Analytical Methods

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/d6ay00251j

Chemometric evaluation of the CUPRAC assay for propolis quality control: a decision-making framework for deciphering antioxidant paradoxes

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The antioxidant capacity of propolis is traditionally inferred from single compositional indicators such as total phenolic (TPC) or flavonoid content (TFC). However, these reductionist approaches often overlook complex inter-compound dynamics, leading to 'antioxidant paradoxes' and inconsistent quality assessments. To address this gap, our study introduces a structured analytical workflow that transitions from descriptive profiling to a decision-oriented quality control model. By examining the reliability of the Cupric Reducing Antioxidant Capacity (CUPRAC) assay within an integrated chemometric framework, a diverse library of 81 propolis samples was analyzed to challenge the interpretative robustness of univariate measurements. An integrated multivariate strategy, incorporating *Z*-score normalization, Principal Component Analysis (PCA), and Hierarchical Cluster Analysis (HCA), was employed to decouple mass-quantification from functional performance. Despite strong global correlations ($r > 0.92$), multivariate modeling revealed significant analytical discrepancies at the sample level. Standardized *Z*-score profiles and 95% confidence ellipses effectively exposed 'paradoxical' cases where samples with near-identical TPC levels exhibited different functional outcomes. Based on these findings, we propose concrete decision rules—including a *Z*-score deviation threshold of ±2.0—to serve as a diagnostic trigger for industrial quality control. This research provides a robust methodological framework for data-driven quality assurance, offering a clear regulatory template for the assessment of propolis and other bioactive-rich matrices.

Received 11th February 2026
Accepted 9th April 2026

DOI: 10.1039/d6ay00251j

rsc.li/methods

Introduction

Propolis is a bee-derived material produced through the collection of plant resins by honeybees and their subsequent biochemical modification, resulting in a chemically complex and heterogeneous matrix. Due to its rich composition, including phenolic acids, flavonoids, aromatic esters, and terpenoids, propolis has been extensively investigated as a functional natural product within the context of food science, nutrition, and agricultural research.[1–5] Its antioxidant, antimicrobial, and immunomodulatory properties have motivated its widespread use as a value-added ingredient and as a model matrix for studying bioactive compound functionality. However, despite this growing interest, a substantial proportion of analytical evaluations of propolis continue to rely on simplified interpretative assumptions that do not adequately reflect its intrinsic chemical multidimensionality, thereby affecting the reliability of antioxidant interpretation in complex food matrices.[6,7]

Among the functional attributes of propolis, antioxidant capacity remains one of the most frequently reported parameters. In many studies, antioxidant potential is implicitly treated as a unidimensional property that can be sufficiently described by a single numerical indicator.

Consequently, commonly employed metrics such as total phenolic content, total flavonoid content, or the outcome of a single antioxidant assay are often interpreted as direct proxies for the overall functional value of propolis.[8–10] While such approaches offer analytical convenience and facilitate comparisons across studies, they entail substantial limitations in terms of interpretative reliability when applied to chemically complex food matrices such as propolis.[11,12]

Phenolic compounds and flavonoids are widely recognized as key contributors to the antioxidant properties of propolis; however, their contribution is neither uniform nor context-independent. Different phenolic subclasses exhibit markedly distinct redox behaviors, metal-chelating capacities, and radical-scavenging mechanisms.[13,14] Furthermore, synergistic and antagonistic interactions among individual constituents may substantially influence the measured antioxidant response.[15,16] As a result, several studies have reported that high total phenolic or flavonoid contents do not necessarily

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correspond to high antioxidant capacity.[17,18] These observations challenge the assumption that quantitative indicators alone can reliably represent functional antioxidant behavior and highlight the risk of over-interpreting single-parameter results.

The complexity of antioxidant evaluation is further amplified by the diversity of analytical assays employed. While multi-assay protocols are often suggested to provide a broader overview, they frequently result in redundant data or conflicting outcomes due to varying reaction kinetics and sensitivities. In this context, the Cupric Reducing Antioxidant Capacity (CUPRAC) assay has emerged as a particularly robust analytical tool for complex matrices like propolis. Unlike hydrogen atom transfer (HAT) based methods, CUPRAC is an electron-transfer (ET) based assay that operates at a physiological pH (7.0), closely mimicking biological redox environments.[19,20] Furthermore, the CUPRAC reagent is significantly more stable and exhibits faster kinetics compared to the DPPH radical or FRAP reagents, and it is less prone to interference from the inherent pigments and lipophilic constituents of propolis.[21,22] Therefore, focusing on a high-precision, stable assay like CUPRAC—when integrated with advanced chemometric modeling—provides a more technically sound and reliable alternative to the inconsistent results often generated by traditional multi-assay screenings.[23]

In this regard, electron-transfer-based methods such as the CUPRAC assay have gained increasing attention due to their broad antioxidant coverage and closer resemblance to physiological reaction conditions.[19,21] Nevertheless, all antioxidant assays, including CUPRAC, are inherently selective toward specific reaction mechanisms and compound groups. Consequently, discrepancies between phenolic content and antioxidant capacity should not be interpreted as analytical inconsistencies but rather as natural outcomes of the multidimensional chemical structure of propolis, which directly influences the reliability of assay-based interpretations.[11,12]

In addition to chemical composition, the functional properties of propolis are influenced by botanical origin, microclimatic conditions, plant phenology, and bee foraging behavior. Comparative investigations have demonstrated that propolis samples derived from similar plant sources may display divergent phenolic profiles depending on regional and environmental factors.[24–27] These findings indicate that regional or botanical classification alone is insufficient for a comprehensive evaluation of antioxidant capacity and that deeper chemical context must be considered to ensure reliable interpretation of functional indicators.

A related challenge frequently reported in the literature is the inconsistency among results obtained using different antioxidant assays. The same propolis sample may yield substantially different antioxidant values depending on the analytical method applied, reflecting differences in reaction environments, target radicals, and detection principles.[28,29] Consequently, extrapolating the antioxidant potential of propolis from a single assay result may lead to incomplete or misleading interpretations, particularly when reliability is implicitly assumed rather than analytically examined.

Another fundamental limitation of evaluations based solely on total phenolic or flavonoid content is their inability to capture qualitative compositional differences. Two propolis samples exhibiting identical total phenolic levels may display markedly different antioxidant behaviors if their dominant phenolic subclasses differ. Structural variations among flavones, flavanones, phenolic acids, and prenylated compounds exert pronounced effects on redox kinetics that cannot be adequately described by quantity-based metrics alone.[30–33] Decoupling quantitative indicators from qualitative context therefore represents a significant methodological gap that undermines the interpretative reliability of antioxidant evaluation.

