Uludag Bee Journal
Uludag Bee J.
e-ISSN: 2687-5594
ISSN 1303-0248
https://dergipark.org.tr/en/pub/uluaricilik
https://uadubj.uludag.edu.tr/

Proline in Honey: A Complementary Biochemical Indicator in Quality, Authenticity, and Adulteration Assessment

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Received: 11.05.2026 Accepted: 07.06.2026 Published:29.07.2026

Abstract

Proline is a major free amino acid in honey and has long been used as a biochemical indicator of honey quality, maturity, and authenticity. Its association with the biochemical contribution of bees during nectar transformation gives it a distinctive role among conventional quality parameters. However, proline concentration is influenced by botanical origin, geographical conditions, pollen content, bee-feeding practices, harvest time, maturation, storage, and heat treatment. Therefore, proline should not be used as a standalone criterion for honey quality or adulteration. This review evaluates the biological origin of proline in honey, its role in quality and maturity assessment, its limitations, its relevance to botanical and geographical origin determination, and its variation in honeys from Türkiye. Analytical approaches for proline determination, including spectrophotometric, chromatographic, mass spectrometric, kit-based, sensor-based, and multi-analytical systems, are also discussed. The literature indicates that low proline levels may indicate counterfeit honey, intensive sugar feeding, or insufficient maturation. However, reliable interpretation requires combined evaluation with HMF, diastase activity, electrical conductivity, sugar profile, C4 sugar ratio, amino acid profile, phenolic/mineral content, isotopic analyses, and chemometric methods. Proline should therefore be considered a complementary indicator within multi-analytical honey authentication systems.

Keywords: Adulteration**,** Honey, Proline, Quality, Turkish honeys

INTRODUCTION

Honey is a natural and complex food product produced by honey bees through the collection of plant nectars, plant secretions, or excretions of plant-sucking insects, followed by transformation with bee-specific enzymatic and biochemical contributions, reduction of water content, and maturation in honeycombs. Therefore, honey is not merely a sweet product rich in fructose and glucose, but a biological matrix shaped by botanical source, geographical environment, bee contribution, maturation process, harvest time, processing, and storage conditions. Although sugars and water constitute the major components of honey, minor constituents such as organic acids, minerals, enzymes, phenolic compounds, volatile compounds, proteins, and free amino acids provide valuable information on its quality, authenticity, and origin (Puścion-Jakubik *et al.* 2020, Soares *et al.* 2017, Ulberth 2016).

The high economic value of honey, consumer perception of it as a natural product, and price differences based on botanical or geographical origin make it vulnerable to adulteration and mislabelling. Honey fraud can generally be evaluated under two main categories: interventions related to the production process and misrepresentation of origin. Direct addition of sugar syrups, intensive feeding of bee families with sugar syrups during the main nectar flow, harvesting of immature honey, excessive heat treatment, and filtration are among the main production-related problems. In contrast, blending low-value honeys with high-value monofloral or regional honeys, mislabelling botanical or geographical origin, and marketing mixed honeys under a different declared origin constitute major authenticity-related problems (Soares *et al.* 2017, Ulberth 2016).

Several parameters have long been used to assess honey quality and authenticity, including moisture, hydroxymethylfurfural (HMF), diastase activity, free acidity, electrical conductivity, sugar profile, C4 sugar ratio, and proline content. These parameters provide complementary information on honey maturity, processing history, storage conditions, botanical source, and potential adulteration. However, the natural composition of honey may vary widely depending on botanical and geographical conditions, which limits the assessment of quality or adulteration based on a single parameter. Therefore, current approaches increasingly emphasize the

combined interpretation of conventional quality parameters with chemical, isotopic, spectroscopic, chromatographic, and chemometric data (Puścion-Jakubik *et al.* 2020, Tsagkaris *et al.* 2021).

Within this framework, proline plays a distinctive role in assessing honey quality and authenticity. As one of the predominant free amino acids in honey, proline has long been considered an important biochemical parameter because of its association with bee-derived contributions during nectar transformation and its use as an indicator of honey maturity. Low proline levels may, in some cases, indicate insufficient maturation, intensive sugar feeding, or weakening of the honey's biochemical integrity. Nevertheless, proline concentration can be affected by several factors, including botanical origin, geographical conditions, pollen content, bee-feeding status, harvest time, heat treatment, and storage. For this reason, proline should not be interpreted as an absolute standalone decision criterion.

In recent years, a major trend in honey analysis has been a shift from interpreting proline solely as a conventional quality parameter based on threshold values to considering it as a complementary biochemical indicator within multi-analytical systems. Sugar and amino acid profiles, phenolic markers, isotopic analyses, spectroscopic approaches, LC-HRMS/metabolomic methods, and machine learning-supported models provide a more comprehensive framework for evaluating honey authenticity and origin. This transition does not diminish the importance of proline; rather, it positions it as a strategic indicator of quality to be interpreted within an appropriate analytical context.

Previous reviews have mostly focused on general honey quality parameters, floral markers, authenticity problems, or modern analytical methods used for adulteration detection. However, focused reviews that integrate the biological origin of proline, its role in honey maturity and naturalness assessment, its limitations in adulteration interpretation, its variability in Turkish honeys, and its complementary position within modern multi-analytical authentication systems remain limited. Therefore, this review aims to position proline not as an isolated threshold-based criterion, but as a complementary biochemical indicator to be interpreted alongside conventional quality parameters, botanical and geographical context, and advanced analytical approaches.

The aim of this review is to re-evaluate the role of proline in honey quality, maturity, authenticity, botanical/geographical origin, and adulteration assessment in light of current literature. The biological origin of proline, its strengths and limitations, reported proline values in Turkish honeys, analytical methods for proline determination, and its complementary role in modern multi-analytical authentication systems are discussed through a concept-oriented approach.

