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ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

METHOD VALIDATION FOR DETERMINATION OF KYNURENIC ACID CONTENT IN NATURAL PRODUCTS BY RP-HPLC-UV

Doğal Ürünlerdeki Kynurenik Asit İçeriğinin RP-HPLC-UV ile Belirlenmesine Yönelik Yöntem Doğrulama

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ABSTRACT

Kynurenic acid (KYNA) is a metabolite with pharmacoactive properties found primarily in chestnut honey, linden, and other honeys. Considering the anti-inflammatory and immunosuppressive functions of KYNA, it can be seen that it has bidirectional effects on biological pathways. For this reason, determining and knowing the amount of honey, an important natural product can have a high impact on health. Studies on the detection of this metabolite in both natural products and animal tissues are ongoing, and it is important to develop fast and easily applicable methods. Within the scope of this study, a new method was created using an ultraviolet detector (RP-HPLC-UV) in reverse-phase high-performance liquid chromatography to determine the KYNA content of natural products (honey, chestnut pollen, and chestnut flowers) quantitatively in a short time. According to the data obtained, LOD and LOQ values were found to be 0.030 µg/mL and 0.092 µg/mL, respectively. At the same time, solutions of KYNA prepared in ultrapure water (UPW), 70% EtOH, EtOH and MeOH solvents were analyzed in this method, and it was found that UPW was the best solvent. The findings of this research can contribute significantly, particularly in the application of measuring KYNA, to distinguishing the botanical origin of chestnut honey.

Keywords: Kynurenic acid, Chestnut honey, HPLC-UV

ÖZ

Kynurenik asit (KYNA) başta kestane balı olmak üzere ıhlamur ve diğer bazı ballarda bulunan farmakoaktif özelliğe sahip bir metabolittir. KYNA'nın antiinflamatuar ve immünosüpresif fonksiyonu düşünüldüğünde biyolojik yolaklar için çift taraflı bir etkiye sahip olduğu görülebilir. Bu sebeple de önemli bir doğal ürün olan ballardaki miktarının belirlenmesi ve bilinmesi sağlık üzerinde yüksek bir etkiye sahip olabilir. Bu metabolitin hem doğal ürünlerde hem de hayvan dokularında tespitine yönelik çalışmalar devam etmekte olup, hızlı ve kolay uygulanabilir metotların geliştirilmesi önem arz etmektedir. Bu çalışma kapsamında doğal ürünlerin (bal, kestane poleni ve kestane çiçeği) KYNA içeriğinin kantitatif olarak kısa sürede belirlenmesi amacıyla ters faz yüksek performanslı sıvı kromatografisinde ultraviyole dedektörü (RP-HPLC-UV) kullanılarak yeni bir metot geliştirilmiştir. Elde edilen verilere göre LOD ve LOQ değerleri sırasıyla 0,030 µg/mL ve 0,092 µg/mL olarak bulunmuştur. Aynı zamanda KYNA'nın ultra saf su (UPW), %70 EtOH, EtOH ve MeOH çözücülerinde hazırlanmış çözeltileri bu metotta analiz edilmiş ve UPW 'un en uygun çözücüsü olduğu görülmüştür. Bu araştırmanın bulguları, özellikle kestane balının botanik kökeninin ayırt edilmesinde uygulanması açısından KYNA'nın ölçülmesine önemli ölçüde katkıda bulunabilir.