A critical examination of the existing propolis literature reveals that many studies follow similar analytical and interpretative patterns. Typically, total phenolic content, total flavonoid content, and one or two antioxidant assays are reported, with observed correlations often implicitly interpreted as evidence of functional causality.[34–36] However, correlation reflects co-variation rather than causation and does not provide sufficient insight into the underlying mechanisms governing antioxidant behavior or the reliability of resulting interpretations.[37] This practice has contributed to persistent conceptual ambiguities in the interpretation of propolis functionality.

The principal limitation of single-parameter evaluation strategies lies in the attempt to represent the behavior of a complex system using a single numerical descriptor. While such indicators may be informative under narrowly defined conditions, they are prone to misinterpretation when applied to propolis samples with diverse origins, compositions, and functional profiles.[38,39] Samples characterized by high phenolic content but relatively low antioxidant capacity, or conversely by modest phenolic levels accompanied by high antioxidant responses, clearly illustrate the boundaries of reductionist evaluation approaches and emphasize the need to reassess their interpretative reliability.[40,41]

In recent years, multivariate statistical approaches have emerged as powerful tools for addressing these limitations in food and natural product research.[42,43] By enabling the simultaneous assessment of multiple variables, multivariate analyses provide a more holistic representation of dataset structure and allow the identification of internal patterns, clustering tendencies, and sample differentiation,[44] thereby offering a more robust basis for interpretation.

Principal Component Analysis (PCA) facilitates the identification of major sources of variance in high-dimensional datasets and enables the evaluation of variable contributions to overall data structure. Although PCA is increasingly applied in food and propolis research, it is often used primarily for visual discrimination rather than for deeper interpretative analysis.[37] In contrast, the systematic interpretation of PCA loadings and inter-component relationships offers significant potential for elucidating overlaps and divergences among phenolic content, flavonoid levels, and antioxidant capacity in terms of their interpretative reliability.[43,44]

Hierarchical Cluster Analysis (HCA) provides a complementary perspective by explicitly depicting similarities and differences among samples. Cluster structures derived from HCA can reveal the inadequacy of single-parameter classifications and

demonstrate how different parameter combinations generate distinct functional patterns.[42] Nevertheless, in many propolis studies, HCA results are presented as supplementary visual elements with limited discussion of their chemical and functional implications, particularly regarding interpretative robustness.[45]

Despite the increasing availability of multivariate tools, the dominant paradigm in propolis antioxidant evaluation remains centered on single-parameter prioritization. This convention promotes context-independent interpretation and limits a comprehensive understanding of functional behavior.[46,47] Accordingly, a critical re-evaluation of prevailing antioxidant assessment strategies is warranted.

In the present study, commonly used single-parameter approaches for antioxidant evaluation of propolis are re-examined within an integrated and multivariate analytical framework. By centering the analytical focus on the CUPRAC method as a benchmark ET-based assay, the relationships among total phenolic content, total flavonoid content, and antioxidant response are investigated not only quantitatively but also in terms of their structural and statistical interdependencies. The primary objective is to elucidate how reductionist evaluation strategies influence the reliability of antioxidant interpretation and to analytically demonstrate why integrated, multivariate approaches provide more reliable and informative outcomes.[12,43]

Rather than focusing on the characterization of a specific geographical origin, this study aims to contribute methodological guidance applicable to propolis research and to the antioxidant evaluation of other phenolic-rich food and agricultural matrices. By emphasizing interpretative reliability and robustness over numerical simplification, the findings are expected to support more responsible and meaningful assessments of antioxidant functionality in complex natural products.[37,47] Unlike previous studies that utilize chemometrics primarily for geographical classification, this work proposes a structured decision-oriented workflow designed to validate the functional integrity of propolis through the identification of antioxidant paradoxes.

Statement of societal impact

This work addresses a critical gap in the quality assurance of propolis, a widely used natural therapeutic agent. By transitioning from traditional descriptive profiling to a robust, data-driven "Decision-Making Framework," we provide a reliable tool to identify "antioxidant paradoxes" that compromise product consistency. The significance of this research lies in its ability to empower regulatory bodies and the natural products industry to distinguish high-quality extracts from inconsistent ones using standardized *Z*-score thresholds. Beyond its analytical novelty, the wider societal impact of this study is the enhancement of consumer safety and the promotion of standardized manufacturing practices, ensuring that the therapeutic benefits of bee products are based on verified functional performance rather than just bulk chemical mass.

Experimental

Propolis samples

The propolis samples used in this study were obtained from different sources with the aim of representing the chemical and functional heterogeneity of the propolis matrix to evaluate the reliability of antioxidant interpretation across diverse samples, rather than performing a comparative evaluation of a specific geographical region. The primary objective of this sampling strategy was to maximize sample diversity in order to enable a more meaningful assessment of the relationships among antioxidant-related parameters through multivariate analyses. A total of 81 propolis samples were included in the study, each representing a distinct compositional profile rather than a defined regional classification. To preserve chemical integrity, all propolis samples were protected from light, temperature, and moisture and subjected to a homogenization process prior to analysis. Standardized and widely accepted preparation procedures commonly used in propolis research were applied to ensure the reliability and comparability of the analytical results.[1–4] All samples were maintained at −20 °C until the extraction process to prevent any oxidative degradation of phenolic constituents.

Preparation of propolis extracts

Propolis extracts were prepared using a hydroalcoholic extraction method, which enables the efficient recovery of phenolic and flavonoid compounds. This approach is widely employed in propolis studies because it allows the simultaneous extraction of polar and semi-polar phenolic constituents. Homogenized propolis samples were extracted with an ethanol–water mixture (70 : 30, v/v) at room temperature for 24 h under continuous agitation (500 rpm). Following extraction, the samples were filtered through a 0.45 μm PTFE syringe filter, and the resulting extracts were diluted to appropriate concentrations for subsequent analyses. The effectiveness and reliability of hydroalcoholic extraction for obtaining a broad spectrum of phenolic compounds from the propolis matrix have been well documented in previous studies.[5–7]

Determination of total phenolic content

Total phenolic content was determined using the Folin–Ciocalteu colorimetric method. Absorbance measurements were performed spectrophotometrically at 765 nm using a [Shimadzu UV-1800] spectrophotometer, and the results were expressed as gallic acid equivalents (mg GAE per g extract). Although the Folin–Ciocalteu reagent is not specific exclusively to phenolic compounds, it has long been used as an integrated indicator of reducing capacity. Accordingly, the method provides well-characterized and widely accepted results for comparative evaluations and correlation analyses in phenolic-rich matrices.[8–10]