Quality and Authenticity Framework

The concepts of quality, naturalness, and authenticity in honey are closely related, but they do not have the same meaning. Quality refers to whether honey meets the physicochemical, enzymatic, and compositional characteristics defined by legislation and international standards. Naturalness refers to the preservation of the natural bee- and plant-derived production process, the absence of externally added substances, and the retention of honey's natural constituents. Authenticity, on the other hand, refers to whether honey is consistent with its declared botanical or geographical origin. Therefore, honey assessment should not be based solely on standard quality parameters, but should also consider production practices, floral source, geographical origin, processing history, and possible adulteration practices together (Bogdanov *et al.* 1999, Puścion-Jakubik *et al.* 2020, Ulberth 2016). According to the Turkish Food Codex Honey Communiqué, honey is defined as a natural product produced by honey bees through the collection of plant nectars, secretions of living parts of plants, or excretions of plant-sucking insects, followed by transformation with bee-specific substances, reduction of water content, and maturation in honeycombs. The communiqué states that no food ingredient, including food additives, or any external substance may be added to honey, and that honey should be free from organic or inorganic substances not naturally present in its composition (Turkish Food Codex Honey Communiqué 2020). This definition emphasizes that honey should be evaluated not only by its chemical composition, but also by the naturalness of its production process.

Moisture, hydroxymethylfurfural (HMF), diastase activity, free acidity, electrical conductivity, sugar profile, C4 sugar ratio, and proline content are widely used in honey quality control. Each of these parameters reflects a different aspect of honey: moisture is related to maturation and fermentation risk; HMF to heat treatment and storage history; diastase activity to enzymatic integrity; electrical conductivity particularly to the differentiation of blossom and honeydew honeys; sugar profile to the basic carbohydrate composition; and C4 sugar ratio to the addition of sugars derived from maize or sugar cane. Proline is one of the main biochemical indicators used to assess bee contribution, maturity, and naturalness (Bogdanov *et al.* 1999, Puścion-Jakubik *et al.* 2020, Ulberth 2016). However, compliance with quality parameters does not always prove that honey is authentic or correctly labelled. For example, honey that complies with regulatory limits for moisture, HMF, or diastase activity does not necessarily have the declared botanical or geographical origin. Similarly, because some sugar syrups can be formulated to resemble honey in terms of fructose and glucose composition, adulteration cannot always be detected solely from the basic sugar profile. Therefore, assessing honey's

naturalness and authenticity requires a multi-parameter approach that goes beyond regulatory limits and considers the biological and analytical integrity of the product. The limits defined in the Turkish Food Codex Honey Communiqué provide an important framework for this multi-parameter assessment. In general, HMF content is limited to a maximum of 40 mg/kg, diastase number to a minimum of 8, free acidity to a maximum of 50 meq/kg, and C4 sugar ratio to a maximum of 7%. For proline, the general minimum limit is 300 mg/kg; however, different botanical sources have specific limits: at least 180 mg/kg for rapeseed, linden, citrus, lavender, and eucalyptus honeys; at least 120 mg/kg for rosemary and acacia honeys; and at least 500 mg/kg for chestnut honeys (Turkish Food Codex Honey Communiqué Annexes 2020). These differences clearly show that botanical origin should be considered when interpreting proline values. Within this framework, honey quality and authenticity assessment should not be reduced to a single parameter. Each indicator reflects a different characteristic of honey, but no single indicator can fully explain all aspects of quality, naturalness, and origin. The main parameters commonly used in the assessment of honey quality, naturalness, and authenticity, together with their interpretation areas, are summarized in Table-1. Therefore, proline provides a more reliable basis for interpretation when evaluated together with HMF, diastase activity, electrical conductivity, sugar profile, C4 sugar ratio, and origin-related parameters.

Table 1. Main parameters used in the assessment of honey quality, naturalness, and authenticity (Bogdanov et al. 1999, Puścion-Jakubik et al. 2020, Turkish Food Codex Honey Communiqué Annexes 2020, Ulberth 2016).

Parameter	Property assessed	Purpose of interpretation
Moisture	Maturity, fermentation risk	Assessment of harvest and storage suitability
HMF	Heat treatment, storage	Indicator of excessive heating and/or prolonged storage
Diastase	Enzymatic integrity	Assessment of heat treatment and freshness
Free acidity	Fermentation/spoilage tendency	Quality control
Electrical conductivity	Mineral content, honey type	Differentiation of blossom and honeydew honeys
Sugar profile	Carbohydrate composition	Evaluation of basic composition and possible sugar anomalies
C4 sugar ratio	Addition of C4-derived sugars	Detection of maize/cane sugar adulteration
Proline	Bee contribution, maturity, naturalness	Biochemical quality indicator
Pollen/marker analyses	Botanical and geographical origin	Authenticity assessment

Source and Biological Meaning of Proline in Honey

Free amino acids in honey are minor constituents in terms of quantity, but they provide important information about the biological formation process of the product. These amino acids may originate from both plant-derived and bee-derived sources. Nectar and pollen are the main plant-derived sources, whereas bee-derived contribution is associated with enzymes and other biochemical components added during the transformation of nectar into honey. Therefore, the free amino acid profile of honey can be considered not only a chemical compositional feature, but also a biochemical trace reflecting the formation process of honey (Hermosín *et al.* 2003, Janiszewska *et al.* 2012).

Proline is one of the most prominent free amino acids in honey. Since it constitutes a substantial proportion of total free amino acids in many honey types, it occupies a central position in the amino acid composition of honey. However, the predominance of proline is not uniform across all honey types; in some floral sources, phenylalanine, tyrosine, or other amino acids may become more prominent. This indicates that proline is an important indicator within the general amino acid composition of honey, but its biological meaning should always be interpreted in relation to botanical and environmental context (Hermosín *et al.* 2003). Proline is a cyclic amino acid in which the side chain is bonded to the amino nitrogen, giving it a distinctive structural feature among proteinogenic amino acids (Figure-1).