Anahtar Kelimeler: Kynurenik asit, Kestane balı, HPLC-UV

GENİŞLETİLMİŞ ÖZET

Amaç: Kinurenik asit (KYNA), C-4'te bir hidroksi grubu ile ikame edilmiş kinolin-2-karboksilik asit olan bir kinolinmonokarboksilik asittir. Yapılan bazı çalışmalarda, KYNA, özellikle kolit, kolon tıkanıklığı veya ülserasyonla ilgili olarak gastrointestinal sistemin çeşitli patolojilerinde olumlu özelliklere sahip olabileceği ifade edilmiştir. Temel olarak gastrointestinal sistemdeki olumlu özellikleri ve hipermotiliteyi azaltma yeteneği KYNA'nın gıdalardan alımı konusunda daha geniş bir araştırma yapılması ihtiyacını ortaya çıkarmaktadır. Örneğin ısırgan otu veya sarı kantaron, her ikisi de KYNA açısından zengin maddelerdir, sıklıkla sindirim sistemi hastalıklarının semptomlarını azaltmak için kullanılır. Bunların yanı sıra günlük hayatta tüketilen bal arısı ürünlerinden propolis ve ballarda da (kestane, ıhlamur vs.) yüksek KYNA içeriği olduğu bildirilmiştir. KYNA'nın biyokimyası ve biyolojik fonksiyonları göz önüne alındığında bal arısı ürünleri başta olmak üzere bazı doğal ürünlerdeki miktarının tespit edilmesi önem arz etmektedir. KYNA'nın tespitine yönelik çeşitli yöntemler önerilmiş ve çalışılmıştır. Ancak bu yöntemlerin bazılarında gerek analiz öncesi hazırlığın uzun olması gerekse analiz için gerekli olan ekipmanın kolay bulundurulamaz olması bazı zorlukları da yanında getirmektedir. Bu sebeple özellikle ballar başta olmak üzere doğal ürünlerdeki KYNA içeriğinin belirlenmesi için hızlı ve uygulanabilir bir yöntemin oluşturulması ve validasyonunun yapılması bu çalışmada amaçlanmıştır.

Gereç-Yöntem: Uygulanacak analizde örneklerin hazırlanmasında kullanılacak olan çözücünün belirlenmesi için farklı çözücüler ile analizler yapılmıştır. Aynı zamanda geliştirilecek olan metotta kullanılacak olan dalga boyunun seçimi için KYNA standardının 200-800 nm dalga boyu aralığında spektrum taraması yapılmıştır. Çalışmamız kapsamında çeşitli arı ürünleri ve bitki kökenli örnekler için KYNA içeriğinin belirlenmesine yönelik RP-HPLC-UV sisteminde hızlı ve kolay uygulanabilir metot geliştirmesi yapılmış ve validasyon çalışmaları kapsamında kesinlik, doğruluk, geri kazanım, bağıl hata, LOD ve LOQ gibi parametreler incelenmiştir.

Bulgular: Arı ürünleri ve diğer bazı bitki kökenli örneklerde KYNA içeriğin tespiti için UPW uygun çözücü olarak belirlenmiş ve MeOH, %70 EtOH ve EtOH ile hazırlanan standartların kromatogramlarındaki pikte omuzlanma veya genişleme olduğu gözlenmiştir. Metotta kullanılmak üzere KYNA'ya ait dalga boyunun belirlenmesi için yapılan spektrum taraması neticesinde 330 nm'nin analizde kullanılabileceği görülmüştür. Oluşturulan analiz metoduna ait validasyon parametrelerine bakıldığında aynı gün ve farklı günlerde geri kazanım değerleri sırasıyla %98,254 ve %97,762 olarak bulunmuştur. Standarda ait kalibrasyon grafiğinin R^2 değeri 0,999 ve LOD-LOQ değerleri sırasıyla 0,030-0,092 µg/mL olarak tespit edilmiştir. Analiz edilen örnekler içinden de CH5'in KYNA içeriğinin en yüksek olduğu belirlenmiştir.

Sonuç: Çalışmamız kapsamında KYNA analizinin özellikle balların botanik kökeninin değerlendirilmesine ve ayırt edici bir belirteç olarak kullanılmasına katkı sağlanması amaçlanmıştır. Bu bağlamda uygun maliyetli, kolay uygulanabilir ve minimum numune hazırlama adımları gerektiren bir yöntem geliştirilmiştir. Bu sayede kestane balları da dahil bazı arı ürünleri ile bitki kökenli bazı örneklerde KYNA analizinin karakterizasyonunun literatüre ve paydaşlara fayda sağlayabileceği düşünülmektedir.