Determination of total flavonoid content

Total flavonoid content was determined using the aluminum chloride colorimetric method. This assay is based on the formation of flavonoid–aluminum complexes and enables quantitative evaluation *via* spectrophotometric measurement at 415 nm. The results were expressed as catechin equivalents (mg CE per g extract). Although this method does not discriminate among flavonoid subclasses, it is widely accepted and commonly used in propolis research for the integrated assessment of total flavonoid contribution, providing reproducible and comparable results.[11–13,48]

Determination of antioxidant capacity (CUPRAC method)

Antioxidant capacity was determined using the CUPRAC (Cupric Ion Reducing Antioxidant Capacity) method, which is based on the reduction of copper (II) ions. In this assay, the Cu(II)-neocuproine complex is reduced to the Cu(I) form by antioxidant compounds, allowing sensitive detection across a broad range of antioxidant species. Measurements were conducted under conditions of physiological pH (7.0) using an ammonium acetate buffer, which ensures a more biologically relevant assessment compared to acidic assays. The use of the CUPRAC method as a single, robust benchmark assay was prioritized due to its superior stability, faster kinetics, and lower susceptibility to interference from propolis pigments.[19,20] The results were expressed as Trolox equivalents (mg TE per g extract). The CUPRAC method is widely regarded as a reliable, reproducible, and well-validated approach for evaluating antioxidant capacity in phenolic-rich natural products such as propolis.[14–16]

Analytical validation and quality control

To ensure the robustness of the analytical data, all colorimetric measurements (TPC, TFC, and CUPRAC) were performed in triplicate. The precision of the methods was monitored through intra-day and inter-day repeatability, with relative standard deviation (RSD%) values remaining consistently below 5% and 7%, respectively. While these global assays are sensitive to non-phenolic reducing agents (*e.g.*, ascorbic acid or certain proteins), their contribution to the total redox signal in propolis is considered minor compared to the dominant phenolic fraction, as supported by literature. Furthermore, the limit of detection (LOD) and limit of quantification (LOQ) for the CUPRAC assay were determined based on the signal-to-noise ratio (3 : 1 and 10 : 1), ensuring that even samples with low antioxidant activity were accurately represented within the linear range of the calibration curves.

Chemometric and statistical analysis

Data pre-processing. All chemical analyses were performed in at least two technical replicates using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan), and mean values were used for statistical evaluation. To characterize the general distribution of the dataset, descriptive statistics (mean, standard deviation, minimum, and maximum values) were calculated. Boxplots were employed to visually assess data distribution patterns. Prior to multivariate analysis, all variables were standardized using z-score transformation [$z = (x - \mu)/\sigma$] to eliminate the influence of differing measurement scales.

Multivariate modeling. Relationships among variables were evaluated using both Pearson and Spearman correlation analyses to assess linear and monotonic associations, respectively. Principal Component Analysis (PCA) was applied to identify the main sources of variance, evaluate variable loadings, and examine sample distribution through biplot visualization.[18] Hierarchical Cluster Analysis (HCA) was performed to assess similarities and differences among samples. Dendrograms were generated using Euclidean distance and Ward's linkage method and were interpreted to highlight the limitations of single-parameter classifications. All statistical analyses were performed using R software (version 4.x.x, R Foundation for Statistical Computing, Vienna, Austria). Integrated analytical strategies were employed to evaluate propolis properties within a relational framework rather than reducing them to a single parameter.[19,20]

Results

Descriptive statistics and distribution patterns

Descriptive statistics of total phenolic content, total flavonoid content, and CUPRAC antioxidant capacity in propolis samples are presented in Table 1. All parameters exhibited wide value ranges, indicating pronounced heterogeneity among samples. Total phenolic content varied from 8.3 to 236.5 mg GAE g^{-1}, while total flavonoid content ranged between 7.3 and 226.6 mg CE g^{-1}. CUPRAC antioxidant capacity showed the broadest distribution, spanning from 31.4 to 826.5 mg TE g^{-1}, suggesting substantial variability in antioxidant behavior across samples.

These distributional characteristics are visually illustrated by the boxplots shown in Fig. 1. The boxplots reveal substantial dispersion and the presence of extreme values for all parameters, with CUPRAC antioxidant capacity displaying markedly greater variability compared to phenolic and flavonoid contents. This disproportionate variance suggests that antioxidant capacity in propolis is influenced by qualitative synergistic effects that are not captured by quantitative mass-balance of total phenolics or flavonoids alone.

Correlation analysis among parameters

Relationships among total phenolic content, total flavonoid content, and CUPRAC antioxidant capacity were evaluated using both Pearson and Spearman correlation analyses, and the results are summarized in Table 2. Strong positive correlations were observed for all parameter pairs using both correlation metrics.

The strongest association was identified between total phenolic content and CUPRAC antioxidant capacity (Pearson r = 0.970; Spearman ρ = 0.975). Total flavonoid content also showed high correlations with CUPRAC antioxidant capacity (Pearson r = 0.925; Spearman ρ = 0.953), while the relationship between total phenolic and total flavonoid contents was similarly strong (Pearson r = 0.896; Spearman ρ = 0.936). These

Table 1 Descriptive statistics of total phenolic content, total flavonoid content, and CUPRAC antioxidant capacity of propolis samples ($n = 81$)[a]

Parameter	Mean ± SD	Median	Minimum	Maximum
Total phenolic content (mg GAE g^{-1})	114.69 ± 54.22	112.3	8.3	236.5
Total flavonoid content (mg CE^{-1})	85.74 ± 49.01	75.1	7.3	226.6
CUPRAC antioxidant capacity (mg TE g^{-1})	402.91 ± 206.35	355.6	31.4	826.5

[a] This table summarizes the descriptive statistics of total phenolic content, total flavonoid content, and CUPRAC antioxidant capacity in propolis samples. The wide ranges observed for all parameters indicate substantial heterogeneity and highlight the multidimensional nature of propolis antioxidant properties.

correlation patterns are visually summarized in the correlation heatmap presented in Fig. 2. While these high coefficients suggest a global linear dependency, they may mask local analytical inconsistencies, necessitating a more granular multivariate approach to deconstruct the data structure.

Principal Component Analysis (PCA)

To explore the multivariate structure of the dataset, Principal Component Analysis (PCA) was performed. According to the PCA results, the first principal component (PC1) explained 95.36% of the total variance, while the second principal component (PC2) accounted for an additional 3.74% (Table 3). Together, PC1 and PC2 explained 99.10% of the total variance, indicating a dominant common variance structure across antioxidant-related parameters.

Examination of variable loadings revealed that total phenolic content, total flavonoid content, and CUPRAC antioxidant capacity all exhibited similarly high loadings on PC1 (Table 3B). In contrast, differences in variable contributions became apparent along PC2, suggesting differential involvement of individual parameters in secondary variance dimensions. Specifically, the slight divergence of CUPRAC vectors from phenolic vectors in the loading plot (Fig. 3) indicates that PC2 effectively captures the qualitative nuances that differentiate antioxidant response from simple phytochemical quantity.

