptive results presented above indicate that saffron tissues share a common metabolomic background but differ in quantitative composition. While some compounds such as anthocyanins display near-binary presence across tissues, the overall compositional structure is dominated by quantitative gradients involving multiple compound classes, justifying the use of continuous projections. To quantify these shifts, botanicals were mapped within a tissue-anchored chemical space using multivariate modelling.

3.2.1 Projection of botanicals along the stigma–petal axis

Principal component analysis (PCA) of $\log_{10}(1 + x)$ -transformed profiles revealed clear separation of anatomical reference parts (Fig. 4A). Stigma samples were characterized by crocin-dominant signatures, whereas petals and stamens exhibited higher relative contributions of flavonoids and anthocyanins. Most botanicals clustered near the stigma reference region, consistent with the crocin-dominant pattern observed in the heatmap (Fig. 3).

To quantify compositional displacement, botanicals were projected onto the direction defined by stigma and petal centroids (Fig. 4B). This projection (t_{feature}) provides a continuous metric ranging from stigma-like to petal-like profiles. A complementary projection in PCA space (t_{PCA}) showed strong agreement with the full feature-space metric.

As expected, given their label information, S7A and S7B exhibited the largest displacement toward the petal direction, followed by S9 samples, indicating measurable enrichment in non-stigma-associated compounds consistent with the presence of non-stigma-derived material. In contrast, the remainder of botanicals remained closer to the stigma reference along this axis, despite the occasional localized non-stigma signals observed in the heatmap (Fig. 3).

3.2.2 Residual non-stigma structure

To investigate compositional variation not captured by the primary stigma–petal gradient (Fig. 4), profiles were decomposed into directional and orthogonal components (Fig. 5). The residual fraction was high for most botanicals, indicating that a substantial portion of their compositional variability is not fully explained by the dominant stigma–petal axis.

Residual alignment toward a composite non-stigma reference, defined as the mean of petal (P1–P2) and stamen profiles, revealed distinct structural patterns. S4A and S4B showed the strongest residual alignment, indicating a non-stigma component closely resembling whole-flower composition. In contrast, S7 samples displayed strong directional displacement but comparatively weak residual alignment, suggesting that their deviation is largely captured by the primary stigma–petal gradient. S9 samples exhibited intermediate behavior.

Together, directional and residual analyses distinguish botanicals that deviate along the stigma–petal continuum from those characterized by broader whole-flower-like compositional signatures.

4. Discussion

Quality assessment of botanical ingredients is inherently complex. Unlike synthetic products, raw plant materials and extracts are chemically heterogeneous systems whose complexity may arise from biological differences, cultivation conditions, harvesting time, processing steps, and blending strategies related to authentication issues. In high-value matrices such as saffron, the availability of extensive collections of authenticated reference materials is often limited. Under these conditions, quality evaluation must rely on clearly defined internal benchmarks rather than large, supervised training sets and for saffron is mandatory to consider also the eventuality of auto-adulteration, that is the voluntary or accidental addition of floral parts other than stigmas.

The chemical profiling performed in this study first established a structured anatomical reference space based on stigma, petal, and stamen tissues, necessary to manage their eventual blending in products declaring to contain only the stigmas. These floral parts exhibited clear quantitative asymmetries, particularly the predominance of crocins in stigmas and the enrichment of flavonoids and anthocyanins in non-stigma tissues. At the same time, a substantial proportion of metabolites was shared across these parts. This overlap limits the discriminative power of single-marker approaches and highlights the need for multivariate representations of compositional balance.

By anchoring the multivariate space to anatomical centroids, botanicals could be positioned relative to a defined internal reference. Projection along the stigma–petal axis quantified displacement within the dominant gradient of variation.

Most botanicals remained close to the stigma reference, while S7 showed the largest shift toward the petal direction and S9 a more moderate displacement. As described in Table 1, these samples declared or suggested in their technical information the presence of petals or at least of flavonoids characteristic of these plant parts such as kaempferol. These shifts were stable under compound resampling and macro-class block resampling, indicating that the observed gradients reflect coordinated variation rather than isolated fluctuations.

Residual analysis provided a complementary perspective by isolating compositional components not captured by the dominant stigma–petal gradient. After removal of the stigma-aligned component,

residual vectors were evaluated relative to a whole-flower proxy defined by the combined petal and stamen reference profiles.

