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Metabolomic Differences Are Not Automatically Chemotypes: An Evaluation Framework for Separating Genotype, Geography, Phenology, Processing, and Analytical Batch Effects in Medicinal Plants

Kristian Eriksen[1]*, Solveig Berg[1], Magnus Dahl[2]

[1] Department of Metabolomics and Chemotype Evaluation, Faculty of Pharmacy, University of Oslo, Oslo, Norway.
[2] Department of Genotype–Environment–Phenology Integration, Faculty of Pharmacy, University of Bergen, Bergen, Norway.

***E-mail** ✉ kristian.eriksen@farmasi.uio.no

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ABSTRACT

Metabolomics can resolve striking chemical differences among medicinal-plant samples, but multivariate separation does not by itself identify the biological meaning of those differences. Similar clustering patterns may arise from inherited variation, population structure, geography, developmental stage, harvest timing, tissue composition, post-harvest processing, or analytical batch effects. This article develops an original evaluation framework for deciding when metabolomic differentiation can support a chemotype claim and when it should instead be interpreted as context-dependent or technically contingent variation. The analysis distinguishes three questions that are often collapsed: whether the observed profile is analytically credible, whether the chemical pattern is biologically persistent, and whether its most plausible source is sufficiently stable and source-linked to justify chemotype terminology. Evidence from plant metabolomics, chemophenetics, medicinal-plant development, processing studies, spatial metabolomics, and analytical quality-control research is integrated to identify recurrent confounders and evidential boundaries. The proposed framework treats chemotype assignment as a graded inference requiring defined source material, coherent chemical differentiation, challenge against plausible alternatives, and replication across the contexts most capable of generating the original separation. It also separates feature-level recurrence from compound-level confirmation and biological persistence from analytical reproducibility. The framework is not a validated classification algorithm and does not prescribe universal numerical thresholds. Its purpose is to make chemotype claims more falsifiable, more comparable across studies, and less vulnerable to overinterpretation of attractive but context-bound metabolomic separation.

Keywords: Chemotype, Medicinal plants, Metabolomics, Genotype–environment interaction, Phenology, Processing

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Introduction

Plant metabolomics can make chemically heterogeneous material appear unusually orderly. Principal-component scores, hierarchical clusters, supervised classifiers, or heat maps may separate accessions, locations, harvests, processed products, or experimental runs with high visual clarity. Yet the meaning of that separation depends on how plant material was authenticated and sampled, how metabolites were extracted and measured, how signals were identified, and how analytical uncertainty was handled. Methodological guidance for plant metabolite profiling therefore emphasizes that a profile is not a self-interpreting biological object: source documentation, acquisition conditions, structural assignment, and reporting all constrain the claims that can be made from it [1]. The central problem addressed here is consequently not whether medicinal plants differ chemically, but what kind of difference has actually been demonstrated.

Plant metabolic diversity is generated by interacting genetic, developmental, physiological, and environmental processes, and metabolomics captures the combined output of those processes rather than a single causal layer [2]. This makes chemical phenotypes useful but potentially ambiguous. A cluster may represent a stable biosynthetic phenotype, a population-specific metabolic background, an acclimatory response, a transient ontogenic state, or a mixture of these. Statistical discrimination cannot resolve those alternatives by itself because methods optimized to find covariance among samples are not causal tests of why that covariance arose. The same apparently clean separation can therefore carry very different biological meanings depending on experimental design and replication.

For medicinal plants, the distinction matters because chemotype labels may be carried forward into germplasm selection, pharmacognostic comparison, authentication, cultivation strategy, quality-control specifications, or assumptions about chemical consistency. A term that implies relatively persistent biological differentiation should therefore not be assigned merely because a statistical model separates groups. Nor should statistical strength substitute for chemical interpretation: a highly discriminating feature set may contain metabolites reflecting developmental status, post-harvest transformation, or analytical behavior rather than a stable source-linked phenotype.