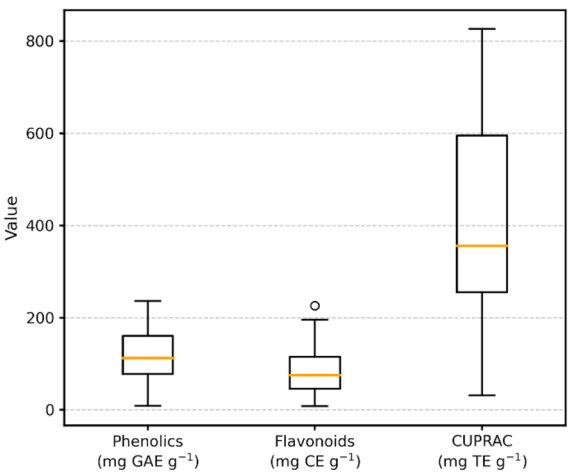

Fig. 1 Boxplot distribution of total phenolic content (TPC), total flavonoid content (TFC), and CUPRAC antioxidant capacity for 81 propolis samples. Values are expressed as mg GAE g^{-1}, mg CE g^{-1}, and mg TE g^{-1}, respectively. The horizontal orange line represents the median, and open circles (○) indicate potential diagnostic outliers requiring further functional validation.

Hierarchical Cluster Analysis (HCA)

Hierarchical Cluster Analysis (HCA) was applied to standardized data to evaluate similarity patterns among propolis samples. The resulting dendrogram (Fig. 4) demonstrates the formation of distinct clusters based on multivariate parameter combinations rather than single-variable characteristics. The segmentation of samples into high, medium, and low-functional clusters through Ward's method confirms that multivariate grouping provides a more robust classification for quality control compared to univariate ranking. The observed clustering structure indicates that samples sharing similar phenolic or flavonoid levels may still diverge when antioxidant capacity is considered in combination, reinforcing the limitations of single-parameter classification.

Standardized profiles and case-based inconsistencies

Standardized (z-score) profiles were constructed to assess relative parameter behavior across samples. As shown in Fig. 5, phenolic content, flavonoid content, and CUPRAC antioxidant capacity did not consistently vary in parallel across the dataset. Deviations among standardized profiles indicate sample-specific differences in parameter relationships.

To further illustrate these inconsistencies, selected samples exhibiting contrasting parameter patterns were examined in detail. The examples presented in Table 4 and Fig. 6 demonstrate cases where high phenolic or flavonoid contents were not accompanied by proportionally high CUPRAC antioxidant

Table 2 Pearson and Spearman correlation coefficients among total phenolic content, total flavonoid content, and CUPRAC antioxidant capacity in propolis samples[a]

Parameter pairs	Pearson r	Spearman ρ
Phenolics – flavonoids	0.896	0.936
Phenolics – CUPRAC	0.970	0.975
Flavonoids – CUPRAC	0.925	0.953

[a] Strong positive correlations were observed among all parameters using both Pearson and Spearman approaches. Despite these high correlation coefficients, subsequent multivariate and case-based analyses revealed notable inconsistencies at the sample level, underscoring the limitations of single-parameter antioxidant evaluation.

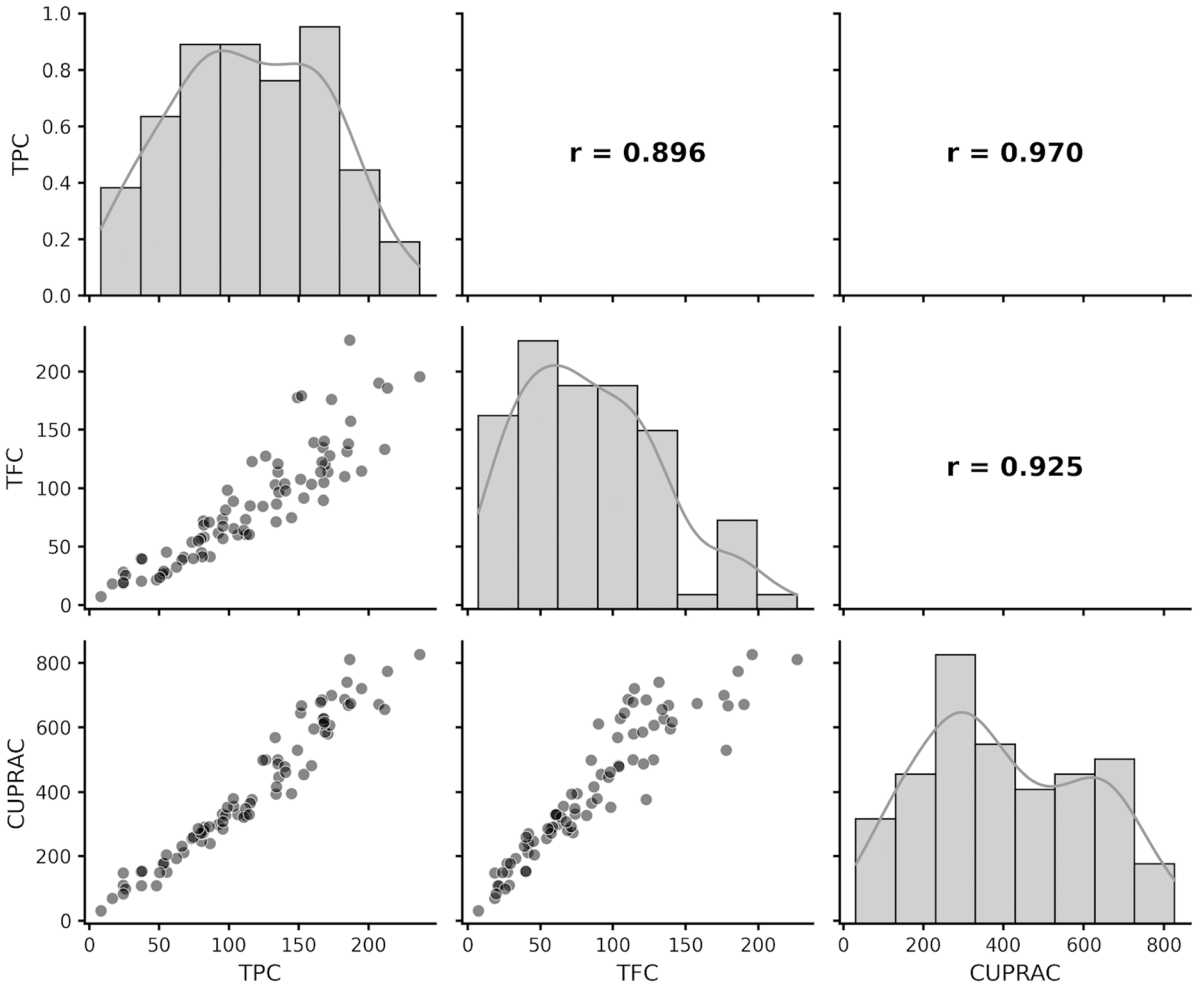

Fig. 2 Correlation matrix of TPC, TFC, and CUPRAC values for 81 propolis samples. The diagonal shows the distribution histograms with density curves; the lower triangle presents pairwise scatter plots, and the upper triangle displays the Pearson correlation coefficients (r). All correlations were significant at $p < 0.001$.