This analysis revealed heterogeneous residual alignment across botanicals. S4 samples exhibited the strongest residual alignment with the whole-flower reference, indicating a structured non-stigma component resembling combined non-stigma tissues. In contrast, S7 samples, despite showing substantial displacement along the stigma–petal axis, displayed comparatively weak residual alignment, suggesting that their variation is largely captured by the primary directional gradient linked to the presence of petals. S9 samples showed intermediate residual alignment, which may be in accordance with their label information clearly describing, albeit in a qualitative manner, the presence of kaempferol.

These findings indicate that compositional differences among botanicals may arise through distinct mechanisms: directional shifts along the stigma–petal continuum or broader whole-flower-like residual patterns. However, alternative explanations such as differential extraction efficiency, processing-induced degradation, or formulation effects cannot be excluded and may contribute to the observed compositional patterns.

Decomposition of the residual whole flower-like patterns further clarified the structure of these deviations. Residual components were predominantly flavonoid-associated in most samples. In S4, the residual was almost entirely driven by flavonoids, consistent with its stamen-oriented alignment. In S7, both flavonoids and anthocyanins contributed to the residual component. Crocins, although dominant in absolute abundance, did not account for the directional residual structure observed in these samples.

Taken together, the combined projection and residual analyses show that compositional displacement can be quantified along anatomically interpretable directions even when stigma-derived chemistry dominates the overall profile. Rather than imposing categorical labels, the framework evaluates alignment with an internal benchmark and resolves direction-specific deviations within a shared metabolomic background. Furthermore, despite its reduced size, the sample group of botanicals evaluated showed a remarkable trueness to their label and technical information, with products declaring the presence of the sole stigma well placed among the stigma centroid and the two (S7 and S9) with different description positioning themselves with congruent deviations toward the stigma-petal axis,

From a methodological perspective, this study illustrates that high-resolution chemical mapping coupled with structured multivariate modelling can support quality-oriented evaluation under conditions of limited reference availability, with a specific focus on auto-adulteration. By explicitly defining the analytical space and evaluating robustness through resampling strategies, it is possible to detect and characterize subtle compositional variation within a single botanical species. The approach may be extended to other botanical matrices where intrinsic chemical overlap and limited standardization represent practical constraints.

Beyond the specific case of saffron, the proposed framework illustrates how biologically anchored projections combined with residual decomposition can provide interpretable structure in small-sample metabolomics settings, where conventional classification approaches are not feasible.

4. Conclusions

This study shows that a multivariate framework anchored on single floral parts can be used to evaluate compositional consistency of saffron-based botanicals relative to anatomically defined reference materials also under n-small conditions. By defining a structured chemical space and quantifying directional displacement within it, botanicals can be positioned against an internal benchmark without relying on large, supervised training sets.

Within the current study, most products remained closely aligned with the stigma-dominant reference profile. A subset of samples exhibited reproducible displacement along anatomically interpretable directions. Projection along the primary stigma–petal gradient identified petal-oriented shifts in selected botanicals, while residual analysis revealed additional direction-specific deviations toward stamen or petal profiles after removal of the dominant stigma component. These deviations were primarily associated with flavonoid-related metabolites.

In practice, this approach could support routine quality control by monitoring raw plant materials and extracts consistency against an internal tissue-specific benchmark and by identifying products that diverge from the expected chemical balance. In particular, the approach is particularly suited for scenarios characterized by limited reference availability and high intrinsic chemical overlap, where evaluation cannot rely on large classification models. The same strategy may be applicable to other botanical systems characterized by intrinsic chemical variability and constrained standardization, where comprehensive reference libraries are not available and evaluation must rely on a limited but well-defined set of internal standards.

Declarations

Author contributions

HG: Writing - Original Draft, Conceptualization, Methodology, Formal Analysis, Investigation. **GC:** Writing - Original Draft, Conceptualization, Methodology, Formal Analysis, Investigation. **MB:** Resources. **RB:** Writing - Review & Editing, Resources, Supervision. **CD:** Writing - Original Draft, Conceptualization, Resources, Supervision, Project administration, Funding acquisition.

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Data Availability Statement

Raw compound-level abundance matrix is provided as supplementary file. Raw LC–TWIMS–HRMS instrument data are not publicly available due to project-related data restrictions but may be made available by the corresponding author upon reasonable request. **Conflict of Interest** The authors declare no competing financial interests or personal relationships that could have influenced the work reported in this paper.

Ethics declaration: not applicable.