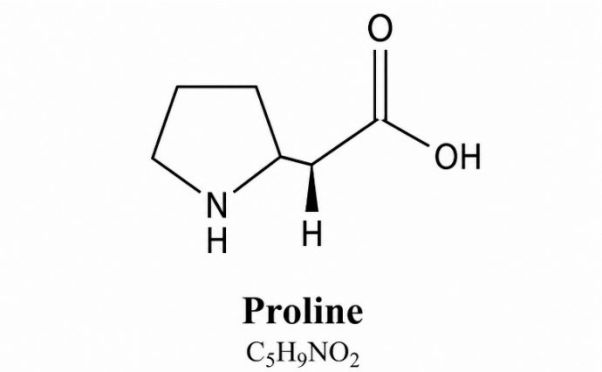

Figure 1. Chemical structure of proline.

The presence of proline in honey is associated with plant-derived sources such as nectar and pollen, but it is also largely explained by the bee contributions during nectar processing. Nectar may contain

varying amounts of amino acids depending on plant species, environmental conditions, and secretion period. Pollen is a richer source of amino acids and may contribute to the formation of the free amino acid profile of honey. However, a substantial part of the proline in honey is considered to be added by bees during the transformation of nectar into honey. Therefore, proline is a distinctive compound that simultaneously reflects plant-derived inputs from nectar and pollen and the biochemical contribution of bees (Flanjak *et al.* 2016, Janiszewska *et al.* 2012).

Bee-derived contribution affects not only the sugar composition of honey, but also its protein, enzyme, and amino acid profiles. During the transformation of nectar into honey, bees reduce water content, secrete enzymes that convert sucrose into glucose and fructose, contribute to organic acid formation, and make the product suitable for storage in honeycombs. Enzymes and free amino acids added by bees during this process shape not only the chemical composition of honey but also its biological maturity. In this context, proline can be considered a meaningful indicator reflecting the biochemical processing activity of bees on nectar and the transformation process involved in honey formation.

Proline concentration is not determined solely by bee-derived contribution. Nectar flow intensity, the sugar and amino acid composition of nectar, pollen content, botanical source of honey, condition of the bee family, harvest time, and duration of nectar processing by bees may also affect the proline content of honey. Therefore, the biological meaning of proline should not be reduced to a single source. A more accurate interpretation is that proline is a biochemical indicator located at the intersection of plant-derived sources, bee contribution, and the maturation process.

The variability in proline and total amino acid contents among honeys of different botanical and geographical origins suggests that this compound is also related to honey origin. However, in this section, the main point is not the discriminatory power of proline in botanical origin determination, but rather its ability to reflect the multi-source biological formation of honey. Proline and the general amino acid profile are further discussed in the following sections in relation to botanical/geographical origin and Turkish honeys.

In conclusion, proline is not merely an amino acid parameter measured in honey. Together with components derived from nectar and pollen, it also reflects the biochemical contribution of bees during the transformation of nectar into honey. Therefore, proline should be considered one of the key indicators of honey formation, bee contribution, and biological maturity. This biological basis also provides a starting point for understanding the role of proline in assessing honey quality, maturity, authenticity, and adulteration.

Strengths and Limitations of Proline

Proline has long been used as one of the main biochemical indicators for assessing honey quality, maturity, and naturalness. Its importance lies mainly in its predominance among free amino acids in honey, its association with the biochemical contribution of bees during nectar transformation, and its link to honey maturation. Therefore, proline is not merely a compositional parameter but also a complementary indicator that provides information about the natural formation process, bee contribution, and the biochemical integrity of honey. However, proline should not be interpreted independently of botanical origin, production conditions, bee-feeding practices, harvest time, storage, and heat treatment.

One of the main strengths of proline in honey quality assessment is its ability to provide discriminative information between natural honey and honey adulterated or biochemically weakened by sugar syrup feeding. Intensive feeding of bee families with sugar syrups during the main nectar flow may not always clearly change the basic sugar composition of honey, because some syrups may resemble honey in terms of fructose and glucose profile. In such cases, proline becomes a more complementary indicator than basic sugar parameters such as sucrose, fructose, or glucose alone.

Guler *et al.* (2007) reported that proline, electrical conductivity, and sucrose were important variables for differentiating pure blossom honey from honeys obtained from bee families fed with sucrose syrup. Similarly, Guler *et al.* (2017) reported that proline, electrical conductivity, free acidity, vitamin and mineral contents, and enzyme activity were affected by sugar source and syrup level in honeys from bee families fed different industrial sugars. They also suggested that proline values below 300 mg/kg in pure blossom honey may serve as a warning indicator for excessive feeding with some sugar syrups. These findings indicate that proline may carry an important biochemical signal, particularly in cases of indirect adulteration related to bee feeding.

In a comparison of natural multifloral honeys and honeys obtained from bees fed with sucrose syrup, Nisbet *et al.* (2018) reported that proline levels were higher in natural honeys than in adulterated honeys. They also found that total phenolic and flavonoid content, as well as several mineral elements, were higher in natural honeys. This suggests that proline becomes more significant in naturalness assessment when evaluated alongside other biochemical indicators, such as phenolic content, antioxidant capacity, and mineral profile.