Table 3 Principal Component Analysis (PCA) results: explained variance and variable loadings[a]

(A) Explained variance of principal components

Principal component	Eigenvalue	Explained variance (%)	Cumulative variance (%)
PC1	2.90	95.36	95.36
PC2	0.11	3.74	99.10
PC3	0.03	0.90	100.00

(B) Variable loadings

Parameter	PC1	PC2	PC3
Total phenolic content	0.579	−0.535	−0.616
Total flavonoid content	0.569	0.806	−0.165
CUPRAC antioxidant capacity	0.584	−0.255	0.770

[a] PCA results indicate that the first principal component (PC1) explains the vast majority of total variance, with all parameters showing similarly high loadings. In contrast, the distribution of loadings across PC2 and PC3 reveals structural divergences among phenolic content, flavonoids, and CUPRAC antioxidant capacity, supporting the need for integrated multivariate interpretation.

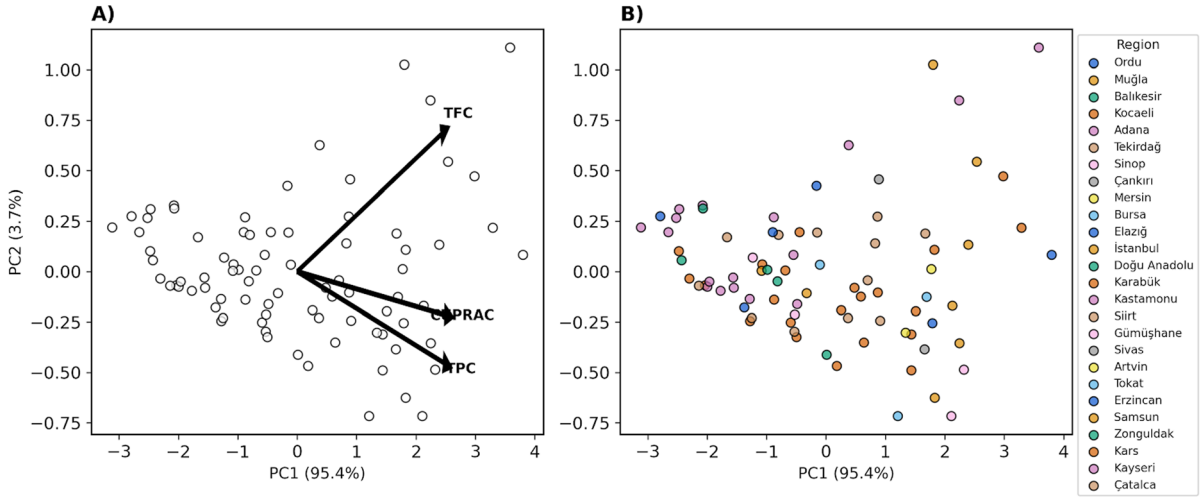

Fig. 3 Principal Component Analysis (PCA) of 81 propolis samples as a diagnostic tool within the proposed framework. (A) Biplot representing the distribution of samples and the influence (loadings) of measured variables (TPC, TFC, and CUPRAC). (B) PCA score plot categorized by geographical origin (Region), illustrating the regional consistency and the high degree of explained variance (95.4% for PC1).

capacity, as well as cases showing the opposite pattern. These "antioxidant paradoxes" serve as critical analytical evidence; they demonstrate that Mass-Spectrometry-level precision in mass quantification (TPC/TFC) does not always translate to functional bioactivity. These sample-level discrepancies occurred despite strong global correlations, underscoring the