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Figures

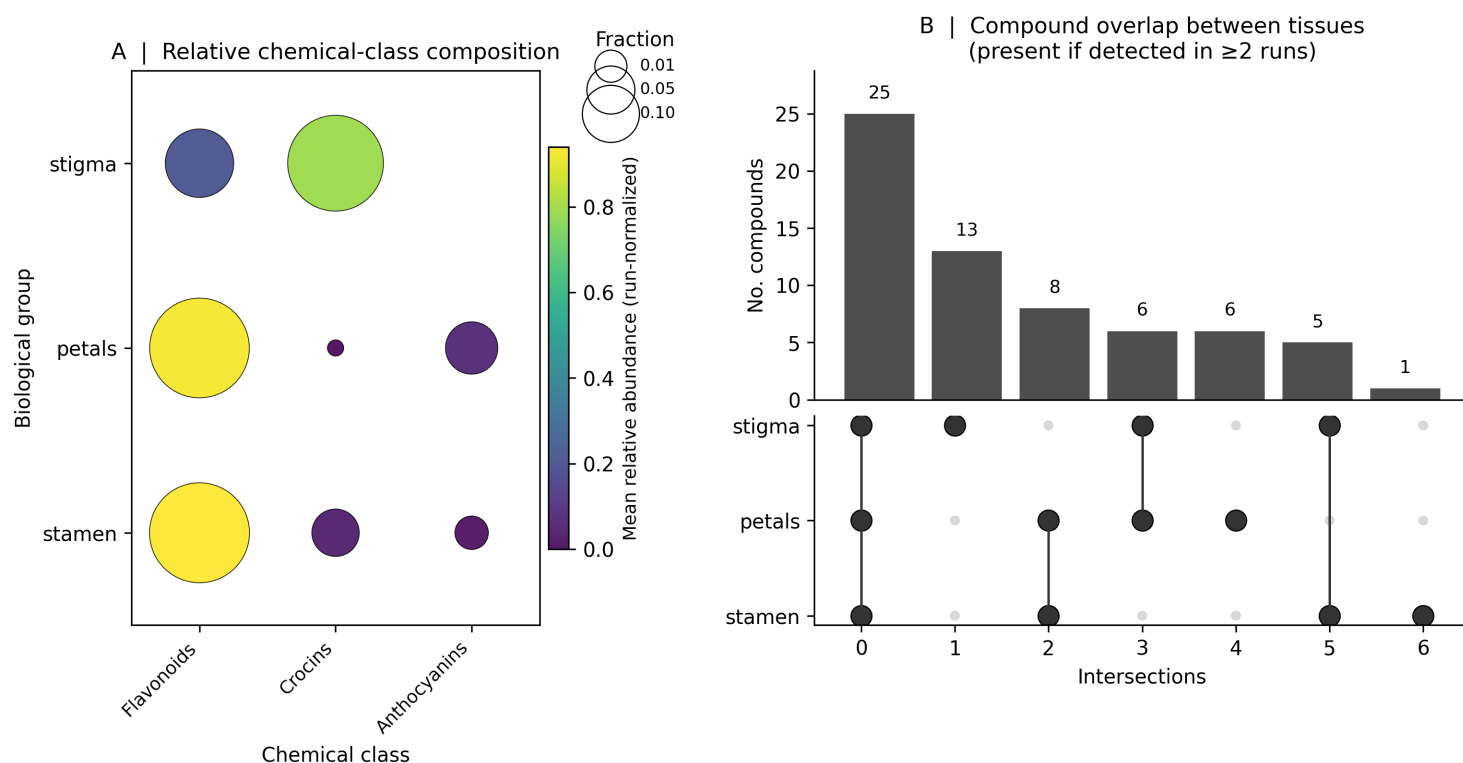


Figure 1

a) Bubble Plot showing chemical class composition of anatomically defined reference saffron flower parts. Bubble size and colour indicate mean run-level relative abundance within each tissue group

(stigma, petals, stamen). **b)**UpSet plot showing compound overlap across anatomical reference materials. Compounds were considered present within a flower part if detected in at least two runs. Bars report intersection sizes across stigma, petals, and stamen.