INTRODUCTION

Kynurenic acid is a quinoline monocarboxylic acid that is quinoline-2-carboxylic acid substituted with a hydroxy group at C-4. Kynurenic acid (KYNA) is a tryptophan metabolite and has been reported to be present in living systems. Since its discovery in dog urine in 1853 (Liebig 1853), it has been found in insects (Smirnov et al. 2006), mammals (Moroni et al. 1988), plants (Starratt and Caveney 1996), and biological fluids and organs (Kuc et al. 2006, Kuc et al. 2008). Although the main product of kynurenine catabolism is nicotinamide adenosine dinucleotide (NAD+), KYNA is also produced due to the activity of kynurenine aminotransferases (Adams et al. 2012, Schwarcz et al. 2012). While the physiological functions of all TRP metabolites have not yet been elucidated, it is known that certain TRP metabolites, such as kynurenic acid (KYNA) and quinolinic acid (QA), exhibit bioactivity. It has been demonstrated that KYNA is an antagonist of ionotropic glutamate receptors (Mok et al. 2009, Alt et al. 2004) and alpha 7 nicotinic receptors (Hilmas et al. 2001). Since these two receptors are predominantly expressed in the brain, it has been indicated that KYNA is present in the brain (Moroni et al. 1988). In addition, it has been found that KYNA is an agonist of G-protein-coupled GPR35 receptors. It is noted that GPR35 receptors are primarily located in the gastrointestinal

system (Wang et al. 2006). In some studies, KYNA has been suggested to have positive properties in various pathologies of the gastrointestinal system, particularly colitis (Varga et al. 2010), colon obstruction (Walczak et al. 2011), or ulceration (Glavin and Pinsky 1989).

It has been reported that bee products, especially honey, are important in the pathophysiology of the gastrointestinal system and that this effect is due to various components (Coşkun and Coşkun 2022). Essentially, the positive properties in the gastrointestinal system and the ability to reduce hypermotility call for further extensive research on the intake of KYNA from foods (Varga et al. 2010, Kaszaki et al. 2008). For example, both nettle and St. John's Wort have been reported to be rich in KYNA and are frequently used as remedies to alleviate symptoms of digestive system disorders (Turski et al. 2011). In addition, it has been reported that propolis and honey consumed in daily life, including chestnut and linden honey, also have a high KYNA content (Turski et al. 2016, Pavlin et al. 2023).

Considering the biochemistry and biological functions of KYNA, determining its quantity in certain natural products, especially bee products, becomes crucial. Various methods have been proposed and studied for the detection of KYNA. However, some of these methods pose challenges, such as prolonged pre-analysis preparation (Turski et al. 2016) or the difficulty in obtaining the required equipment for analysis (Fukushima et al. 2022). Therefore, this study aims to establish and validate a rapid and applicable method for determining the KYNA content in natural products, especially honey.

MATERIALS AND METHODS

Samples

Honey samples were obtained from experienced beekeepers in different regions of Türkiye. Pollen samples were sourced from experienced beekeepers in Artvin and Trabzon. Chestnut flowers were collected from chestnut trees in the Trabzon province. All samples studied are from the year 2022.

Preparation for Analysis

An absorbance scan of the KYNA standard within the wavelength range of 200-800 nm was conducted to determine the wavelength to be used in the method. Additionally, various solvents are employed for extraction in natural products and bee products. The selection of an appropriate solvent for the analysis is as crucial as the analysis itself. In the new method to be employed for the determination of KYNA in natural products and bee products, different solvents, namely UPW, MeOH, EtOH, and 70% EtOH were used to dissolve standard KYNA (Sigma Aldrich) for analysis, aiming to reveal the effects of these solvents. All samples to be analyzed were extracted in UPW (1/5, w/v) and then filtered through ordinary filter paper (Turski et al. 2016). It was then analyzed by passing through 0.45 µm membranes. Each sample underwent three injections for robust analysis.

RP-HPLC-UV Condition

High-Performance Liquid Chromatography (HPLC) utilizing a UV detector (Elite La Chrom Hitachi, Japan), was employed for analyses. The method involved a reverse phase C18 column (250 mm x 4.6 mm, 5 µm) and utilized acetonitrile and water in an isocratic program. For RP-HPLC-UV analysis, the mobile phase comprised ultrapure water (UPW) and acetonitrile (Carlo Erba, France) in a ratio of 91:9, containing 20 mM ammonium acetate (Isolab, Germany) and 35 mM acetic acid (Merck, Germany). The sample injection volume was set at 20 µL, maintaining a column temperature of 25 °C, a flow rate of 0.7 mL/min, and an analysis duration of 15 minutes. The wavelength for analysis was set to 330 nm.