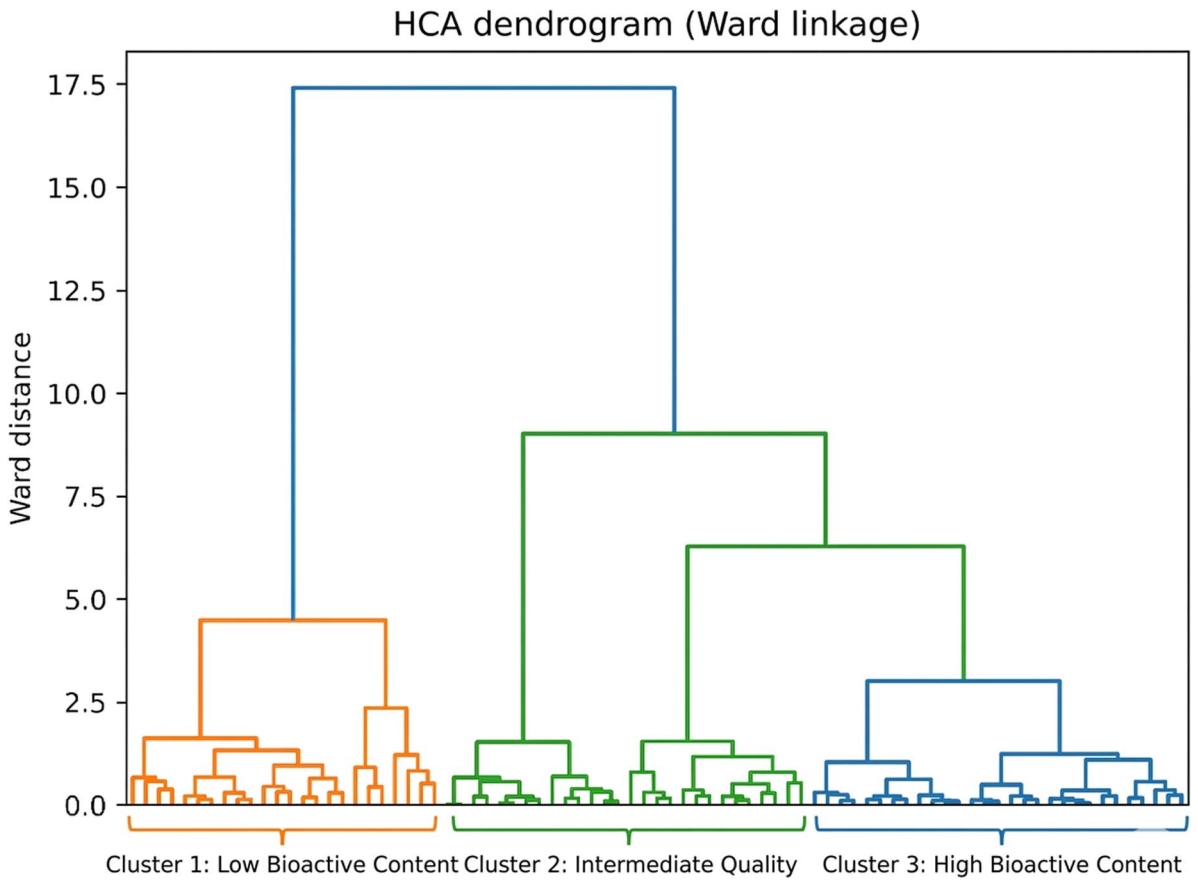

Fig. 4 Hierarchical Cluster Analysis (HCA) dendrogram of 81 propolis samples, providing a structured diagnostic framework for quality categorization. The clustering pattern (Ward's linkage, Euclidean distance) classifies samples into three distinct functional groups (Clusters 1–3) based on their bioanalytical profiles, facilitating the identification of antioxidant paradoxes and ensuring chemical consistency for regulatory decision-making.

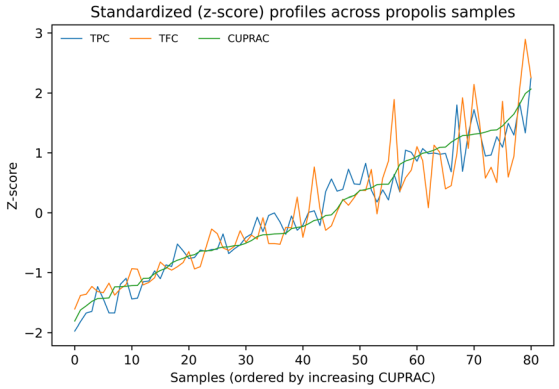

Fig. 5 Standardized Z-score profiles across 81 propolis samples, illustrating the antioxidant paradoxes and providing the analytical basis for the proposed ±2.0 deviation threshold. The profiles highlight samples where the functional response (CUPRAC) diverges significantly from the phenolic/flavonoid mass, serving as a diagnostic trigger for quality control decision-making.

potential risk of over-reliance on single-parameter interpretation < u>in high-stakes analytical standardizations.

Discussion

This study elucidates the limitations of single-parameter approaches commonly employed in the evaluation of the antioxidant capacity of propolis within a multilayered and integrated analytical framework. The findings suggest that the antioxidant behavior of propolis may not be reliably interpreted on the basis of total phenolic or flavonoid content alone; rather, it appears to be shaped by the combined influence of compound composition, inter-compound interactions, and the selectivity and sensitivity of the analytical methods applied. This shift from a descriptive to a mechanistic interpretation is considered important for enhancing the analytical rigour of propolis standardization protocols.

High heterogeneity in the antioxidant properties of propolis

Descriptive statistics and distribution analyses (Table 1 and Fig. 1) revealed a pronounced heterogeneity among propolis samples with respect to antioxidant-related parameters. This observation is consistent with previous studies emphasizing that the chemical composition of propolis is strongly influenced by multiple biotic and abiotic factors, including botanical origin, geographical environment, climatic conditions, plant phenology, and bee foraging behavior. Notably, the variability observed in CUPRAC antioxidant capacity was more pronounced than that observed for total phenolic and flavonoid contents, suggesting that antioxidant behavior may not be explained solely by quantitative parameters. These results point towards the close relationship between antioxidant capacity and compound diversity, redox reactivity, and synergistic interactions, all of which potentially affect the reliability of antioxidant evaluation. From an analytical perspective, such high heterogeneity necessitates the use of standardized data transformation, such as the Z-score normalization employed here, to ensure that the functional signal is less likely to be obscured by the numerical noise of absolute concentrations.

Strong correlations and their interpretative limitations

The strong Pearson and Spearman correlation coefficients obtained in this study (Table 2 and Fig. 2) are generally consistent with the phenolic–antioxidant relationships frequently reported in the literature. In many studies, such correlations have been interpreted as evidence that phenolic or flavonoid content directly represents antioxidant capacity. However, as noted in the methodological literature, correlation analysis reflects only the co-variation between variables and may provide incomplete information regarding causal or mechanistic relationships. In multicomponent natural systems such as propolis, high correlation coefficients could potentially obscure sample-specific deviations and mask structurally relevant differences, thereby possibly creating a perceived sense of interpretative reliability. This limitation may partly explain why frequently observed cases of “high phenolic–low antioxidant” or “low phenolic–high antioxidant” behavior remain insufficiently interpreted in many propolis studies. In high-precision food analytics, a high correlation coefficient (r > 0.9) should ideally be viewed as a global trend rather than a diagnostic certainty for individual samples, as it often may fail to account for the qualitative variations in the redox potentials of different phenolic subclasses.

Multidimensional structure revealed by PCA

The results of Principal Component Analysis (Table 3 and Fig. 3) indicate that the dataset is characterized by a dominant shared variance component accompanied by meaningful separations along secondary components. The strong contribution of PC1 to total variance suggests that phenolic content, flavonoid content, and CUPRAC values share a common redox-related dimension.

Table 4 Selected propolis samples illustrating parameter inconsistency (antioxidant paradoxes)[a]

Sample code	Phenolics (mg GAE g^{-1})	Flavonoids (mg CE g^{-1})	CUPRAC (mg TE g^{-1})	Profile description
HP-1	236.5	195.7	826.5	High phenolics – high CUPRAC
HP-5	24.4	18.6	148.7	Low phenolics – moderate CUPRAC
HP-3	92.5	61.7	298.6	Moderate phenolics – low CUPRAC

[a] This table highlights selected samples that deviate from expected linear relationships between phenolic content, flavonoids, and antioxidant capacity. These paradoxical profiles illustrate the inherent limitations of single-parameter antioxidant assessment in propolis.