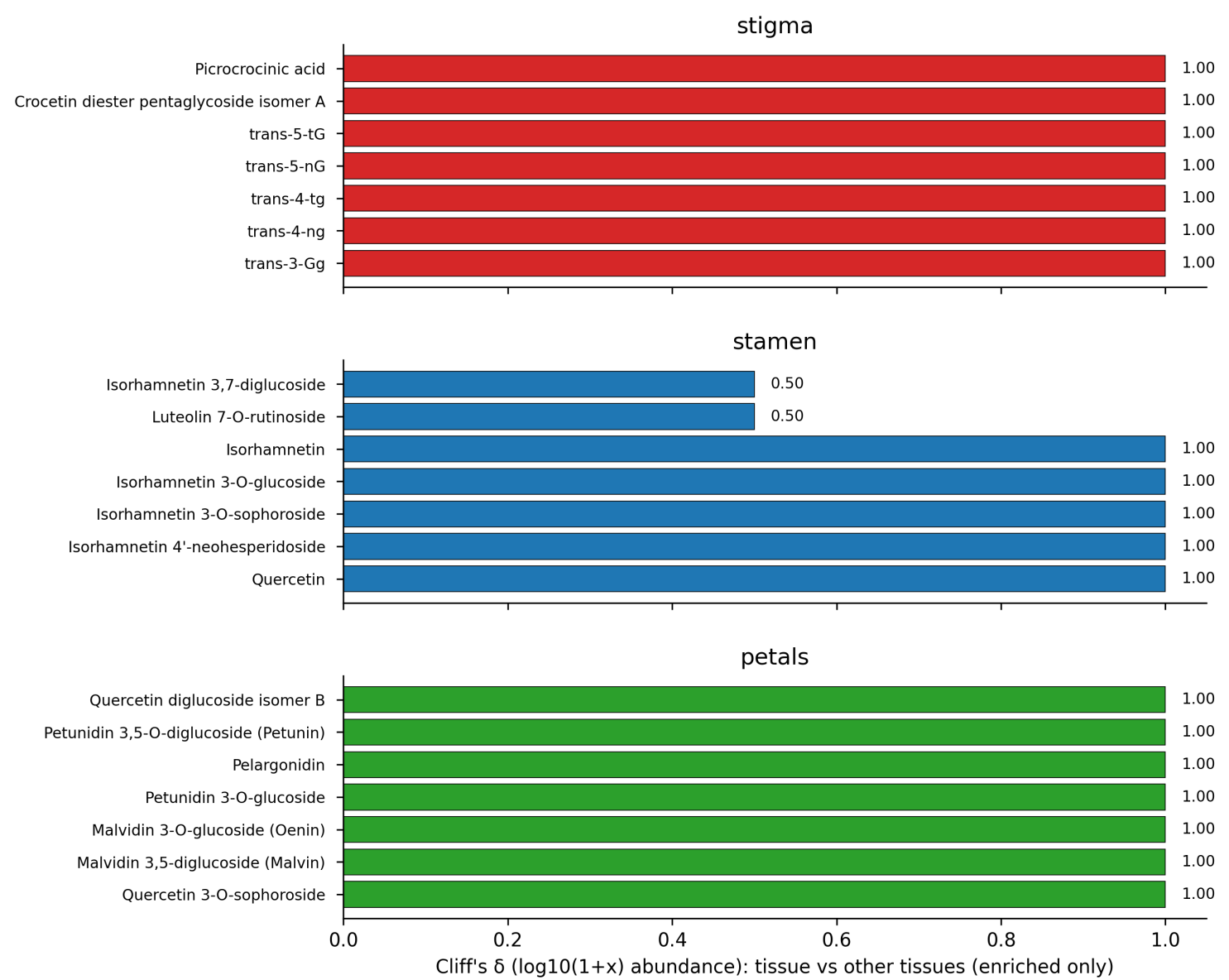


Figure 2

Top flower part-enriched compounds in anatomical references. Enrichment was quantified using Cliff's δ (part vs all other reference parts) on log10(1+x) intensities; only enriched compounds above threshold are shown.