The ambiguity is not theoretical. In *Valeriana jatamansi*, both genotype and environment influenced volatile chemical phenotype, demonstrating that a named or observed chemical type can be jointly structured by inherited and contextual factors [3]. At the same time, mass-spectrometric data can acquire systematic time-dependent signal drift, so even technically generated structure can masquerade as sample-class structure if acquisition order and quality-control information are not handled appropriately [4]. These two forms of variation differ fundamentally: one is biological but potentially plastic, whereas the other is analytical. Both, however, can produce reproducible-looking separation within a single dataset.

This article therefore proposes an evaluation framework in which “chemotype” is treated as an evidence-bounded inference rather than a synonym for a metabolomic cluster. The framework asks whether the observed pattern is chemically coherent, analytically admissible, persistent under relevant resampling, and resistant to alternative explanations arising from genotype, geography, phenology, tissue composition, processing, and batch structure. It does not assume that these factors are mutually exclusive, nor that every study can experimentally decompose them. Instead, the proposed logic grades what can defensibly be claimed from the design actually available. The goal is to preserve the usefulness of chemotype terminology while preventing a strong biological label from outrunning the evidence that produced it.

What a chemotype claim implies

A defensible chemotype claim begins before multivariate analysis. Plant source material must be sufficiently characterized for chemical comparison to have a stable referent; otherwise, apparent chemical classes may partly reflect uncertain species identity, undocumented provenance, heterogeneous plant parts, or poorly specified source material. Guidance for phytochemical research has therefore emphasized rigorous characterization and documentation of plant material as a prerequisite for interpretable chemical work [5]. Botanical identity is necessary, however, not sufficient: two correctly identified samples may still differ because of genotype, developmental state, environment, processing, or analytical history.

Chemotype should also be distinguished from broader chemical classification. Chemophenetic reasoning uses specialized metabolites as informative biological characters, but chemical resemblance and taxonomic or genetic identity are not interchangeable [6]. A metabolomic pattern can carry taxonomic information without establishing an intraspecific chemotype, and an intraspecific chemotype can be meaningful without defining a separate taxon. Accordingly, the claim must specify the biological level at which chemical differentiation is being interpreted and avoid allowing an analytical grouping method to define that level retrospectively.

There are clear examples in which named chemotypes correspond to coherent specialized-metabolite differences. In *Agastache rugosa*, pulegone- and estragole-dominant chemotypes were associated with distinct volatile and biosynthetic profiles, supporting the biological reality of chemically differentiated types in that system [7]. Conversely, phenolic-acid patterns in *Euphrasia* have been used as chemotaxonomic markers for species discrimination, illustrating a related but conceptually different use of chemical variation [8]. These examples caution against treating all chemically discriminable groups as the same kind of biological category. Marker dominance, taxonomic discrimination, and broad metabolome separation answer different questions even when each produces visually convincing classes.

Metabolomics can also operationalize candidate chemotypes from broad fingerprints. In *Eucommia ulmoides*, core germplasm accessions were grouped using metabolomic information and discriminating markers [9]. Yet in tansy, parental genotype and pre-existing chemotype labels explained nonidentical portions of metabolic variation, showing that a chemotype category need not capture the full structure of inherited metabolic differentiation [10]. A broad metabolomic signature may consequently contain both the compounds conventionally defining a chemotype and additional variation arising from genotype or context.

The proposed framework therefore treats a dataset-derived chemotype as provisional until its chemical coherence, persistence, and plausible competing explanations have been examined. "Chemotype" should describe a sufficiently stable and interpretable chemical phenotype; it should not function as a post hoc honorific for any statistically separable cluster. This formulation deliberately avoids a universal concentration threshold, distance metric, or classifier-performance cutoff because the approved evidence does not establish one across medicinal-plant systems.

Sources of genuine biological differentiation

The first competing explanation for metabolomic separation is genuine biological differentiation that is real but not necessarily chemotypic. Population history and genetic structure can organize plant chemistry. Diverse *Camellia sinensis* populations, for example, show structured metabolite signatures associated with population-level diversity [11]. Environmental conditions can simultaneously reshape specialized metabolism within a genetic background. In American ginseng, cold-associated changes in ginsenoside accumulation occurred alongside alterations in regulatory features during plant growth and development [12]. Thus, a metabolomic difference may be biologically authentic while remaining environmentally responsive.