Method Validation

In the concentration range of 1.563 to 50 µg/mL, calibration curves were constructed, each composed of six data points replicated three times. Repeatability, accuracy, and detection limits were thoroughly investigated to validate the developed method in this study. Evaluation of validation parameters involved the calculation of relative standard deviation through the plotting of calibration curves. Limits of detection (LOD) and limits of quantification (LOQ) were established utilizing LOD = 3.3 × SD / m and LOQ = 10 × SD / m, where 'm' denotes the slope, and 'SD' represents the standard deviation at the lowest level of the calibration curves.

RESULTS

In studies analyzing KYNA in various samples, it is observed that different wavelengths are used (Sousa et al. 2021). Therefore, wavelength scanning was performed in present study, and it was decided that the optimum wavelength for our method is 330 nm (Fig 1). Various trials were conducted using different solvents for kynurenic acid prior to method validation. Optimal results were observed when kynurenic acid was dissolved in ultrapure water (UPW) (Fig 2). The comparative analysis of values obtained under identical conditions using alternative solvents revealed the following order: UPW > 70% Ethanol (70%EtOH) > Methanol (MeOH) > Ethanol (EtOH).

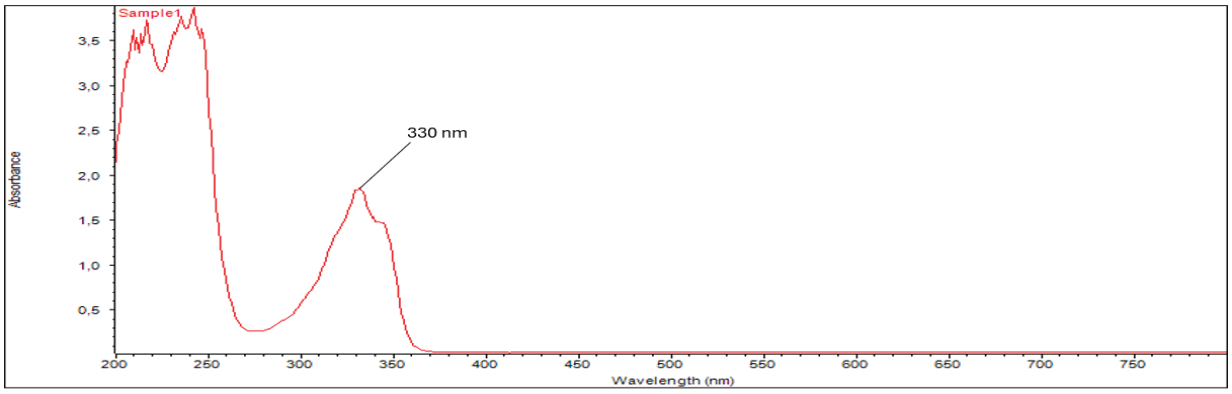

Figure 1. KYNA's wavelength scanning spectrum.

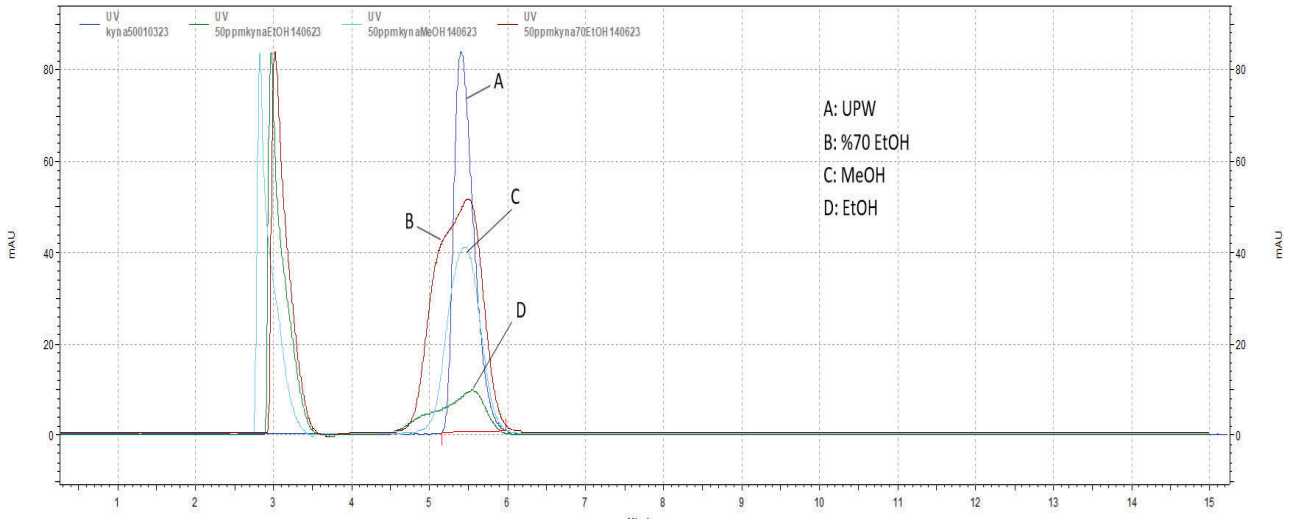

Figure 2. Chromatogram of the kynurenic acid standard in different solvents.