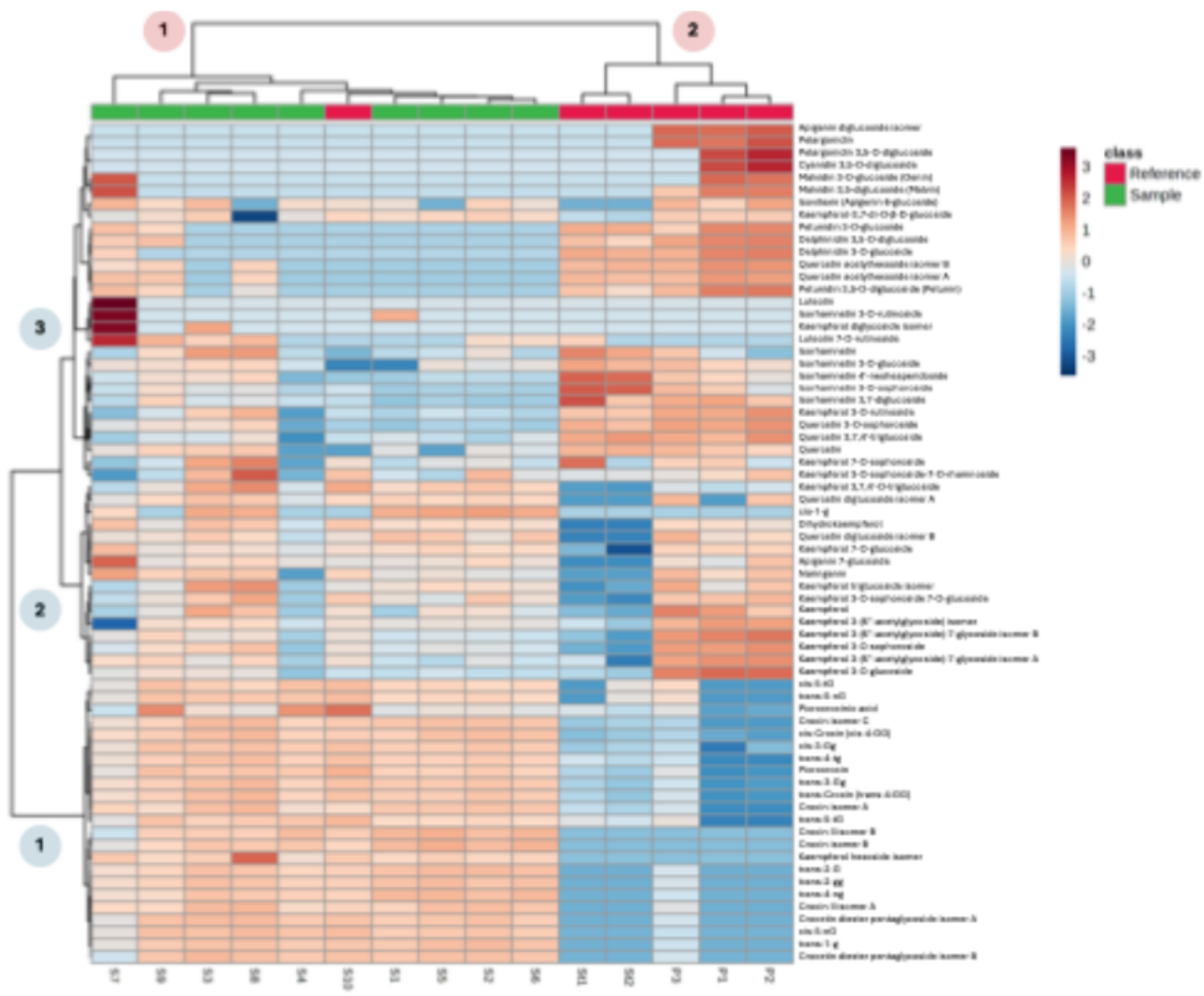


Figure 3

Heatmap obtained for the annotated compounds across all the samples. Abundance values have been log-transformed and averaged for replicates.