Geography intensifies this interpretive problem because it is rarely a single causal variable. Seabuckthorn berries from different Himalayan regions displayed substantial metabolomic diversity by GC-MS [13], but "geographical origin" can bundle population genetics, altitude, soil, climate, cultivation history, and local phenology. Regional discrimination therefore establishes a location-associated chemical pattern; it does not, without additional design, identify which component of location generated the pattern or whether the pattern would persist after transplantation, propagation, or repeated collection under different conditions.

Development and harvest timing provide an even clearer challenge to automatic chemotype language. *Fritillaria hupehensis* showed stage-dependent metabolomic and transcriptomic changes relevant to harvest period [14], while *Scutellaria baicalensis* exhibited coordinated metabolomic and proteomic differences across growth stages [15]. Widely targeted metabolomics of *Dioscorea opposita* likewise revealed substantial chemical reorganization across harvest stages within a defined cultivar context [16]. Taken together, these studies demonstrate that distinct metabolomic profiles can emerge across developmental or harvest stages within plant material [14-16]. Such differences are biologically meaningful but may represent temporal states rather than persistent types.

For chemotype evaluation, the important distinction is therefore not "biological versus nonbiological" but "persistent differentiation versus context-dependent biological plasticity." Genotype, population background, geography, environment, and phenology can all produce genuine chemistry. The proposed framework requires each to be considered according to the claim being made. A pattern that survives relevant environmental or temporal challenge is stronger evidence for a stable chemical phenotype than one observed only at a single site or harvest point, but persistence must be demonstrated rather than assumed.

The principal biological sources of metabolomic separation and the inference each permits at this stage are organized in **Table 1.**

Table 1. Biological sources of metabolomic differentiation and their implications for chemotype interpretation

Source of differentiation	What the pattern may represent	Why it can resemble a chemotype	Required interpretive restraint
Genetic or population background	Inherited biosynthetic differentiation	Stable chemical clustering among lineages	Define the chemical phenotype rather than equating population identity with chemotype
Environment	Plastic metabolic response to external conditions	Repeatable separation within one setting	Do not infer heritability from environmental reproducibility
Geography	Combined regional genetic and environmental structure	Strong origin-associated discrimination	Treat location as a composite exposure until major components are separated

Phenology or harvest stage	Ontogenic or seasonal metabolic state	Large within-species chemical shifts	Do not convert temporal states into persistent types
Genotype–environment interaction	Context-dependent expression of inherited potential	Chemical rankings may differ among environments	Test whether the proposed type survives relevant environmental challenge
Biological persistence	Recurrence across justified resampling conditions	Strengthens source-linked interpretation	Treat persistence as evidence requiring demonstration, not as an assumption

Sources of apparent metabolomic separation

Not all separation that matters chemically originates in the living plant. Processing can substantially transform the measured chemical state after harvest. Direct-injection mass-spectrometric profiling distinguished Moutan Cortex from its processed products using chemical markers [17], and widely targeted metabolomics showed extensive chemical changes across black-tea processing stages [18]. Such results may be highly reproducible and pharmacognostically important, but they describe processing states. Unless the question explicitly concerns processing-defined material, they should not be reinterpreted as evidence of an inherited chemotype. A reproducible post-harvest transformation is still a different causal category from persistent biosynthetic differentiation in the source plant.

Sampling position can create a similar problem before processing begins. Spatial metabolomics of *Salvia miltiorrhiza* demonstrated marked localization of metabolites across tissues and anatomical regions [19]. Consequently, unequal proportions of root zones, tissues, ages, or organs can generate sample-class separation even when all material derives from the same genotype. This problem is especially important when powdered medicinal material obscures anatomical composition after collection. A sampling effect can therefore be both biologically genuine and unsuitable as evidence for a stable whole-plant chemotype.