Another important use of proline is assessing honey maturity. Honey maturation is a dynamic process involving the reduction of nectar water content, the enzymatic conversion of sucrose into glucose and fructose, the development of enzymatic activity, and

the preparation of honey for storage in honeycombs. During this process, bee-derived enzymes and free amino acids shape the biochemical character of honey. Therefore, proline can be considered a supportive indicator of maturation. Czipa *et al.* (2012) reported that proline accounted for a significant proportion of total amino acids and could be affected by storage, whereas Zhang *et al.* (2021) showed that moisture, sugars, proteins, phenolic compounds, proline, and enzyme activities changed together during the natural maturation of rapeseed honey. However, the same literature also indicates that the relationship between proline and maturation is not necessarily linear or sufficient on its own for all honey types.

At this point, the limitations of proline should be considered alongside its strengths. Although many studies have reported lower proline concentrations in adulterated honeys or honeys obtained from bee families intensively fed with sugar syrups, the interpretation of proline values is not always straightforward. A low proline concentration may indicate adulterated or artificial honey, excessive sugar feeding, or insufficient maturation; however, it may also occur naturally in some honeys depending on botanical origin, geographical conditions, nectar composition, pollen contribution, and the duration of nectar processing by bees. Therefore, low proline should be considered a warning signal rather than direct evidence of adulteration.

Similarly, a high proline concentration should not be interpreted as an absolute guarantee of honey quality or authenticity. Some honey types may naturally contain high proline levels due to their floral source, darker colour, higher mineral content, or stronger bee-derived biochemical contribution, but this does not eliminate the need to evaluate other quality and authenticity parameters. In addition, the relationship between proline and honey maturation may not be linear in all botanical sources. Although maturation generally involves water reduction, enzymatic transformation, and biochemical enrichment, proline may vary together with other components rather than acting as an independent maturity marker.

These interpretation challenges highlight the need to evaluate proline within a multi-parameter framework. Proline is most informative when interpreted alongside moisture, HMF, diastase activity, electrical conductivity, sugar profile, C4 sugar ratio, amino acid profile, phenolic and mineral content, isotopic data, and chemometric classification models. Thus, proline should be regarded as a complementary biochemical indicator that increases interpretive power when combined with other analytical evidence, rather than as a standalone criterion for judging honey quality, maturity, authenticity, or adulteration.

The wide variation in proline content among natural honeys is one of the main factors limiting its standalone interpretation. Janiszewska *et al.* (2012) reported that proline was the dominant amino acid in many honey types, but different botanical origins could not be clearly distinguished based only on amino acid composition. Similarly, Carratù *et al.* (2011) showed that although the amino acid profile provides information on botanical origin, variation within the same botanical source and overlaps among different floral types limit its discriminatory power when used alone. Therefore, proline is valuable in origin-related assessment, but it should not be used as an independent floral or geographical marker.

The use of proline as an indicator of maturity should also be interpreted carefully. Zhang *et al.* (2021) reported that, during maturation, moisture, sucrose, and maltose decreased, whereas glucose, fructose, total protein, and some enzyme activities increased. However, they also emphasized that proline does not always show a clear correlation with immature honey. Therefore, although proline is a valuable supportive parameter in maturity assessment, it should be evaluated together with moisture, enzyme activities, protein content, sugar profile, phenolic compounds, and volatile compounds.

Processing history is another important limitation in interpreting proline. Heat treatment and storage may affect not only conventional quality parameters such as HMF and diastase, but also the free amino acid profile. Adnan *et al.* (2014) reported decreases in proline and threonine levels in some honey samples after heat treatment. This indicates that proline concentration may vary not only with botanical origin, naturalness, or maturity, but also with post-harvest processing conditions. Therefore, when low or altered proline levels are observed, the post-harvest history of honey should also be considered.

Proline is also insufficient as a standalone decision tool for adulteration assessment. Sugar syrup addition or feeding bees with sugar may reduce proline levels; however, the effect depends on the type of adulteration, the sugar source used, the application level, and the honey's initial composition. Gün and Karaoğlu (2024) reported that when moisture, pH, free acidity, proline, diastase, colour, electrical conductivity, HMF, sugar profile, and C4 sugar analyses were evaluated individually, they were limited in detecting honeys adulterated with different levels of glucose–fructose and maltose corn syrups. However, adulteration could be detected more successfully when these parameters were evaluated together using PCA. This clearly shows that the real value of proline emerges within multi-parameter and chemometric approaches.

A similar limitation applies to isotopic analyses. Carbon isotope ratio analyses are powerful tools, especially for detecting C4 sugar syrups; however, C3 syrups and low-level indirect adulteration may not always be reliably detected. Guler *et al.* (2014) showed that low-level feeding applications could not always be detected using official isotopic criteria in honeys obtained from bee families fed with different

commercial syrups. Although advanced approaches such as EA/LC-IRMS can improve analytical sensitivity, isotopic findings provide a more reliable assessment when interpreted together with proline and other quality parameters (Elflein and Raezke 2008, Guler *et al.* 2014). Taken together, these findings indicate that low proline levels may signal compromised biochemical integrity, insufficient maturation, or the need for sugar syrup interventions. However, botanical and geographical variation, maturity level, heat treatment, storage, and different types of adulteration limit the standalone interpretation of proline.

Another important limitation is the establishment of universal proline threshold values. Regulatory limits, such as the general minimum value of 300 mg/kg defined in the Turkish Food Codex Honey Communiqué, are practical and necessary for routine quality control; however, they should not be interpreted as absolute biological boundaries applicable to all honey types (Turkish Food Codex Honey Communiqué Annexes 2020). Proline concentration may vary markedly according to botanical origin, geographical production area, nectar source, pollen contribution, bee processing duration, maturity level, and post-harvest conditions. The existence of different legal proline limits for specific botanical sources also supports the view that proline has strong botanical variability. Therefore, proline thresholds should be considered warning limits within a broader quality and authenticity assessment, rather than standalone criteria for accepting or rejecting honey authenticity.