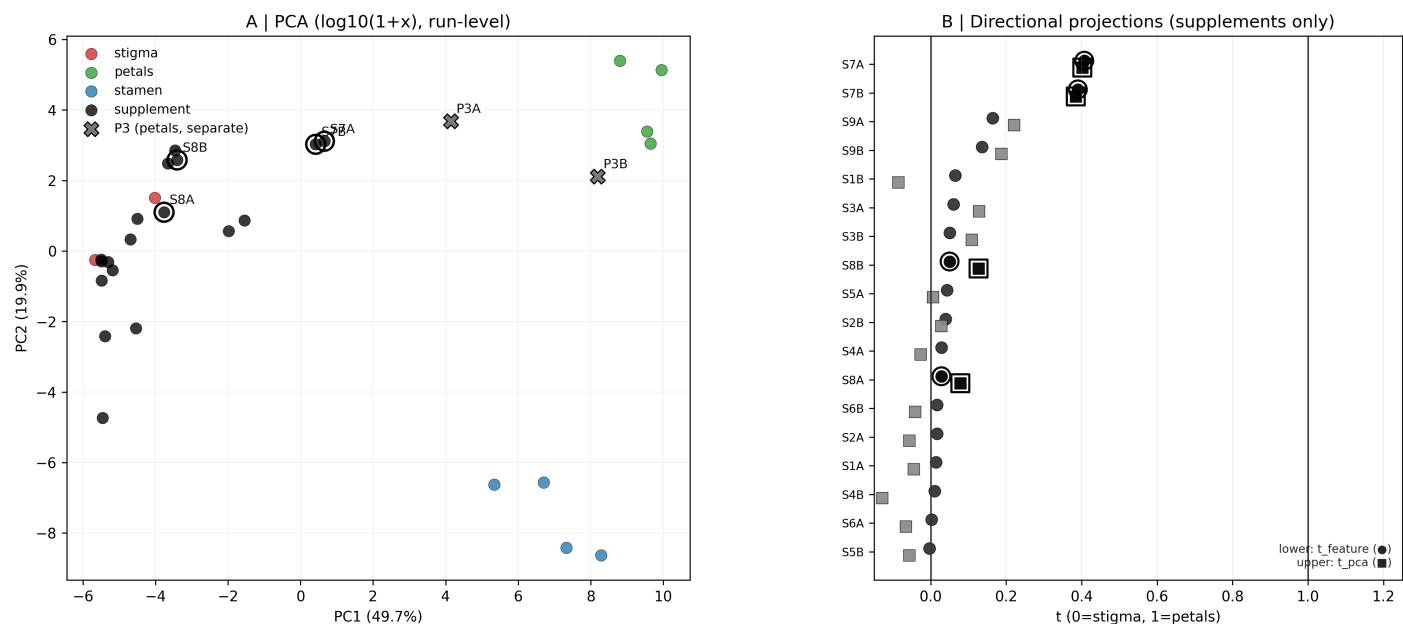


Figure 4

Directional displacement toward petals in saffron botanicals. (A) Principal component analysis (PCA) of log10(1+x)-transformed run-level data including anatomical reference tissues and botanicals. (B) Directional projections of botanicals along the stigma–petal axis computed in PCA space (t_{PCA}) and in the full transformed feature space (t_{feature}).

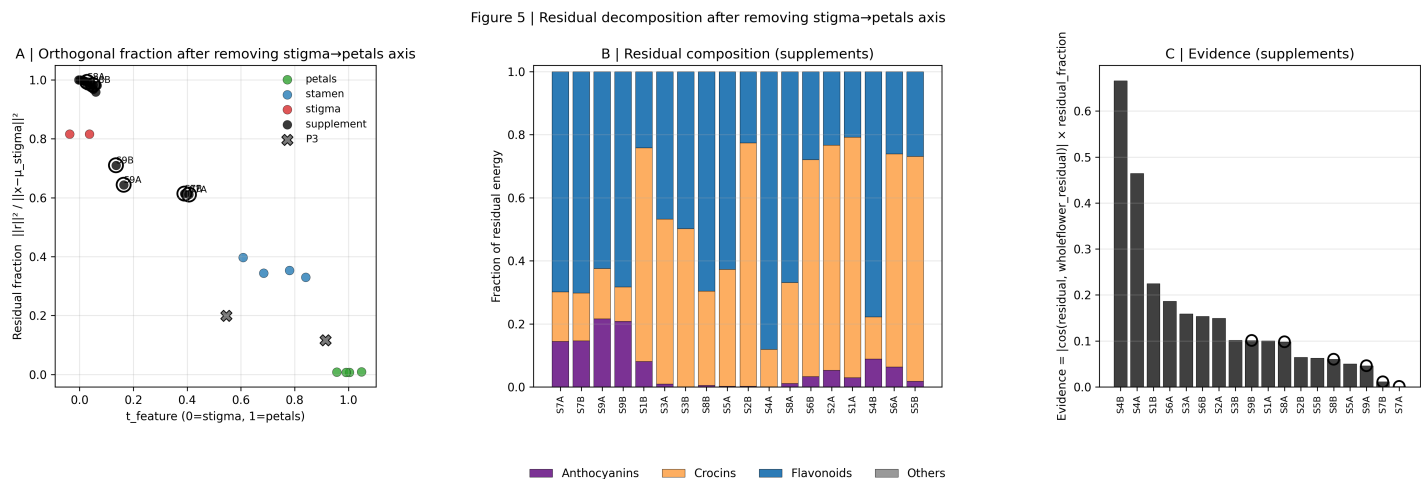


Figure 5

Directional components aligned with the stigma–petal axis were removed to isolate residual compositional structure in log10(1+x)-transformed data. Panel A shows directional displacement (t_{feature}), panel B the magnitude of the orthogonal residual component (residual_fraction), and panel C the residual alignment toward a whole-flower proxy defined by combined petal and stamen references.

Supplementary Files

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