Analytical batch structure is more problematic because it can generate separation without any corresponding biological difference. Large-scale metabolomics requires explicit batch assessment because systematic shifts in feature abundance and inter-feature relationships can arise between analytical batches; methods such as CordBat were developed to reduce such discrepancies while attempting to preserve biological concordance [20]. Correction, however, cannot substitute for design. If all samples from one biological class are run in one batch and all samples from another class in a second batch, biological class and analytical batch are inseparable from the resulting data. Post hoc normalization cannot establish which explanation is correct when the design contains no independent information with which to separate them.

Quality-control samples are essential but are not a talisman against this problem. A scoping review of untargeted LC-MS studies found that pooled QC samples were widely used but not consistently exploited or reported in ways that demonstrate how data quality was evaluated [21]. More recent QC frameworks likewise emphasize multiple dimensions—including missingness, drift, carryover, outliers, and related technical behavior—rather than a single acceptance statistic [22]. The implication for chemotype work is stringent: visual separation becomes interpretable only after the analytical process itself has earned credibility. Conversely, successful technical QC does not establish that the surviving biological pattern is a chemotype; it only removes or constrains one class of alternative explanation.

The proposed framework therefore treats processing, spatial sampling, and analytical batch as distinct alternatives rather than a single category of "noise." Processing may create a real and reproducible chemical state; tissue composition may reveal authentic within-plant organization; batch effects may be wholly technical. Their common feature is narrower: each can yield convincing group separation without establishing a persistent biological chemotype. Before chemotype terminology is used, the source of separation must be aligned with the biological level the term is intended to describe, and plausible nonchemotypic explanations must remain visible rather than being erased by the strength of multivariate discrimination.

Separating persistent from context-dependent patterns

A metabolomic pattern becomes more informative when it persists under conditions capable of challenging the explanation originally assigned to it. Persistence, however, is not equivalent to repeated statistical separation. It first requires measurements whose identities and quantitative behavior are sufficiently secure to support comparison. Best-practice guidance for mass-spectrometry metabolomics emphasizes annotation confidence,

quantitative limitations, experimental design, replication, and transparent reporting because uncertainty at these levels propagates directly into biological interpretation [23]. Reappearance of the same unresolved feature across runs is therefore weaker evidence than reproducible change in a chemically characterized metabolite measured under controlled analytical conditions.

Natural-product extracts add another difficulty: a detected feature is not automatically a confirmed constituent, and signal abundance is not automatically proportional to chemical abundance. Approaches that combine untargeted profiling with semiquantitative assessment illustrate why composition claims require attention to annotation confidence, detector response, artefacts, and chemical confirmation [24]. The proposed framework consequently separates three forms of recurrence: analytical recurrence of a feature, chemical recurrence of an identified constituent or coherent constituent pattern, and biological recurrence of that pattern across relevant resampling contexts. Only the third directly addresses persistence of a proposed chemotype, and it remains conditional on the first two being credible.

The appropriate challenge depends on the original explanation. A geographically separated profile should be retested across seasons, sites, or propagation conditions where feasible; a harvest-associated pattern should be followed across developmental windows; a genotype-associated pattern should be examined in more than one relevant environment; and a putative biological class should not remain aligned with analytical batch. Failure of a pattern under one challenge does not prove that the original observation was erroneous. It may instead identify the context within which that chemical phenotype exists. The proposed distinction is therefore between persistent, context-bounded, and analytically unresolved patterns rather than between “real” and “false” differences.

Table 2 summarizes the analytical contrasts needed to distinguish persistence from context dependence without assuming that any single test is universally decisive.

Table 2. Analytical challenges for distinguishing persistent chemical phenotypes from context-dependent metabolomic separation

Observed separation	Competing explanation to challenge	Informative comparison	Interpretation if separation weakens
Between genotypes or accessions	Environment or genotype–environment interaction	Same genetic material across relevant environments	Chemical phenotype is environmentally conditional
Between geographic origins	Population structure, climate, soil, management, phenology	Repeated sites/seasons or common-condition material where feasible	Geography is an association, not an isolated cause
Between harvest groups	Developmental or seasonal state	Repeated developmental windows	Difference is temporally bounded
Between raw or processed materials	Post-harvest transformation	Matched starting material across controlled processing states	Separation is processing-defined
Between tissues or sampled fractions	Spatial chemical heterogeneity	Standardized anatomical sampling	Difference depends on sampled composition
Between analytical groups	Drift or batch structure	Randomized allocation, bridge/QC samples, cross-batch replication	Biological attribution remains unresolved

These distinctions are integrated visually in **Figure 1**, which shows how evidence for chemical differentiation must pass through alternative-explanation challenges and explicit validation boundaries before a chemotype interpretation is strengthened.