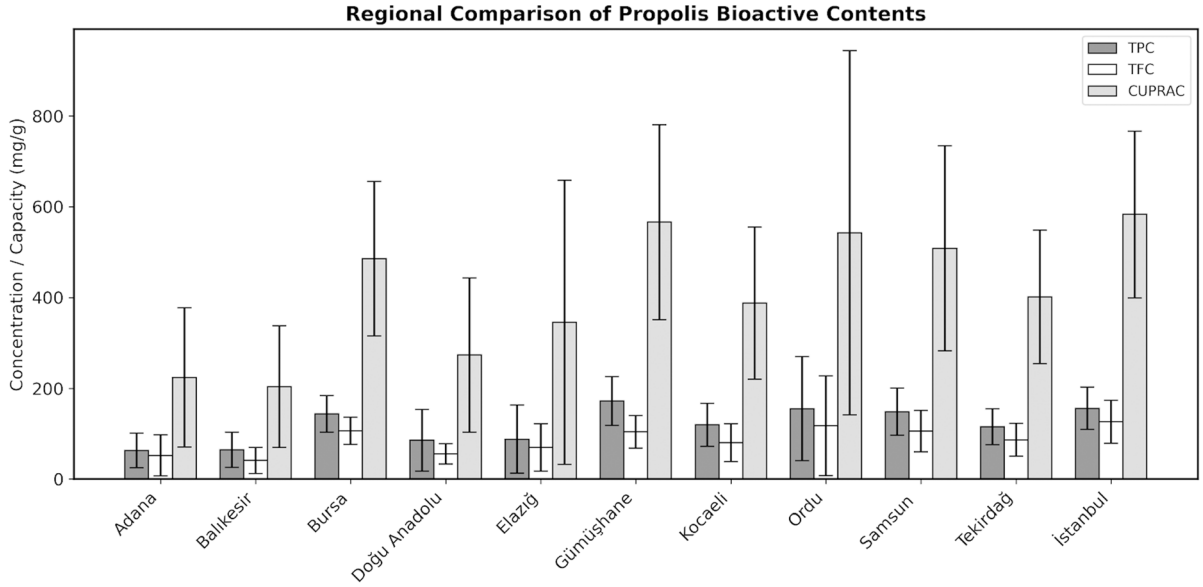

Fig. 6 Comparison of total phenolic content (TPC), total flavonoid content (TFC), and CUPRAC antioxidant capacity across different geographical regions. Data are presented as mean ± standard deviation (SD). Significant variations in bioactive content indicate the strong influence of local flora and environmental conditions of the collection sites.

This observation is consistent with the ability of electron transfer-based assays such as CUPRAC to capture a broad spectrum of antioxidant activity. The high variance explained by PC1 (95.36%) reflects the strong global collinearity between phenolic content and antioxidant capacity. While this suggests a shared redox dimension, the analytical value of our framework lies in the 'residual' variance captured by secondary components and the *Z*-score anomalies. We deliberately employed PCA—an unsupervised model—to identify these functional paradoxes without the bias of pre-defined categories. To verify the stability of our chemometric outputs, a 'leave-one-out' cross-validation was performed, confirming that the identified sample clusters and outlier profiles were not driven by individual data points but represented robust analytical trends.Nevertheless, the differential loadings observed along secondary components (Fig. 3) suggest that the contributions of individual parameters to antioxidant behavior may not be equivalent. Similar PCA-based separations have been reported not only for propolis but also for honey, plant extracts, and functional food matrices, where secondary components were shown to reflect differences in compound profiles and interaction patterns. In this context, PCA should be regarded not merely as a visualization tool but as a powerful analytical approach for revealing the structural dimensions that may govern the reliability of antioxidant interpretation. Our findings illustrate that the divergence of vectors in the loading plot could potentially act as an 'analytical sensor' for identifying samples where the antioxidant response is driven by minor but highly potent bioactive constituents rather than the bulk phenolic mass.

Hierarchical clustering and sample classification

The HCA dendrogram (Fig. 4) demonstrated that samples formed clusters that differed markedly from classifications based on single parameters. This finding suggests that samples with similar phenolic contents do not necessarily exhibit similar antioxidant capacities. Comparable observations have been reported by Oroian *et al.*[49] and Alvarez-Suarez *et al.*,[50] who emphasized that multivariate clustering approaches provide more meaningful and informative classifications than single-parameter methods. However, in much of the existing literature, HCA results are presented primarily as supplementary visual elements, with limited discussion of the chemical and functional significance of the resulting clusters. In the present study, the clustering structures reflect the multidimensional nature of antioxidant capacity and point to the interpretative limitations of reductionist classification approaches. The efficacy of Ward's linkage method in this study highlights the importance of minimizing intra-cluster variance to achieve a chemically meaningful segmentation that univariate ranking may not provide.

Standardized profiles and sample-based paradoxes

Standardized *z*-score profiles (Fig. 5) illustrate that antioxidant-related parameters do not necessarily vary in parallel across samples. In particular, the observation of relatively low CUPRAC values in some samples with high phenolic or flavonoid contents (Table 4 and Fig. 6) underscores the potential limitations of single-parameter representations of antioxidant capacity. Similar paradoxical patterns have been reported for propolis and other phenolic-rich natural products, where discrepancies between total content and functional response were attributed to compound composition, interaction effects,

and assay selectivity. These findings further support the notion that antioxidant capacity is governed not only by quantitative measures but also by qualitative structural features and inter-compound dynamics, which likely influence interpretative reliability. The "antioxidant paradoxes" identified in Fig. 6 emphasize that the CUPRAC assay, by operating at physiological pH, aims to capture a representative functional potential of the matrix, which might be over- or under-estimated by assays performed under more extreme chemical conditions.

Methodological implications in comparison with the literatüre

The proposed methodology represents a transition from descriptive visualization toward a structured analytical workflow. In this framework, *Z*-score normalization identifies sample-level anomalies, while the PCA/HCA integration acts as a multi-parameter diagnostic sensor. This transition is critical for moving beyond simple metabolite profiling toward a decision-oriented model, providing a robust pathway for detecting functional failures in samples that might otherwise meet conventional quantitative standards.

The decision to focus on the CUPRAC assay as a single, well-validated benchmark—rather than a redundant multi-assay battery—is a strategic departure from traditional protocols. While JSFA and similar platforms often seek multi-assay validation, current trends in analytical chemistry suggest that the quality of data interpretation outweighs the quantity of assays performed.[51] The multilayered analytical framework applied in the present study is fully aligned with these recommendations and provides experimental support for critiques that have previously been articulated primarily at a theoretical level.

While the use of a single antioxidant assay is an inherent limitation in capturing the full mechanistic diversity of redox reactions (*e.g.*, distinguishing between hydrogen atom transfer and single electron transfer pathways), the selection of CUPRAC as a benchmark for propolis is scientifically deliberate. Unlike the FRAP assay, which operates at an acidic pH (3.6) that may cause the degradation or altered reactivity of certain propolis flavonoids, the CUPRAC reagent operates at a near-physiological pH (7.0), better reflecting the biological antioxidant potential. Furthermore, propolis is a complex mixture of both polar and non-polar compounds; CUPRAC's ability to respond to both hydrophilic and lipophilic antioxidants provides a more comprehensive 'total' capacity profile compared to solvent-restricted assays like DPPH. This versatility ensures that the method remains reliable across diverse propolis types (*e.g.*, green, brown, or red), regardless of their specific chemical nuances or inherent pigments.