In conclusion, proline is a valuable, yet context-dependent, biochemical indicator for assessing honey quality, maturity, and naturalness. It reaches its highest analytical value when interpreted together with sugar profile, C4 sugar ratio, HMF, diastase activity, electrical conductivity, phenolic/mineral content, amino acid profile, pollen analysis, isotopic analyses, and chemometric methods. Therefore, proline should not be treated as a standalone decision-making parameter, but rather as a complementary component of a multi-parameter quality and authenticity assessment. The multidimensional role of proline in honey quality, maturity, authenticity, and adulteration assessment is summarized in Figure-2.

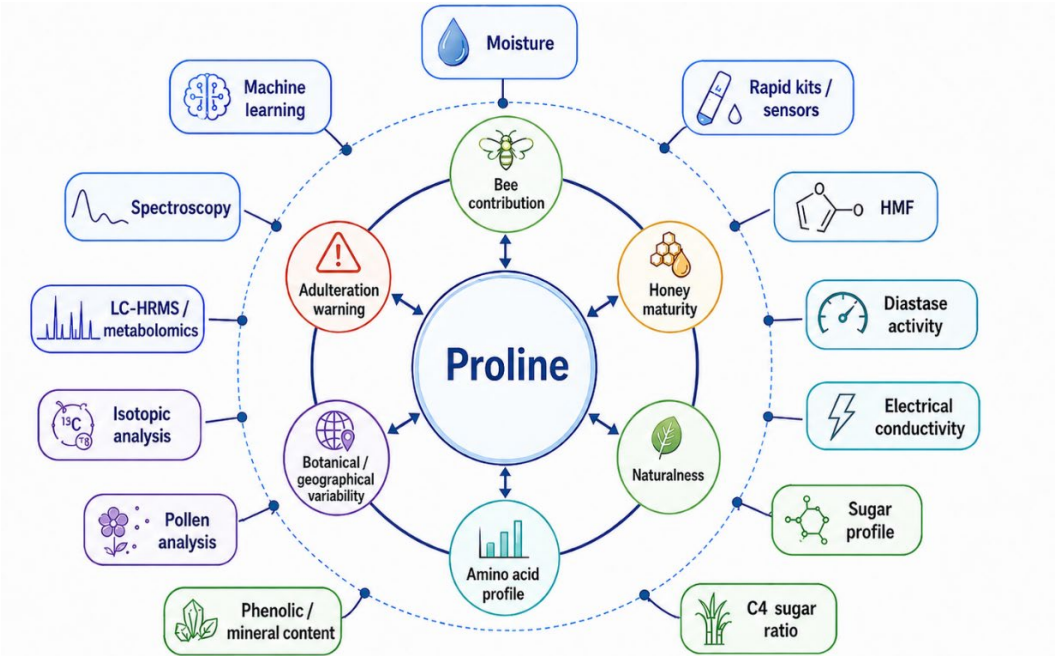

Figure-2. Multidimensional role of proline in honey quality, maturity, authenticity, and adulteration assessment. The scheme summarizes the complementary role of proline in multi-parameter, data-driven honey authentication systems.

Botanical/Geographical Origin and Turkish Honeys

The botanical and geographical origin of honey is among the main factors determining its sensory properties, chemical composition, market value, and labeling reliability. This is particularly important for monofloral or regional honeys, which often have higher economic value and therefore require verification of whether they truly represent the declared floral or geographical source. Although melissopalynological analysis, sensory evaluation, and basic physicochemical parameters have traditionally been used for origin determination, a single method is not always sufficient because of the natural variability and complex chemical structure of honey (Anklam 1998, Kaškonienė and Venskutonis 2010).

Conventional quality parameters used in origin assessment provide valuable information on compliance with standards, but they may be limited in their ability to directly explain botanical or geographical origin. Indicators such as HMF,

moisture, enzyme activity, electrical conductivity, sugar profile, and acidity provide information on honey maturity, processing history, or honey type. However, verification of declared floral or regional origin often requires more detailed chemical fingerprints.

Therefore, the combined evaluation of different marker groups, including amino acids, phenolic compounds, volatile compounds, mineral profile, sugar fractions, and isotopic indicators, provides a more reliable approach for origin determination. Proline and the general free amino acid profile are not definitive, standalone indicators for botanical and geographical origin determination; however, their discriminatory value increases when combined with phenolic compounds, mineral content, sugar profile, and chemometric analyses. Selected studies on the use of proline and related amino acid/phenolic markers in botanical and geographical origin determination are summarized in Table-2.

Table 2. Selected studies on the use of proline and related markers in botanical and geographical origin determination

Reference	Country/ honey type	Markers evaluated	Analytical/statistical approach	Main contribution
Hermosín *et al.* (2003)	Spanish honeys	Amino acid profile, proline	HPLC	Showed that the amino acid composition may vary according to botanical origin
Cotte *et al.* (2004)	Different floral honeys	Amino acid profile	HPLC	Provided differentiation for some floral types and showed a complementary role in detecting sugar syrup addition
Kaškonienė and Venskutonis (2010)	Review	Volatile compounds, phenolics, carbohydrates, amino acids	Marker-based evaluation	Emphasized the need for a multi-marker approach rather than relying on a single marker
Kečkeš *et al.* (2013)	Serbian unifloral honeys	Free amino acid profile	PCA, LDA	Demonstrated the potential of amino acid profiles for floral origin differentiation
Sun *et al.* (2017)	Chinese unifloral honeys	Amino acid profile	RP-HPLC, PCA, clustering, discriminant analysis	Supported botanical origin classification using amino acid data
Wen *et al.* (2017)	Chinese floral honeys	Proline, phenolic compounds	LDA	Showed that the combined use of proline and phenolic markers improves discrimination power
Tarapatskyy *et al.* (2021)	Polish honeys	Phenolic acids, minerals, proline, sugars	Chemometric analysis	Indicated that proline gains value within a multi-component chemical fingerprint

Turkish honeys provide an especially important field of evaluation in this context. Türkiye has a rich flora, diverse climatic zones, wide geographical variation, and strong beekeeping potential, allowing the production of honeys with highly diverse botanical and regional characteristics. This diversity directly affects the sugar profile, mineral content, electrical conductivity, HMF and diastase values, as well as the proline content of honey. Therefore, proline in Turkish honeys should not be evaluated solely on a single threshold value, but rather in conjunction with botanical origin, production region, harvest year, processing history, and other quality parameters.