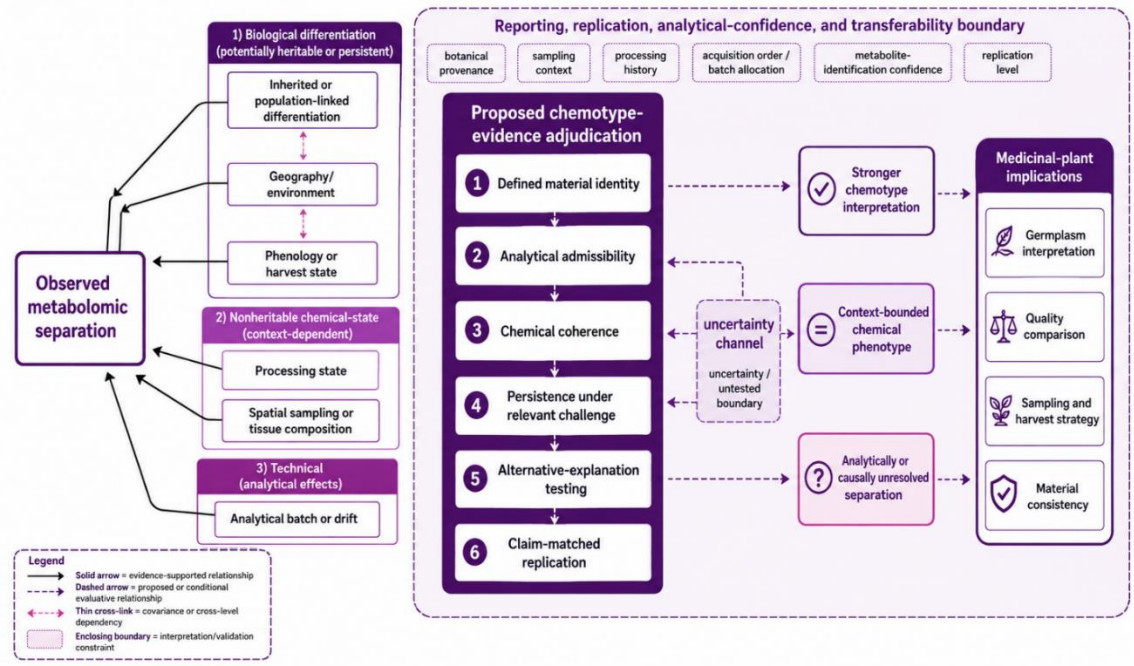

Figure 1. metabolomic separation and defensible chemotype interpretation: a proposed conditional evidence framework

Criteria for defensible chemotype assignment

The proposed framework treats chemotype assignment as a graded evidential judgment rather than a statistical label. First, the biological material must have a sufficiently defined identity and sampling provenance for the claimed phenotype to have a reproducible referent. Second, the analytical dataset must be admissible: separation should not depend on obvious drift, uncontrolled batch structure, poor QC behavior, or uncertain signal processing. Third, the chemical phenotype itself should be interpretable at the level claimed. Plant-profile quality, metabolite annotation, and composition assessment all constrain whether a set of discriminating signals can legitimately be described as a named chemical phenotype [1, 23, 24].

Fourth, persistence should be tested against the alternative explanation most capable of producing the observed pattern. This requirement is deliberately claim-specific. Geography-associated differentiation requires a different challenge from harvest-stage differentiation; genotype-associated separation requires a different challenge from processing-associated separation. The framework does not demand that every source of variation be experimentally eliminated. Instead, untested plausible alternatives lower the strength and scope of the chemotype claim. Where genotype and environment cannot be separated, for example, the appropriate conclusion may be a stable location-associated or cultivation-associated chemical phenotype rather than an inherited chemotype.