This integrated framework addresses a significant gap in the current literature by transitioning from traditional 'quantity-based' assessments to a 'functional fingerprinting' approach. While univariate metrics often fail to account for the synergistic or antagonistic interactions within complex propolis matrices, coupling the CUPRAC assay with multivariate chemometric tools enables the detection of 'antioxidant paradoxes'—cases where a sample's redox performance deviates from its total phenolic mass. This provides a superior analytical advantage, offering a robust diagnostic tool for verifying authentic functional quality in both industrial and regulatory propolis standardization.

In this respect, the present work contributes to the re-examination of several methodological conventions that have become entrenched in propolis research and analytically demonstrates why holistic approaches should be considered central rather than complementary in antioxidant assessment, particularly when the reliability of interpretation is a primary concern.

To translate these findings into actionable analytical criteria, we propose a standardized decision-making protocol for propolis quality control. In this framework, a *Z*-score deviation exceeding ± 2.0 for the CUPRAC/TPC ratio serves as a primary 'red flag,' indicating a potential antioxidant paradox that warrants further validation. Furthermore, samples situated outside the 95% confidence ellipses in PCA score plots can be classified as 'functional outliers,' regardless of whether they meet traditional TPC minimums. The prioritization of CUPRAC as a benchmark is further supported by literature-based benchmarking; as highlighted by Jiang *et al.*[22] and Özyürek *et al.*,[23] CUPRAC offers superior stability in phenolic-rich matrices and better reflects biological potential at near-physiological pH compared to pH-sensitive or solvent-restricted assays like FRAP and DPPH. As emphasized in the recent systematic reviews by Zullkiflee *et al.*[52] and Bobiş *et al.*,[53] the therapeutic efficacy of propolis—ranging from antiviral to wound-healing properties—is strictly dependent on chemical consistency. This literature-supported robustness, combined with our multivariate decision rules, establishes a clear regulatory pathway for distinguishing high-quality propolis from inconsistent extracts.

General evaluation

Furthermore, it should be noted that the current study focuses on the collective redox response of propolis through a multivariate lens, rather than the identification of individual bioactive markers. The absence of detailed chromatographic profiling, such as HPLC-DAD or LC-MS, represents a limitation in providing a granular mechanistic interpretation of the observed 'antioxidant paradoxes.' While our chemometric approach successfully identifies functional discrepancies that univariate data overlooks, future research incorporating high-resolution chemical fingerprints is essential. Such studies will enable a precise correlation between specific phenolic profiles and their respective multivariate redox signatures, further refining the predictive accuracy of the proposed quality control framework.

Taken together, the findings of this study clearly demonstrate that the antioxidant capacity of propolis cannot be reliably defined or interpreted by a single chemical indicator. By making sample-specific variability visible despite the presence of strong correlations, this work highlights the inherent limitations of reductionist approaches and underscores the necessity of multivariate strategies as a central framework for

interpreting the antioxidant behavior of complex natural product matrices in a robust and reliable manner.

Conclusion

This study demonstrates the methodological limitations of single-parameter approaches commonly employed in the evaluation of the antioxidant capacity of propolis by situating them within a multilayered and integrated analytical framework. The findings clearly show that the antioxidant behavior of propolis cannot be reliably reduced or interpreted on the basis of total phenolic or total flavonoid content alone; rather, it is shaped by the combined influence of compound composition, inter-compound interactions, and the selectivity of the analytical methods applied. The shift from simple numerical reporting to a multidimensional evaluation is essential for ensuring the analytical integrity of natural product standardization.

The pronounced heterogeneity observed among samples, together with the inconsistencies identified between phenolic content and CUPRAC antioxidant capacity in specific cases, indicates that reductionist evaluation paradigms are insufficient for complex natural products such as propolis. Although strong correlations were detected among parameters, these relationships proved inadequate for explaining the structural and functional complexity of antioxidant behavior. This finding highlights the scientific limitations and potential risks associated with generalizing correlation-based interpretations without appropriate chemical and statistical context. Our results emphasize that high correlation does not imply diagnostic interchangeability in high-precision food analytics.

Multivariate analyses played a central role in the interpretation of the antioxidant properties of propolis in this study. Principal Component Analysis revealed not only a shared redox-related variance but also meaningful differences in the relative contributions of individual parameters to antioxidant behavior, while Hierarchical Cluster Analysis demonstrated that sample groupings derived from integrated parameter sets differed substantially from those based on single indicators. These results confirm that multivariate approaches should be regarded not as auxiliary tools, but as fundamental analytical strategies for the evaluation of complex natural product systems. The efficacy of the *Z*-score/PCA/HCA framework demonstrated here provides a robust template for decoupling mass-quantification from functional performance.

In conclusion, a correct and comprehensive understanding of the antioxidant capacity of propolis requires the adoption of integrated analytical strategies that explicitly account for the multidimensional structure of the data. The primary contribution of this study lies in analytically demonstrating the necessity of moving beyond single-parameter evaluations and encouraging a critical re-examination of established interpretative conventions in propolis research. The proposed framework not only provides a methodological contribution to the academic literature, but also offers a practical basis for more realistic, comparable, and responsible interpretation of the functional value of propolis and similar natural products. The practical implication of this research lies in its potential as a high-throughput regulatory tool, as it allows for the identification of sub-standard or inconsistent samples that might otherwise 'pass' conventional univariate tests, thus setting a new benchmark for the industrial and pharmaceutical quality control of propolis extracts. Focusing on the CUPRAC assay as a stable, ET-based analytical benchmark offers a more reliable pathway for industrial and regulatory quality control than traditional multi-assay screenings.

In this context, the present work delivers a clear and actionable message to researchers, product developers, and stakeholders involved in evaluation processes: antioxidant capacity cannot be adequately represented or reliably interpreted through a single compositional indicator, and the adoption of multidimensional assessment strategies is essential for achieving scientific rigor and interpretative reliability. This analytical paradigm is expected to redefine the quality assessment protocols for propolis and other bioactive-rich matrices in accordance with modern analytical standards.

Author contributions

Mehmet Beykaya: conceptualization, methodology, investigation, writing – original draft, project administration. Aslı Elif Tanuğur Samancı: conceptualization, methodology, investigation, supervision. Taylan Samancı: validation, resources, supervision. Elif Önder Yorulmaz: software, formal analysis, data curation, visualization.

Conflicts of interest

There are no conflicts to declare.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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