Chromatographic methods are commonly used in separation and purification processes. Considering the literature and our experiments, a UPW:ACN (91:9) solution containing 20 mM ammonium acetate and 35 mM acetic acid was used as the mobile phase in isocratic flow. At the optimized conditions, no interference was detected at the retention time corresponding to KYNA, validating the method's suitability for quantifying KYNA.

KYNA exhibited strong linearity with a correlation coefficient of $R^2 \geq 0.999$, while the recovery values for calibration standards were determined as 97.762% and 98.254% for inter-day and intra-day precision, respectively. Accuracy, reflecting the proximity of results to the true value, was evaluated

through absolute and relative error measurements. Absolute error quantifies the disparity between measured and true values, whereas relative error is calculated by dividing the absolute error by the true value. In present study, the relative error for KYNA was observed to be 0.017. Additionally, LOD and LOQ values for detection and quantification limits were determined as 0.030 µg/mL and 0.092 µg/mL, respectively (Table 1).

Table 1. Validation parameters of kynurenic acid

Kynurenic acid	
Linear range (µg/mL)	1.563-50
R^2	0.999
Relative Error	0.017
Limit of Detection (LOD) (µg/ml)	0.030
Limit of Quantification (LOQ) (µg/ml)	0.092
Recovery (%)	
Intra-day	98.254
Inter-day	97.762

As a result of the KYNA content analysis conducted on various honey, pollen, and flower samples using the developed method within the scope of the study, it was observed that the highest content was in chestnut honey (ChH5: 2688.949±0.257 µg/g). KYNA contents of the samples are given in Table 2. It is seen that the KYNA contents of chestnut honey vary between 192.254 and 2688.949 µg/g.

Table 2. Kynurenic acid content of various honey, pollen, and flowers samples

Sample	**Kynurenic acid (µg/g sample)**
Thyme honey (TH)	40.593±0,125
Parsley honey-1 (PH1)	84.977±0,247
Parsley honey-2 (PH2)	41.140±0.175
Lavender honey (LH)	43.169±0.163
Flower honey (FH)	45.194±0.109
Cedar honey (CeH)	47.463±0.118
Chestnut-oak honey (COH)	151.661±0.198
Chestnut honey-1 (ChH1)	192.254±0.252
Chestnut honey-2 (ChH2)	866.780±0.268
Chestnut honey-3 (ChH3)	1773.919±0.202
Chestnut honey-4 (ChH4)	1377.809±0.295
Chestnut honey-5 (ChH5)	2688.949±0.257
Chestnut honey-6 (ChH6)	930.548±0.226
Thyme honey (TH)	48.554±0.106
Oak honey (OH)	42.275±0.164
Astragalus honey (AH)	2.954±0.105
Chestnut pollen-1 (ChP1)	777.162±0.213
Chestnut pollen-2 (ChP2)	57.951±0.137
Chestnut flower (ChF)	15.245±0.116

The KYNA contents in chestnut pollen were observed to be 57.951 and 777.162 µg/g. Additionally, it has been observed that honey samples derived from various botanical origins also contain KYNA, albeit at relatively lower levels (Fig 3). Within the scope of present study, it was observed that the chestnut flower analyzed had the lowest KYNA content (15.245 µg/g).