Fifth, replication should occur at the level at which generalization is intended. Repeating injections addresses analytical repeatability, repeating extractions addresses sample-preparation variability, resampling plants addresses biological variability, and repeating sites or seasons addresses environmental transportability. These are not interchangeable demonstrations of reproducibility. A chemotype designation becomes stronger when the same chemically coherent pattern survives the levels of replication relevant to its proposed biological meaning.

Table 3 converts these principles into a manuscript-responsive decision sequence. The decision states are proposed and should be treated as an evaluative framework requiring prospective testing rather than as validated thresholds.

Table 3. Proposed decision sequence for evidence-bounded chemotype assignment

Decision stage	Question	Evidence supporting progression	If evidence is insufficient
Material definition	Is the biological source and sampled material unambiguous?	Authenticated identity, provenance, plant part and sampling context	Restrict or defer the claim
Analytical admissibility	Is separation robust to QC, drift and batch assessment?	Acceptable analytical behavior and nonconfounded acquisition	Treat separation as analytically unresolved

Chemical coherence	Does the group differ through interpretable constituents or constituent patterns?	Qualified identities and reproducible chemical pattern	Retain feature-level classification only
Persistence	Does the pattern survive relevant temporal, environmental or sampling challenge?	Recurrence under claim-relevant resampling	Classify as context-bounded
Alternative-explanation test	Have plausible nonchemotypic causes been challenged or bounded?	Competing causes experimentally tested or explicitly constrained	Reduce chemotype confidence
Replication and scope	Is recurrence demonstrated at the level to which the claim generalizes?	Independent biological/contextual replication	Limit the stated scope
Interpretive outcome	What label matches the accumulated evidence?	Convergent support across preceding stages	Use provisional, context-bounded or unresolved terminology

Implications for medicinal-plant metabolomics

Medicinal-plant metabolomics already uses chemical profiles to address origin, cultivation, harvest, processing, authentication, and quality-related questions [25]. The proposed framework does not diminish those applications; it narrows the inference attached to each. A profile that reliably distinguishes origins can remain useful for provenance testing even when the causal basis of the regional difference is unresolved. A harvest-stage signature can guide collection strategy without becoming a chemotype. A processing signature can support product discrimination without being attributed to the genetics of the source plant.

This distinction matters for germplasm selection. If a metabolomic group is treated as a stable chemotype when it is actually environment-dependent, selection based on one location or season may fail to reproduce the expected chemistry elsewhere. Conversely, context dependence is not necessarily undesirable. A cultivar whose chemistry responds predictably to harvest stage or environment may be valuable precisely because that response can be managed. The framework therefore separates stability of identity from controllability of chemistry: both can matter to medicinal-material quality, but they answer different scientific questions.

The framework also changes how apparently discordant studies should be compared. Two investigations may both report a “high-metabolite” and “low-metabolite” group while having sampled different organs, seasons, processing states, or analytical platforms. Treating those groups as equivalent chemotypes can manufacture agreement that the underlying designs do not justify. Cross-study synthesis should therefore compare the defining chemical constituents, the biological unit to which the label refers, and the contexts across which the pattern was shown to persist. Where those elements differ, the safer interpretation is convergence in chemical tendency rather than identity of chemotype. This distinction is particularly relevant when medicinal materials move from field collections into cultivation, processing, and standardized-product workflows, because each transition can preserve, attenuate, or replace the variation observed at the preceding stage.

The same logic applies to quality control. Chemotype labels can simplify communication when they summarize a persistent and chemically coherent phenotype, but they can obscure heterogeneity when used as substitutes for measured composition. Where evidence remains incomplete, reporting the observed chemical class together with its sampling, geographic, developmental, processing, or analytical context is more informative than prematurely assigning a biologically stronger name.

Accordingly, the principal practical implication is not that chemotypes should be used less often, but that their evidential content should be made explicit. Candidate chemotypes, context-bounded chemical phenotypes, processing-defined profiles, and analytically unresolved clusters should not be collapsed into a single category merely because all can be separated by the same statistical tools.