Studies conducted in Türkiye show that proline levels may vary widely depending on honey type and region. For example, proline values of 301–977 mg/kg reported for pine honeys from Muğla indicate that proline in honeydew honeys should be interpreted together with parameters such as electrical conductivity and mineral composition. Similarly, proline ranges of 312–986 mg/kg in Astragalus honeys from Ağrı, 369.8–557.5 mg/kg in blossom honeys from Afyonkarahisar, and 281.61–2259.43 mg/kg in different monofloral, polyfloral, and honeydew honeys reveal a wide natural variation in Turkish honeys. This wide range indicates that proline is valuable for quality assessment, but it should not be interpreted in isolation from botanical and regional differences.

In the Turkish literature, proline has been evaluated not only as a quality indicator, but also in relation to origin and classification. Kıvrak (2015) investigated the free amino acid profiles of Turkish unifloral

honeys from different regions and reported that phenylalanine, proline, tyrosine, and isoleucine were among the prominent amino acids. This study is important because it shows that amino acid profiles may help differentiate floral origins. More recent studies have also evaluated proline together with HMF, diastase, C4 sugar ratio, sugar profile, electrical conductivity, melissopalynological analysis, and multivariate statistical approaches.

Studies on commercial and regional honey samples also show that high proline levels alone do not rule out all quality problems. In some commercial honey samples from different locations in Türkiye, high mean proline values have been reported despite non-compliance in parameters such as HMF or diastase. In contrast, in studies on commercial blossom honeys from Konya, Astragalus honeys from Ağrı, and blossom honeys from Afyonkarahisar, proline values evaluated together with sugar profile, C4 sugar ratio, HMF, and diastase values generally indicated compliance with regulatory limits. This shows that proline provides a more reliable interpretation when evaluated alongside a broader set of quality parameters than when used as a single indicator.

Table-3. Reported proline values in Turkish honeys and their main interpretation

Reference	Region/honey type	Number of samples	Proline value	Main finding
Adal *et al.* (2026)	Blossom honeys from Afyonkarahisar	10	369.8–557.5 mg/kg	Proline values were interpreted together with C4 sugar, HMF, diastase, and sugar profile for quality and adulteration assessment.
Guler *et al.* (2007)	Pure blossom honey and honey obtained after sucrose syrup feeding	15	One of the main variables differentiating the groups	Proline, together with electrical conductivity and sucrose, helped differentiate natural and adulterated honey.
Çınar and Ekşi (2012)	Pine honey from Muğla	100	301–977 mg/kg	Wide proline variation was observed in pine honey, supporting the need for botanical and regional interpretation.
Kıvrak (2015)	Seventeen unifloral honey types from Türkiye	58	One of the prominent amino acids	Free amino acid profiling, including proline, supported floral origin differentiation.
Guler *et al.* (2017)	Honeys obtained from bee families fed with industrial sugars	100	<300 mg/kg may be a warning indicator	Low proline values were associated with excessive sugar feeding when interpreted with other quality parameters.
Nisbet *et al.* (2018)	Natural and sucrose-adulterated multifloral honeys	45	Natural: 202 ± 26 mg/kg; adulterated: 71.1 ± 21.6 mg/kg	Natural honeys had higher proline values than adulterated honeys, supporting its complementary role in authenticity assessment.
Çiftçi and Parlat (2018)	Commercial blossom honeys from Konya markets	5	487.81–699.05 mg/kg	Samples complied with Turkish Food Codex limits for proline and related quality parameters.
Tabay Sümbül (2024)	Astragalus honeys from Ağrı	30	312–986 mg/kg; mean 665.68 mg/kg	Proline values supported the quality characterization of Astragalus honeys.
Özbay *et al.* (2024)	Monofloral, polyfloral, and honeydew honeys	95	281.61–2259.43 mg/kg	A wide natural variation in Turkish honeys was observed, highlighting the effects of botanical and geographical factors.
Kibar (2024)	Different honey samples from Konya	20	678.83 ± 192.16 mg/kg	Proline contributed to machine learning classification models.
Karlıdağ and Solak-Amet (2025)	Commercial honeys from different locations in Türkiye	9	1003.43 ± 316 mg/kg	High proline alone did not ensure overall quality, confirming the need for multi-parameter evaluation.
Çetintaş (2025)	Honeys from Muğla	20	1742 mg/kg in cedar honey	Multivariate analysis supported origin differentiation using proline and related quality parameters.

Studies addressing proline in relation to adulteration and indirect sugar feeding in Türkiye are also noteworthy. Guler *et al.* (2007) and Guler *et al.* (2017) showed that sugar syrup feeding practices may affect proline, electrical conductivity, free acidity, enzyme, mineral, and sugar profiles of

honey. Nisbet *et al.* (2018) reported that proline, together with phenolic content, flavonoids, antioxidant activity, and mineral profile, could be useful in differentiating natural multifloral honeys from honeys adulterated through sucrose feeding. These studies indicate that, in the Turkish literature, proline has been considered not only within the framework of regulatory limits, but also as a complementary indicator reflecting the biochemical integrity of honey.