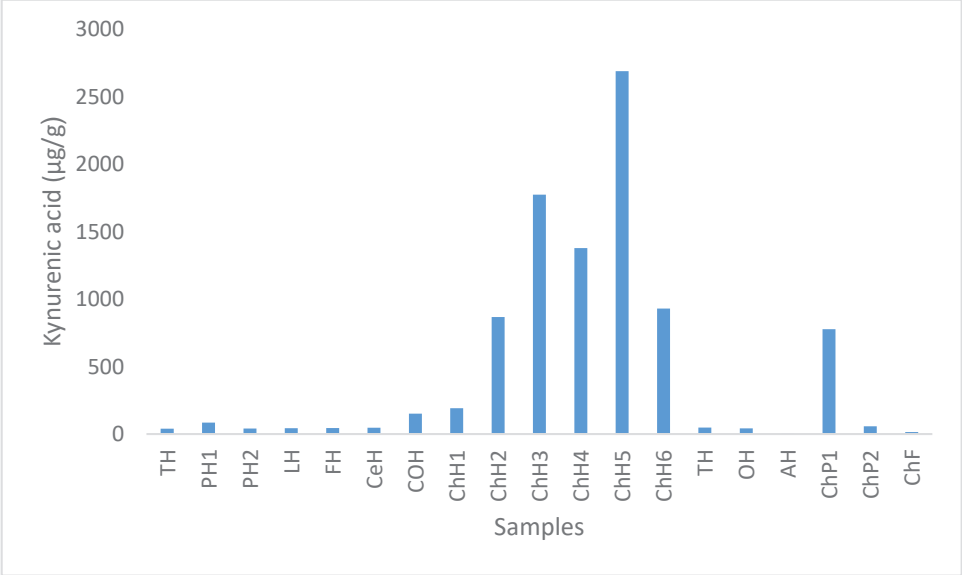

Figure 3. Visualization of the kynurenic acid levels in the samples.

DISCUSSION

Kynurenic acid (KYNA) is a tryptophan metabolite that exhibits a range of positive effects, including anti-inflammatory and antioxidative activities. It has been stated that the levels of tryptophan metabolites are important for assessing the stage of neurological disorders and could be evaluated for clinical diagnosis (Fukushima et al. 2022). It has been reported that KYNA is an agonist of the GPR35 receptors located in the gastrointestinal system (Wang et al. 2006), and it has been stated to have a positive effect on various gastrointestinal system pathophysiologies (Walczak et al. 2011, Wirthgen et al. 2018). Considering the bioactive properties of KYNA, its intake through the diet is also considered significant. According to the literature, some foods rich in KYNA can be listed as follows: nettle, St. John's Wort, certain parts of the chestnut tree, and chestnut honey samples (Turski et al. 2011, Pavlin et al. 2023).

The determination of KYNA content in foods has become important, especially in complementary medicine. In this context, the development of simple and easily applicable methods for determining KYNA content is crucial. Within the scope of our study, a rapid and easily applicable method using RP-HPLC-UV has been developed and validated. The wavelength specific to the analyte was first determined for the analysis conditions of the developed method. For this purpose, a spectrum scan of standard KYNA was performed, and 330 nm was identified as the optimum wavelength. Sousa et al. (2021) and Kim et al. (2022) reported conducting analyses at wavelengths of 344 nm and 240 nm, respectively, in their studies. Meanwhile, Beratta (2009) adjusted the UV-DAD detector to 327 nm in their study to conduct to analyze KYNA.

Subsequently, to determine the ideal solvent for analysis, a KYNA standard prepared in different solvents was analyzed using the established method. However, negative aspects such as shoulder formation or peak broadening were encountered with solvents other than UPW. Therefore, it was decided to use UPW both as the solvent for the standard substance and for the extraction of samples. Kim et al. reported a special extraction method for determining the KYNA content in chestnut honey samples, stating that the KYNA content in samples left to stand for 6 hours in 10% EtOH was higher (Kim et al. 2022). In their KYNA analysis studies, Sousa et al. (2021) and Lan-Gan et al. (2009) reported using distilled water to prepare their standard stock solutions. Although the use of distilled water has been reported, we also identified the potential of different solvents in our study.

In a study, a method was developed using HPLC-UV/FD, and ammonium formate/formic acid was employed as the buffer solution for the mobile phase

(Sousa et al. 2021). In another study, trifluoroacetic acid was used to create the mobile phase (Beretta et al. 2009). Lan-Gan reported using zinc acetate and sodium acetate in the mobile phase containing 6% ACN in their KYNA analysis study conducted using a fluorescence detector (Lan-Gan et al. 2009). In present study, to eliminate interferences other than the analyte and to accurately determine the analyte peak, we used ammonium acetate and acetic acid as buffers in our mobile phase containing 9% ACN.