Reporting and replication requirements

Evaluation is possible only when the information needed to reconstruct alternative explanations is reported. Untargeted metabolic phenotyping therefore benefits from explicit documentation of analytical QA/QC procedures and their observed performance, as emphasized by mQACC recommendations [26]. For medicinal plants, this analytical information should be accompanied by botanical identity, provenance, sampled organ or

tissue, developmental or harvest state, cultivation or collection context, processing history, extraction protocol, acquisition order, randomization, batch allocation, and the level of metabolite identification supporting the biological claim.

Pooled QC samples, multidimensional QC assessment, and explicit QA/QC reporting provide complementary evidence for the analytical auditability of untargeted metabolomics data [21, 22, 26]. Their functions should nevertheless remain distinct. QC injections can reveal drift or instability; diagnostic frameworks can expose carryover, missingness or outliers; transparent reporting allows readers to judge whether corrective procedures were appropriate. None independently resolves confounding between biological classes and analytical batches.

Replication should likewise be described by type rather than by a single sample-size label. Technical replicate injections, independent extractions, multiple plants, repeated harvests, seasons, sites, populations, and analytical batches address different uncertainty sources. The proposed framework therefore recommends a replication-by-claim principle: the stronger the biological scope implied by "chemotype," the more directly the replication design should challenge the contexts in which that phenotype could disappear or be replaced by an alternative explanation.

Reporting should also make confounding inspectable before readers reach the multivariate results. Sample identifiers should permit reconstruction of biological group, collection context, extraction sequence, and analytical batch without requiring inference from figures. Acquisition order and randomization should be stated, especially when class membership could align with run order. When correction or normalization is applied, the variable being corrected, the information used to estimate it, and the evidence that relevant biological structure was not simply removed should be described. For proposed chemotypes, a compact provenance-to-analysis record is more useful than a long instrument description that omits how biological samples were distributed across batches. These requirements do not guarantee reproducibility; they make the conditions of reproducibility visible and allow later studies to reproduce or deliberately challenge the original claim.

No universal replication number follows from the selected evidence. Species biology, chemical heterogeneity, propagation system, analytical platform, and intended claim differ too greatly for one threshold to be defensible. What should be universal is transparency about which levels were replicated, which were not, and which unresolved dimensions limit transferability. Such reporting turns absence of evidence into an explicit boundary rather than allowing it to disappear behind a categorical chemotype label.

Conclusion

Metabolomic separation is evidence of chemical differentiation, not an automatic diagnosis of chemotype. In medicinal plants, the same multivariate pattern can arise from inherited variation, population structure, environment, geography, phenology, tissue composition, processing, or analytical batch effects, and several of these causes may operate simultaneously. A useful chemotype concept must therefore carry more information than membership in a statistical cluster.

The evaluation framework proposed here organizes that inference around defined material identity, analytical admissibility, chemical coherence, persistence, alternative-explanation testing, and claim-matched replication. Its central distinction is between a persistent chemical phenotype and a context-bound or technically unresolved pattern. This distinction preserves biologically meaningful plasticity rather than treating it as noise, while also preventing technical reproducibility from being mistaken for biological stability.

The framework is intentionally nonnumerical. It provides no universal distance cutoff, abundance threshold, classifier performance criterion, or minimum replication count, because the evidence does not establish such values across medicinal-plant systems. Nor does it establish causal inheritance merely by excluding several alternatives. Its role is to make the reasoning behind chemotype assignment explicit, graded, and falsifiable.

A further boundary is causal attribution. Even a persistent, reproducible chemical phenotype may remain mechanistically unexplained, and the framework does not convert stability into proof of a particular genetic pathway or environmental driver. It instead identifies the strongest interpretation warranted by the available design and makes the remaining causal uncertainty explicit.

Used in this way, chemotype can remain a valuable pharmacognostic and phytochemical concept. Its strongest use is not as a convenient name for any metabolomic difference, but as a bounded interpretation supported by convergent chemical, biological, analytical, and replication evidence. Where that convergence is incomplete, more precise provisional terminology is scientifically stronger than categorical certainty.

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