In recent years, proline has also been used in Türkiye alongside modern analytical and classification approaches. Kibar (2024) evaluated proline, Brix, and colour properties using machine learning and achieved high classification accuracy. Çetintaş (2025) evaluated proline, diastase, HMF, C4 sugar ratio, HPLC sugar profile, and hierarchical clustering analysis in honeys from Muğla and revealed compositional differences associated with botanical and ecological origin. These studies suggest that proline may gain further importance in Turkish honeys not only as a conventional quality control parameter, but also as a component of multivariate classification and authenticity verification systems.

Proline values and related quality parameters reported for different honey types and regions in Türkiye are summarized in Table-3. Overall, proline values in Turkish honeys show a wide range, and this variation appears to be associated with botanical origin, region, honey type, harvest year, storage conditions, and the quality parameters evaluated. Therefore, for the interpretation of proline in Turkish honeys, a multi-parameter assessment system based on botanical and geographical reference data appears more appropriate than a fixed, single-threshold approach.

In conclusion, proline is not a definitive, standalone marker for determining botanical and geographical origin. However, when used together with the free amino acid profile, phenolic compounds, mineral content, sugar profile, electrical conductivity, melissopalynological analysis, and chemometric methods, it becomes a strong complementary parameter. The existing literature on Turkish honeys indicates that proline is valuable in both quality and origin assessment; however, reliable interpretation requires the development of regional and botanical reference databases.

Analytical Methods and Multi-Analytical Authentication Systems

Proline determination in honey is one of the main analytical steps used in quality control, maturity assessment, and interpretation of naturalness. The conventional approach is based on the reaction of proline with ninhydrin to form a coloured complex, followed by spectrophotometric measurement of the resulting colour. Methods proposed by the International Honey Commission (IHC), the Association of Official Analytical Chemists (AOAC), and Ough are commonly used in this context. However, standardization is important because reaction conditions, analysis time, and sample matrix may affect the results. Therefore, studies aimed at optimizing classical spectrophotometric methods have increased in recent years.

From a methodological perspective, this is important because ninhydrin-based spectrophotometric assays do not measure proline as an isolated structural marker but rather depend on controlled colour development under specific reaction conditions. Therefore, variations in heating time, cooling procedure, solvent system, extraction efficiency, calibration approach, and sample matrix may influence the final absorbance response. This makes method optimization and inter-method comparability particularly important when proline values are used not only for routine quality control but also for regulatory interpretation or comparison among studies.

Chromatographic and mass spectrometric methods allow proline to be evaluated not only as a single parameter, but also as part of the free amino acid profile. Approaches such as HPLC, UPLC-MS/MS, and LC-MS/MS provide broader biochemical data for botanical origin, quality, and classification studies. Capillary electrophoresis offers a practical alternative for honey quality control by enabling the simultaneous analysis of proline with major sugars. Recently developed colorimetric kits and microwave sensors also show that proline analysis can be transferred to rapid screening and field-applicable systems. The main conventional analytical methods and emerging approaches used for proline determination are summarized in Table 4. In general, spectrophotometric methods are low-cost and suitable for routine analysis, whereas chromatographic and mass spectrometric methods offer higher sensitivity and multi-amino acid profiling. Kit- and sensor-based approaches are promising as rapid, portable, and potentially production-chain-integrated methods.

In modern honey authentication systems, proline determination should not be considered a standalone analytical step, but rather a component of multi-data-based decision systems. One important part of this transformation is isotopic analysis. Stable carbon isotope ratio analysis is a powerful tool, particularly for detecting sugar syrups derived from C4 plants such as maize and sugar cane. However, C3-derived syrups, low-level indirect adulteration, regional isotopic variation, and some specific honey matrices may complicate interpretation. Therefore, isotopic analyses provide a more reliable basis for authentication when evaluated together with proline, sugar profile, HMF, diastase activity, electrical conductivity, and amino acid profile.

Spectroscopic and imaging-based methods are also gaining increasing importance in honey analysis because they are rapid, often non-destructive, and

suitable for screening large numbers of samples. Techniques such as NIR, MIR, FTIR, UV–Vis, Raman, and hyperspectral imaging can generate spectral or image-based fingerprints related to the chemical composition of honey. When combined with chemometric and machine learning models, these methods can serve as powerful screening tools for adulteration, heat treatment, quality parameters, and differentiation of botanical/geographical origins. However, their reliability depends on large, well-defined calibration sets, appropriate model validation, and databases that represent different honey types.

Table-4. Main analytical methods and emerging approaches for proline determination in honey

Method/approach	Basic principle	Main advantage	Limitation/point to consider	Representative reference
Ninhydrin-based spectrophotometry	Measurement of absorbance resulting from the proline–ninhydrin colour reaction	Suitable for routine analysis and low-cost	Reaction conditions and method standardization are important	Truzzi *et al.* (2014)
Optimized spectrophotometric method	Optimization of reaction time, temperature, and cooling conditions	Analysis time and extraction efficiency can be improved	Optimum conditions may vary depending on honey type	Bayram *et al.* (2023)
HPLC / UPLC-MS/MS / LC-MS/MS	Chromatographic/mass spectrometric analysis of proline and other free amino acids	Provides multi-amino acid profiling	Requires instrumental infrastructure, higher cost, and expertise	Dimins *et al.* (2022); Yang *et al.* (2024)
Capillary electrophoresis	Simultaneous separation of proline and major sugars	Rapid analysis with low sample and reagent consumption	Requires method optimization and validation	Dominguez *et al.* (2016)
Colourimetric kit	Adaptation of the ninhydrin-based colour change into a kit format	Potential for rapid screening and practical use	Field validation and standardization are required	Khakpour *et al.* (2025)
Microwave sensor	Determination of proline/sucrose through dielectric or sensor responses	Non-invasive, rapid, and potentially suitable for field applications	Requires validation with broader honey sample sets	Zonouri *et al.* (2025)

LC-MS, LC-HRMS, metabolomic, and omics-based approaches provide a more comprehensive chemical fingerprint for modern honey authentication. These methods not only detect known markers but also discover new markers through untargeted analysis. In more complex adulteration scenarios, such as syrups formulated to mimic the sugar profile of honey, C3-derived sweeteners, and sugar feeding during production, LC-HRMS and metabolomic approaches provide strong complementary tools to conventional methods. LC-HRMS-based marker studies, such as those reported by Paiano *et al.* (2025), indicate that compounds such as mannose and oligo/polysaccharide markers may become increasingly important in official control systems. Similarly, untargeted LC-MS data, when evaluated with machine learning, can support successful discrimination between authentic and adulterated honey.