It has been reported that chromatographic methods employing UV or fluorescence detectors are commonly used for the determination of TRP and its metabolites, especially in serum and plasma fluids (Fukushima et al. 2022). In a study, analysis was conducted using the HPLC-UV/FD method for the determination of TRP and its metabolites, and UV detection in isocratic flow was reported for KYNA analysis (Sousa et al. 2021). In another study, KYNA contents were analyzed using High-Performance Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (HPLC-ESI-MS) to determine markers related to the botanical origin of honey (Beretta et al. 2008). Turski et al. (2016) stated that they conducted the analysis of KYNA content in various chestnut honeys and parts of chestnut trees fluorometrically in their study. Pavlin et al. (2023) developed a method for the analysis of KYNA content using HPLC-MS/MS and reported its application in various honeys. In present study, we developed a simple and easily applicable method was developed for analyzing KYNA content in various honeys, chestnut pollen, and chestnut flowers using an HPLC device equipped with a UV detector.

The validation of the method developed in present study was conducted, and the LOD-LOQ values were determined to be 0.030-0.092 µg/mL, respectively. In studies aimed at determining KYNA content, it has been reported that some developed methods have LOD and LOQ values of 0.013-0.050 µg/mL (Sousa et al. 2021) and 0.001-0.010 µg/mL (Pavlin et al. 2023), respectively. Although the LOD and LOQ values may vary depending on the detector used, it was found that the obtained values are not significantly different from those reported in the literature.

KYNA can be obtained from various natural sources, including honey and various parts of certain plants. In present study, it is seen that the flora of the regions where chestnut honey is obtained creates significant differences in the KYNA content. It has also been observed that other honeys contain KYNA but at relatively lower levels. (Table 3). It is considered that the KYNA content of chestnut pollen may be related to its botanical origin. In a study conducted using 20 different botanical sources and 44 commercially available Italian honey samples, it has been suggested that chestnut honey exhibits significantly higher levels of KYNA content and could serve as a biomarker for chestnut honey (Beretta et al. 2008). In a study where a combination of HPLC-DAD-ESI MS and NMR techniques was used to analyze KYNA and some derivatives in various commercial honeys, it was emphasized that KYNA and its derivatives could be significant markers in chestnut honeys. Furthermore, in this study, it was suggested that KYNA and its derivatives may possess antinociceptive activity (Beretta et al. 2009).

In Slovenia, KYNA analysis was conducted using HPLC-MS/MS in 129 honey samples obtained from local beekeepers and commercially sourced. It was reported that chestnut and linden honey samples contained high levels of KYNA. Additionally, it was stated that the sample preparation process for this analysis was kept to a minimum, and they avoided complex extractions (Pavlin et al. 2023). It has been reported that KYNA analysis was conducted using HPLC-UV in chestnut honey samples collected from nine different regions of Korea. Additionally, it was stated that the most suitable method for obtaining high KYNA content in honey samples was to extract with 10% EtOH at a ratio of 1:20 for 6 hours (Kim et al. 2022).

Various honey samples collected from different Mediterranean countries and certain parts of the chestnut tree were analyzed for KYNA using HPLC with fluorometric detection. They reported that chestnut honey had the highest KYNA content, followed by the male flowers of the chestnut tree. Additionally, it was noted that the KYNA content was much lower in female chestnut flowers. Based on their findings, they observed that chestnut honey samples from Mediterranean countries could be rich in KYNA (Turski et al. 2016).

Conclusion: Within the scope of our study, the analysis of KYNA aimed to contribute particularly to the assessment of the botanical origin of honey samples and its use as a distinctive marker. It was aimed to overcome the complexity of the extraction conditions in previous studies, the lack of a wide range of uses of the method chosen for detection,

and the need for more information than in the current study for its use. In this context, present study aimed to develop a method that is cost-effective, easily applicable, and requires minimal sample preparation steps. This way, it is thought that the characterization of KYNA analysis in chestnut honey samples and some other related samples could provide benefits to the literature and stakeholders.

Author Contribution: Yakup Kara: analysis, investigation, writing–original draft; Sevgi Kolaylı: review & editing, research planning

Data Availability: The data can be found within the manuscript.

Declaration of interest: The authors state that they have no conflicts of interest to disclose.

Ethics: The research did not involve any animals or human.

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