Machine learning and artificial intelligence-supported approaches are becoming increasingly central in the interpretation of these multi-analytical systems. Methods such as spectroscopy, hyperspectral imaging, LC-MS, gas sensors, colour analysis, sugar profiling, and amino acid profiling generate multidimensional data. Such data may be difficult to interpret using conventional statistical approaches alone. Machine learning models can identify patterns among many variables and provide powerful tools for quality prediction, adulteration detection, botanical origin differentiation, and rapid classification. In this context, proline should no longer be regarded only as a single threshold-based parameter, but as a component of a broader quality model processed together with Brix, colour characteristics, sugar profile, amino acid profile,

spectral data, metabolomic markers, and sensor outputs.

However, the use of advanced analytical platforms does not automatically solve all authenticity problems. Their diagnostic value depends on how well classical indicators, targeted markers, untargeted fingerprints, and statistical models are integrated. In this context, proline can serve as a bridge between conventional quality control and data-driven authentication, providing biologically meaningful information while also being compatible with broader amino acid, spectroscopic, isotopic, and chemometric datasets.

Recent discussions increasingly frame honey fraud as a moving analytical target, emphasizing the need for multi-layer, data-driven, and internationally harmonized authentication frameworks (Schoder 2026). Nevertheless, modern multi-analytical authentication systems also have limitations. Although spectroscopic, metabolomic, and artificial intelligence-supported approaches have high accuracy potential, their performance largely depends on dataset size, sample diversity, validation strategy, and inter-laboratory standardization. Models developed with limited sample sets may not perform as well when tested on independent samples from different botanical origins, geographical regions, or harvest years. Therefore, the transfer of modern methods to official control systems requires large authentic honey databases, independent validation, standardized protocols, open and comparable datasets, and model interpretability.

In conclusion, proline determination remains a classical analytical step in honey quality control, but its meaning has expanded within the current analytical framework. Beyond serving as a quality indicator measured by conventional spectrophotometric methods, proline has become a complementary biochemical parameter, interpreted alongside chromatographic amino acid profiles, simultaneous sugar analyses, isotopic data, spectroscopic fingerprints, LC-HRMS/metabolomic markers, sensor outputs, and machine learning models. Therefore, the strongest future approach in honey authentication will be the integration of classical indicators such as proline with modern multi-analytical and data-driven decision systems.

Conclusion

Proline is a valuable biochemical indicator that has long been used to assess honey quality, maturity, and naturalness. Its association with bee contribution and the transformation of nectar into honey gives it a distinctive position among conventional quality parameters. However, the current scientific value of proline does not arise from its use as a standalone threshold-based criterion, but from its interpretation within the biological and analytical integrity of honey.

The literature reviewed in this study indicates that low proline values may serve as a warning signal for adulterated or artificial honey, excessive sugar feeding, or insufficient maturation. However, proline alone cannot be considered a definitive decision criterion. Since botanical origin, geographical conditions, pollen content, bee contribution, harvest time, storage, heat treatment, and adulteration type may affect proline concentration, this parameter should be evaluated together with HMF, diastase activity, electrical conductivity, sugar profile, C4 sugar ratio, amino acid profile, phenolic/mineral content, pollen information, isotopic data, and chemometric methods.

Data on Turkish honeys indicate that proline levels may vary widely across honey types and regions. Therefore, the development of up-to-date botanical and geographical reference databases for Türkiye is an important need for regional honey characterization, quality control, certification, and interpretation of samples suspected of adulteration.

In the future, the strongest approach in honey authentication will be the integration of proline with conventional quality parameters, LC-MS/MS, LC-HRMS, metabolomics, isotopic analyses, spectroscopic methods, sensor-based systems, and machine learning-supported models. In this context, proline should not be regarded as an outdated parameter that has lost its value, but as a strong complementary indicator in modern honey quality and authenticity assessment when used within an appropriate analytical framework.

Overall, the interpretation of proline in honey should move beyond a simple threshold-based approach. Although legal limits remain useful for routine quality control, the strong influence of botanical origin, geographical conditions, maturity, processing history, storage, and adulteration type limits the use of proline as a universal standalone criterion. Proline retains its practical and scientific value not because it provides an absolute answer alone, but because it strengthens the interpretation of honey quality, maturity, authenticity, and adulteration when integrated with complementary analytical evidence.

AI Declaration: Artificial intelligence tools were used only for linguistic, stylistic, grammatical, and organizational support during manuscript preparation. The scientific content, literature interpretation, critical evaluation, and final responsibility for the manuscript belong entirely to the author.

Author Contribution: The author conceived the review topic, designed the manuscript structure, conducted the literature review, evaluated and interpreted the relevant literature, prepared the tables, wrote the manuscript, revised the content critically, and approved the final version of the manuscript.

Conflict of Interest: The author declares that there is no conflict of interest.

Data Availability: Data sharing is not applicable to this article, as no new data were generated or analysed during the current review.

Ethical Statement: This review article did not involve any experimental study on humans or animals. Therefore, ethics committee approval was not required.

Funding: This review received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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